

Report Title: Test For Chemical Induction of Mutation In Mammalian Cells In Culture: The L5178Y TK+/- Mouse Lymphoma Assay

Test Type: Genotoxicity Study

Conducting Laboratory and Location: Sitek Research Laboratories, Rockville, MD

Test Substance(s): G0539.03 – Octopirox in ethanol tested with and without transferrin

Species: Mammalian Cells

Test Conditions: Thymidine kinase locus of L5178Y TK+/- Mouse Lymphoma cells mutation tested in the absence and presence of Aroclor-induced rat liver S-9. Studies done in the presence and absence of transferrin. Maximum dosages tested with and without activation were specified in an amendment to the protocol.

Results: Positive dose-dependent responses were produced both in the presence and absence of S-9; the test article was less toxic when treatment was performed in conjunction with S-9. No significant difference in the toxic response or mutant frequencies was evident between the cultures treated with or without transferrin in the presence of S-9.

Study #: 0066-2400

Report Date: 9/30/87

QA report/GLP compliance: Yes

Accession #: 34002



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**TEST FOR CHEMICAL INDUCTION OF MUTATION
IN MAMMALIAN CELLS IN CULTURE
THE L5178Y TK+/- MOUSE LYMPHOMA ASSAY**

FINAL REPORT

SITEK's Study Number: 0066-2400

Sponsor's DRD Number: BY0708

Test Article Identification: G0539.03

Test Article Batch/Lot No.: Not Provided

Test Article Description: Off-White Powder

Test Article Storage Conditions: Room Temperature

Test Article Receipt Date: August 4, 1987

Study Initiation Date: August 11, 1987

Report Date: September 30, 1987

Sponsor: The Procter & Gamble Company
P. O. Box 39175
Cincinnati, Ohio 45247

Sponsor's Divisional Toxicologist: Kenneth L. Hintze, Ph.D.

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Paul E. Kirby
Paul E. Kirby, Ph.D.
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Ed T + Ken Hintze
DATE: 10-5-87



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**QUALITY ASSURANCE UNIT'S STATEMENT
OF GENERAL LABORATORY PROCEDURES' COMPLIANCE**

STUDY NO. 0066-2400

SPONSOR'S TEST ARTICLE I.D. G0539.03

To the best of my knowledge this study was performed in compliance with the GLP regulations for nonclinical laboratory studies and the GLP standards for health effects as described in 21 CFR Part 58 and 40 CFR Part 792 and 160, respectively. In this context, the facilities, equipment, personnel, methods, practices, records, controls and reports have been inspected per the QAU standard operating procedures and found to be in compliance with the above regulatory requirements.

The following phases were inspected for this study:

<u>Inspection Date</u>	<u>Critical Phase</u>
<u>8/14/87</u>	<u>Addition of cells to the cloning flasks (Without Activation)</u>
<u>8/14/87</u>	<u>TFT addition and VC dilution and addition to the VC flasks</u>
<u>8/18/87</u>	<u>Removal of test article for the Mutation Assay (With Activation)</u>

Findings Reported to Study Director 8/17/87, 8/20/87
Date

Findings Reported to Management 8/17/87, 8/20/87
Date

Signature Patricia Portal Date 9/30/87

SUMMARY

The Procter & Gamble Company's test article G0539.03 was tested for its potential to induce mutations at the thymidine kinase locus of L5178Y TK+/- Mouse Lymphoma cells. Two separate assays were conducted, one in the absence of exogenous activation and one in the presence of Aroclor-induced rat liver S-9. In addition, another parameter was examined under both test conditions. The L5178Y TK+/- cells were exposed to the test article G0539.03 in conditioned medium that was similar to the medium normally used for the L5178Y TK+/- Assay or, alternatively, in conditioned medium to which another material supplied by The Procter and Gamble Company (G2318.01) was added the day before it was used. The maximum doses tested with and without activation were specified in an amendment to the protocol. Therefore, a Range Finding Test was not performed for the selection of doses.

The results of the assays indicated the following:

1. Positive dose-dependent responses were produced both in the presence and absence of S-9, both with and without treatment in conditioned medium containing G2318.01.
2. The test article was less toxic when treatment was performed in conjunction with S-9.
3. No significant difference in the toxic response or mutant frequencies was evident between the cultures treated with or without G2318.01 in the presence of S-9.
4. The cultures treated in the absence of exogenous activation exhibited a difference in response between cultures treated in medium with G2318.01 from those treated in the absence of G2318.01. The difference was observed over a very narrow range (0.6 ug/ml to 0.18 ug/ml). Treatment in conjunction with G2318.01 over the specified narrow range resulted in a reduction of toxicity and mutant frequency as compared to the sister cultures treated in the absence of G2318.01.

INTRODUCTION

This study was conducted by Paul E. Kirby, Ph.D., Roger M. Brauningner, B.S., and Lee-Chin Law, B.S., from August 11, 1987, to August 31, 1987, at SITEK Research Laboratories. The experimental procedures used to perform this study were essentially those of Donald C. Clive (1), and they are described in detail in the protocol appended to this report.

The purpose of this study was to evaluate the test article G0539.03 for its potential to induce mutations at the thymidine kinase locus in L5178Y TK+/- Mouse Lymphoma cells when exposure was conducted either in normal medium or in medium containing another test material (G2318.01) provided by The Procter and Gamble Company. Two assays were conducted, one in the absence of exogenous activation and one with treatment in the presence of Aroclor-induced rat liver S-9.

MATERIALS AND METHODS

INDICATOR CELLS

Source

The L5178Y TK+/- Mouse Lymphoma cells, clone 3.7.2C were originally obtained from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina, on October 17, 1984. The cells were subcultured, cleansed of TK-/- cells, and cryopreserved in a large number of ampules for L5178Y TK+/- Assays. The cells used for this study were from lot no. 031087. A sample of these cells was reconstituted, tested for mycoplasma contamination, and found to be free of mycoplasma.

Culture Conditions

The L5178Y TK+/- Mouse Lymphoma cells were cultured in F₁₀P (Fischer's Medium for Leukemic Cells of Mice supplemented with Pluronic F68 and heat-inactivated horse serum (10%)) in 50 ml disposable centrifuge tubes at 37 ±1.0°C on a roller drum rotating at approximately 25 rpm. Each culture was gassed with 5% CO₂ and 95% air prior to placement on the roller drum. Each culture was sampled daily to determine cell concentrations, and if the cell concentrations were greater than 0.3x10⁶ cells/ml, the cultures were adjusted to 0.3x10⁶ cells/ml.

Stock Cultures

Stock cultures were grown in F₁₀P in T-75 plastic tissue culture flasks on a shaker rotating at approximately 125 rpm. The cultures were monitored and adjusted when necessary to maintain them in log phase growth. For the Assay, subcultures were cleansed of TK-/- cells by exposure to THMG (thymidine, hypoxanthine, methotrexate and glycine) for 24 hours and grown in THG (THMG less methotrexate) for an additional 24 hours.

CONTROL SUBSTANCES

Positive Controls

Ethyl methanesulfonate (EMS), which induces mutation at the TK locus without metabolic activation, was used at 1.0 and 0.5 ul/ml in the non-activated system. The source and lot number of the EMS used in this study are given below.

Source: Kodak

Lot No.: A11G

7,12-Dimethylbenz(a)anthracene (DMBA), which causes mutation at the HGPRT locus with metabolic activation, was used at 7.5 and 5.0 ug/ml in the activated system. The source and lot number of the DMBA used in this study are given below.

Source: Kodak

Lot No.: C13A

Solvent Controls

The test article dosing solutions were prepared by adding ethanol (Clear Spring Distilling Company, Lot No. 18-1100) to an aliquot of the test article, dissolving it, and then performing a serial dilution using ethanol as the diluent.

DMSO was used to dissolve the positive control EMS. The source and purity of the DMSO batch used in this study are given below:

Source: Fisher Scientific Co.

Lot No.: 864016

CAS Registry Number: 67-68-5

Certificate of Actual Lot Analysis:

Appearance (clear, colorless liquid)	P.T.
Density (grams/ml) at 25°C	1.095
Freezing point	18.0°C
Residue after evaporation	0.002%
Color (APHA)	10
Water	0.05%

The other positive control substance, DMBA, was dissolved in acetone to make the stock solutions. The source and purity of the acetone batch used in this study are given below:

Source: Fisher Scientific Co.

Lot No.: 735031

CAS Registry Number: 67-64-1

Certificate of Actual Lot Analysis:

Density (grams/ml) at 25°C	0.7857
Residue after evaporation	0.0002%
Color (APHA)	5
Water	0.5%

TEST ARTICLE

The test article G0539.03 was received on August 4, 1987, and stored at room temperature. The test article was diluted in ethanol to prepare the dosing solutions.

EXPERIMENTAL PROCEDURES

Determination of Solubility of Test Article

The Sponsor indicated that the test article was soluble in ethanol up to a 10% solution. Therefore, a solubility test was not performed.

Preparation of Test Cultures

Without Activation

The L5178Y TK+/- stock cultures were maintained in log phase growth until used in the Assay. Two pools of cells at 6×10^5 cells/ml were established in media that were prepared the previous day. One medium contained 30% conditioned F₁₀P and 70% fresh F₁₀P. The other medium was the same, except G2318.01 was added to obtain a final concentration of 1000 ug/ml. 10 ml of the cell preparation was dispensed to 50 ml disposable centrifuge tubes resulting in 6×10^6 cells/tube. Each tube was gassed with 5% CO₂ and 95% air, sealed, and placed on a shaker to keep the cells suspended until treated.

With S-9 Activation

The pools of cells were prepared as described above, except the final concentration of cells was 1×10^6 cells/ml and 6 ml of the cell suspensions were dispensed to the centrifuge tubes instead of 10 ml.

Preparation of Metabolic Activation System

The metabolic activation system consisted of Aroclor-induced rat liver homogenate (S-9 fraction) and the cofactor pool. The S-9 fraction was prepared in 0.25M sucrose from Aroclor 1254-induced male Sprague-Dawley rats.

Approximately 1 gram of rat liver was used to make 3 ml of buffered S-9 fraction. Immediately prior to treatment, the S-9 fraction was mixed with the cofactor pool to obtain the S-9 cofactor mixture which was kept in wet ice until used. The S-9 cofactor mixture was prepared in the following proportion per ml of S-9 mix.

6.0 mg	NADP
11.25 mg	DL-Isocitric Acid
0.25 ml	S-9 Homogenate
0.75 ml	F ₁₀ P

The S-9 homogenate was added after the other components had been combined, mixed, the pH adjusted to approximately 6.8, and filter sterilized through a 0.45 uM filter.

Test System Identification

All test cultures were labeled with an indelible pen with a code system which clearly identified the experiment number, activation system, test article, controls and concentrations.

Preparation of Test Article Dosing Solutions

The test article dosing solutions were prepared by dissolving the test article in ethanol and then performing a serial dilution. All test article and control treatments were done under UV-filtered lights to avoid possible problems of photoinactivation.

The stability of the test article under the experimental conditions was not determined by SITEK Research Laboratories.

Range Finding Test

No Range Finding Test was conducted since The Procter and Gamble Company specified the maximum doses to use in performing the Mutation Assay.

Mutation Assay

Treatment Without Activation

The test article was dissolved in ethanol, and a serial dilution was performed as previously described. Cultures containing 10 ml of cells at a concentration of 6×10^5 cells/ml were prepared. These cultures contained 30% conditioned F_{1,0}P (supernatant from the stock cultures) and 70% fresh F₀P or 30% conditioned F_{1,0}P, 70% fresh F₀P and G2318.01 at 1000 ug/ml. Each culture was treated with the appropriate concentration of the test article, solvent or positive control chemical. Immediately after each treatment, the culture was gassed with approximately 5% CO₂ and 95% air. After all of the cultures had been treated, they were placed on a roller drum apparatus and rotated at a speed of 25 \pm 2 rpm in an environment of 37°C. Two solvent control cultures were included in each treatment group. In addition, two sets of cultures were treated with a positive control chemical. As indicated earlier, EMS was the positive control chemical for the non-activated portion of the assay.

Treatment With S-9 Activation

The test cultures were prepared as described in the previous paragraph, except the cultures contained 6 ml of cell suspension at a concentration of 1×10^6 cells/ml. The cultures were prepared in the same media described previously (treatment without activation). The test article was dissolved in ethanol, and a serial dilution was performed. Just prior to the addition of test article, solvent or positive control, 4.0 ml of S-9 mix was added to each

culture. The final concentration of cells in culture was 6×10^5 cells/ml. Immediately after each treatment, the culture was gassed with approximately 5% CO₂ and 95% air. After all of the cultures had been treated, they were placed on a roller drum apparatus and rotated at a speed of 25 \pm 2 rpm in an environment of 37°C. Two solvent control cultures were included in each treatment group. In addition, two sets of cultures were treated with a positive control chemical. As indicated earlier DMBA was the positive control chemical for the S-9 activated portion of the assay.

Expression Period

After a 4-hour exposure period, the cells were pelleted by centrifugation at approximately 1000 rpm for 10 minutes, and the test article was removed by pouring off the supernatant. Two rinses in 10 ml of F₁₀P were performed, followed by resuspension in 20 ml F₁₀P, gassing with approximately 5% CO₂ and 95% air, and incubation at 37°C on a roller drum apparatus set at 25 \pm 2 rpm. Approximately 20 hours and 44 hours post treatment, 1 ml samples were removed from each culture to determine the cell population density of each. The 1 ml sample was placed in a vial containing 19 ml of 0.1% trypsin. The vials were incubated for 10 minutes at 37°C, after which they were placed on an automatic cell counter. Three counts were made, and the average count, corrected for coincidence, was used to determine the concentration of cells per ml for each culture. After the determination of cell numbers, each culture having a population greater than 0.3×10^6 cells/ml was adjusted to 0.3×10^6 cells/ml. At the 20-hour point the final volume after adjustment was 20 ml. For the 44-hour point the final volume was 10 ml.

Cloning for Mutants and Viability

After the 2-day expression period, cultures were selected for cloning based on their SG. Only cultures having a Relative Suspension Growth of 8% or greater were cloned.

For each culture selected for cloning, 200 ml of cloning medium (CM) was prepared. The CM was made by combining the following ingredients in the indicated proportions for each 100 ml of CM.

F ₁₀ P	70.25 ml
Horse Serum	20.0 ml
Sodium Pyruvate (Stock)	1.0 ml
Purified Agar (4% Solution)	8.75 ml

For each culture selected for cloning, 100 ml of CM was dispensed into a flask designated for the addition of the restrictive agent trifluorothymidine (TFT) and therefore the growth of TK-/- cells only, and 100 ml was dispensed into a

flask designated as a Viable Count (VC) flask. The CM in the VC flask was used to culture an aliquot of cells from each culture cloned to approximate the percentage of viable cells in each culture.

The cloning process was as follows:

1. Each TFT and VC flask received 100 ml of CM and each was placed in a shaker incubator set at approximately 125 rpm and 37°C.

2. The cultures designated for cloning were centrifuged at a speed of approximately 1000 rpm for 10 minutes. 8 ml of the supernatant was aspirated and discarded. The cells were resuspended in the remaining volume of supernatant and then added to the appropriate TFT flask. Each TFT flask contained 3×10^6 cells. Each flask was replaced on the shaker incubator (125 rpm, 37°C).

3. After each TFT flask had shaken for at least 15 minutes, a 1 ml aliquot was removed from each, a 1:10 and 1:5 serial dilution was performed, and a 1 ml aliquot of the last dilution was added to the appropriate VC flask. Each VC flask contained approximately 600 cells.

4. After the completion of cell addition to each VC flask, an aliquot of TFT was added to each TFT flask. The concentration of TFT in the culture was approximately 3 ug/ml. Both TFT and VC flasks were replaced on the shaker incubator (125 \pm 2 rpm, 37°C).

5. After at least 15 minutes of mixing, the contents of each flask were dispensed in equal aliquots into three plates. The plates were chilled for 20 minutes in a 4°C environment and then placed in an incubator at 37°C in an atmosphere of approximately 5% CO₂ and 95% air for 10-12 days.

Enumeration of Colonies

After completion of the incubation period, the number of colonies per TFT and VC plate was determined. The colony numbers were determined by counting them with an automatic colony counter. The raw counts were increased by a correction factor determined for SITEK's colony counter (#000027). The correction factor is:

$$\text{Corrected Counts} = \frac{(\text{Raw Count} - 0.6499)}{0.8423}$$

Determination of Mutant Frequency and Induced Mutant Frequency

The Mutant Frequency (MF) of each culture that was successfully cloned was determined as a function of viable cells forming colonies. The calculation was performed as follows:

$$\text{MF Per } 10^6 \text{ Viable Cells} = \frac{\text{Average No. Mutants Per Plate}}{\text{Average No. of Colonies in the Corresponding VC Plates}} \times 200$$

The Induced Mutant Frequency (IMF) was calculated by using the following formula:

$$\text{IMF} = \text{MF of Treated Cultures} - \text{Average MF of Solvent Control Cultures}$$

Determination of Relative Suspension Growth, Relative Cloning Efficiency and Total Growth

Relative Suspension Growth

The Suspension Growth (SG) of each culture was determined using the following formula:

$$\text{SG} = \frac{\text{Day 1 Conc.}}{0.3 \times 10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Conc.}}{\text{Day 1 Adjusted Cell Conc.}}$$

The Relative Suspension Growth (RSG) of each of the test article-treated cultures was determined by calculating its growth relative to the corresponding solvent control cultures' average SG.

$$\text{RSG} = \frac{\text{SG of Treated Culture}}{\text{Average SG of Solvent Controls}} \times 100$$

Relative Cloning Efficiency

The Relative Cloning Efficiency (RCE) was determined for each culture by using the following formula:

$$\text{RCE} = \frac{\text{Average VC Count of Treated Culture}}{\text{Average VC Count of Solvent Controls}} \times 100$$

Total Growth

The Total Growth (TG) of a culture was calculated as follows:

$$TG = \frac{RSG \times RCE}{100}$$

The TG was calculated for each test article-treated culture that was successfully cloned.

Criteria For a Valid Assay

The following criteria were used in evaluating the acceptability of the assay.

Solvent Control Cultures

1. The average Cloning Efficiency of the solvent control cultures should be 50% or higher.
2. The average MF of the solvent control cultures should be less than 150 per 10⁶ viable cells.

Positive Controls

The results for the positive control cultures should be considered acceptable if:

1. The treated cultures have MF's that are three times or greater than the average of their solvent control cultures.
2. Their solvent controls have an average Cloning Efficiency of 50% or greater.

Evaluation of Test Results

The following criteria were used as guidelines in evaluating the results of the assay for a negative, positive or equivocal response. Since it is impossible to write criteria that would apply to every configuration of data generated by the assay, the Study Director is responsible for the ultimate decision in the evaluation of the results.

Criteria for a Negative Response

1. All of the cultures exhibiting TG of 10% and greater have MF's that are less than twice that of the mean MF of the corresponding solvent control cultures, and
2. There is no evidence of a dose-dependent response.

Criteria for a Positive Response

A response is considered positive if at least one culture has an MF that is two times or more greater than the average MF of the corresponding solvent control cultures and the response is dose dependent.

Criteria for an Equivocal Response

A response is considered equivocal if it does not fulfill the criteria of either a negative or a positive response, and/or the Study Director does not consider the response to be either positive or negative.

ARCHIVES

All raw data, information pertinent to this study and study report(s) will be maintained in SITEK Research Laboratories archives.

RESULTS

SOLUBILITY TEST

The Sponsor indicated that the test article was soluble in ethanol up to a 10% solution. Therefore, a solubility test was not conducted by SITEK Research Laboratories.

RANGE FINDING TEST

A Range Finding Test was not conducted since the Sponsor provided the maximum test article concentrations that should be used in the assays.

MUTATION ASSAY

Without Activation

The results of the assay conducted without exogenous activation are presented in Tables 1-3 (Cloning Data) and Tables 4 and 5 (Total Growth Data). These data are also presented graphically in Figure 1. Several cultures treated either with or without the presence of G2318.01 exhibited mutant frequencies that were greater than twofold when compared to the mutant frequencies observed in the concurrent solvent control cultures. However, it should be noted that only cultures treated with highly toxic doses had mutant frequencies that were greater than two times the background.

In regard to the effect of treatment in the presence or absence of G2318.01, there appears to be a significant reduction in mutant frequency and toxicity in cultures treated in the presence of G2318.01 at test article concentrations of 0.6, 0.2 and 0.18 ug/ml. At test article concentrations of 1.0 ug/ml and above, the presence of G2318.01 does not appear to affect the mutant frequency or toxic response.

The positive control EMS produced the expected positive response. The response was not affected by the presence of G2318.01.

With S-9 Activation

The results of the assay conducted in the presence of S-9 activation are presented in the Tables 6-8 (Cloning Data) and Tables 9 and 10 (Total Growth Data). The data are also presented graphically in Figure 2.

Several cultures treated in the presence or absence of G2318.01 exhibited mutant frequencies that were greater than twofold that of the solvent control cultures. However, it should be noted that the RTG of these cultures was 11% or less.

The presence of G2318.01 did not appear to affect the response in mutant frequency or toxicity. The response of cultures treated in the presence of G2318.01 was very similar to the response of cultures treated in the absence of G2318.01.

TABLE 1

LS178Y TK+/- ASSAY - CLONING DATA

Non-Activated Without G2318.01

STUDY DIRECTOR: Paul E. Kirby, Ph.D.

STUDY NUMBER: 0066-2400

EXPERIMENT NO: B-1

TEST DOSES IN: μ g/ml

TEST ARTICLE I.D.: SC-0066

SOLVENT: ETHANOL

TEST ARTICLE CONCENTRATION	COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM)			AVERAGE COLONIES/RM PLATE	COLONIES PER VIABLE COUNT (VC) PLATE			AVERAGE COLONIES/VC PLATE	CLONING EFFICIENCY	MUTANT FREQUENCY (MF)/10 ⁶	INDUCED MF/10 ⁶	RELATIVE TOTAL GROWTH
	1	2	3		1	2	3					
3.0	-2	-2	-2	NA	-2	-2	-2	NA	NA	NA	NA	NA
2.6	-2	-2	-2	NA	-2	-2	-2	NA	NA	NA	NA	NA
2.2	73	57	56	62	86	75	-1	81	40%	153	89	3%
1.8	78	81	82	80	116	119	117	117	59%	137	73	6%
1.4	113	106	116	112	133	116	132	127	64%	176	112	8%
1.0	110	116	122	116	150	131	135	139	69%	167	103	10%
0.60	113	117	129	120	149	163	160	157	79%	153	89	12%
0.20	98	73	87	86	154	161	165	160	80%	108	44	60%
0.18	67	78	82	76	174	168	165	169	85%	90	26	67%
0.16	89	79	80	83	263	272	246	260	130%	64	0	122%
SOLVENT 1	80	75	68	74	266	259	252	259	130%	57	AVE. SOL- VENT MF	
SOLVENT 2	78	56	75	70	189	202	209	200	100%	70	64	

-1-CULTURE LOST
-2-NOT CLONED

TABLE PREPARED BY Ray Brannings DATE: 9/28/87

TABLE 2

L5178Y TK+/- ASSAY - CLONING DATA

Non-Activated With G2318.01

STUDY DIRECTOR: Paul E. Kirby, Ph.D.

STUDY NUMBER: 0066-2400

EXPERIMENT NO: B-1

TEST DOSES IN: ug/ml

TEST ARTICLE I.D.: SC-0066

SOLVENT: ETHANOL

TEST ARTICLE CONCENTRATION	COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM)			AVERAGE COLONIES/RM PLATE	COLONIES PER VIABLE COUNT (VC) PLATE			AVERAGE COLONIES/VC PLATE	CLONING EFFICIENCY	MUTANT FREQUENCY (MF)/10 ⁶	INDUCED MF/10 ⁶	RELATIVE TOTAL GROWTH
	1	2	3		1	2	3					
3.0	-2	-2	-2	NA	-2	-2	-2	NA	NA	NA	NA	NA
2.6	68	63	62	64	108	85	106	100	50%	128	77	5%
2.2	61	63	72	65	108	122	120	117	58%	111	60	7%
1.8	86	73	75	78	116	117	118	117	59%	133	82	7%
1.4	86	81	79	82	117	105	89	104	52%	158	107	7%
1.0	135	117	110	121	152	155	158	155	78%	156	105	15%
0.60	87	66	61	71	258	277	268	268	134%	53	2	94%
0.20	62	78	62	67	259	258	272	263	132%	51	0	113%
0.18	80	72	67	73	265	253	282	267	133%	55	4	107%

	AVE. SOLVENT MF											
SOLVENT 1	67	55	56	59	222	277	245	248	124%	48	48	48
SOLVENT 2	66	73	51	63	247	230	228	235	118%	54	54	51

-1=CULTURE LOST

-2=NOT CLONED

TABLE PREPARED BY:

Ray Brannigan

DATE:

7/28/87

TABLE 3

LS178Y TK+/- ASSAY - CLONING DATA

POSITIVE CONTROL With And Without 62318.01

STUDY DIRECTOR: Paul E. Kirby, Ph.D.

STUDY NUMBER: 0066-2400

EXPERIMENT NO: B-1

EMS DOSES IN: ul/ml

TEST ARTICLE I.D.: SC-0066

SOLVENT: EMS=DMSO

TEST ARTICLE CONCENTRATION	COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM)			AVERAGE COLONIES/RM PLATE	COLONIES PER VIABLE COUNT (VC) PLATE			AVERAGE COLONIES/VC PLATE	CLONING EFFICIENCY	MUTANT FREQUENCY (MF)/10*	INDUCED MF/10*	RELATIVE TOTAL GROWTH
	1	2	3		1	2	3					
EMS Without 62318.01												
1.0	68	67	70	71	19	13	13	15	8%	947	892	1%
0.5	349	368	335	351	123	119	131	124	62%	566	511	33%
SOLVENT 1	60	56	50	55	254	224	221	233	117%	47	AVE. SOL- VENT MF	
SOLVENT 2	81	68	75	75	237	232	244	238	119%	63	55	
=====												
EMS With 62318.01												
1.0	198	218	182	199	30	28	30	29	15%	1329	1273	2%
0.5	352	361	342	352	136	141	-1	139	69%	510	454	31%
SOLVENT 1	62	79	79	73	235	249	263	249	125%	59	AVE. SOL- VENT MF	
SOLVENT 2	59	61	79	66	262	252	235	250	125%	53	56	

EMS=Ethyl methanesulfonate

-1=CULTURE LOST

-2=NOT CLONED

TABLE PREPARED BY: *Page Brauning* DATE: *7/28/82*

TABLE 4

LS178Y TK +/- ASSAY

TOTAL GROWTH DATA

STUDY DIRECTOR: Paul E. Kirby, Ph.D. STUDY NUMBER: 0066-2400 EXPERIMENT NO: B-1

TEST DOSES IN: ug/ml

TEST ARTICLE I.D.: SC-0066

SOLVENT: ETHANOL

CONCENTRATION	CELL CONCENTRATION DAY 1	CELL CONCENTRATION DAY 2	SUSPENSION GROWTH	RELATIVE SUSPENSION GROWTH	AVERAGE COLONIES/ VC PLATE	RELATIVE CLONING EFFICIENCY	RELATIVE TOTAL GROWTH
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Non-Activated Without G2318.01

3.0	0.236	0.174	0.0	0%	NA	NA	NA
2.6	0.282	0.277	0.0	0%	NA	NA	NA
2.2	0.377	0.336	1.4	8%	81	35%	3%
1.8	0.439	0.427	2.1	12%	117	51%	6%
1.4	0.461	0.487	2.5	14%	127	55%	8%
1.0	0.461	0.545	2.8	16%	139	60%	10%
0.60	0.477	0.584	3.1	18%	157	68%	12%
0.20	1.016	1.329	15.0	85%	160	70%	60%
0.18	1.042	1.404	16.3	92%	169	73%	67%
0.16	1.166	1.471	19.1	108%	260	113%	122%
0.14	1.124	1.398	17.5	99%			
0.12	1.134	1.439	18.1	102%			

SOLVENT 1	1.130	1.345	16.9	SOLVENT AVERAGE 17.7	259	AVE. NO. COLONIES 230	
SOLVENT 2	1.081	1.536	18.5		200		

Non-Activated With G2318.01

3.0	0.302	0.290	1.0	6%	NA	NA	NA
2.6	0.434	0.423	2.0	11%	100	41%	5%
2.2	0.441	0.510	2.5	14%	117	48%	7%
1.8	0.430	0.561	2.7	15%	117	48%	7%
1.4	0.463	0.605	3.1	17%	104	43%	7%
1.0	0.555	0.683	4.2	24%	155	64%	15%
0.60	1.068	1.279	15.2	85%	268	111%	94%
0.20	1.091	1.536	18.6	104%	263	109%	113%
0.18	1.083	1.446	17.4	97%	267	110%	107%
0.16	1.125	1.445	18.1	101%			
0.14	1.128	1.422	17.8	100%			
0.12	1.159	1.399	18.0	101%			

SOLVENT 1	1.063	1.475	17.4	SOLVENT AVERAGE 17.9	248	AVE. NO. COLONIES 242	
SOLVENT 2	1.149	1.446	18.5		235		

 -1=CULTURE LOST
 -2=NOT CLONED

TABLE PREPARED BY:

Regina Brumby
 (Signature)

9/20/87
 (Date)

TABLE 5

L5178Y TK +/- ASSAY

TOTAL GROWTH DATA

STUDY DIRECTOR: Paul E. Kirby, Ph.D. STUDY NUMBER: 0066-2400 EXPERIMENT NO: B-1

EMS DOSES IN: ul/ml

TEST ARTICLE I.D.: SC-0066

SOLVENT: EMS=DMSO

CONCENTRATION	CELL CONCENTRATION		SUSPENSION GROWTH	RELATIVE SUSPENSION GROWTH	AVERAGE COLONIES/VC PLATE	RELATIVE CLONING EFFICIENCY	RELATIVE TOTAL GROWTH
	DAY 1	DAY 2					

EMS Without	62318.01						
1.0	0.474	0.476	2.5	15%	15	6%	1%
0.5	1.025	0.953	10.9	63%	124	53%	33%

SOLVENT	DAY 1	DAY 2	SUSPENSION GROWTH	RELATIVE SUSPENSION GROWTH	AVERAGE COLONIES/VC PLATE	RELATIVE CLONING EFFICIENCY	RELATIVE TOTAL GROWTH
SOLVENT 1	1.077	1.395	16.7	17.3	233	235	
SOLVENT 2	1.084	1.488	17.9		238		

=====							
EMS With	62318.01						
1.0	0.572	0.589	3.7	20%	29	12%	2%
0.5	0.830	1.148	10.6	55%	139	56%	31%

SOLVENT	DAY 1	DAY 2	SUSPENSION GROWTH	RELATIVE SUSPENSION GROWTH	AVERAGE COLONIES/VC PLATE	RELATIVE CLONING EFFICIENCY	RELATIVE TOTAL GROWTH
SOLVENT 1	1.084	1.519	18.3	19.1	249	249	
SOLVENT 2	1.144	1.569	19.9		250		

=====

EMS=Ethyl methanesulfonate

-1=CULTURE LOST
-2=NOT CLONED

TABLE PREPARED BY: Ray Brauning 9/28/87
(Signature) (Date)

FIGURE 1

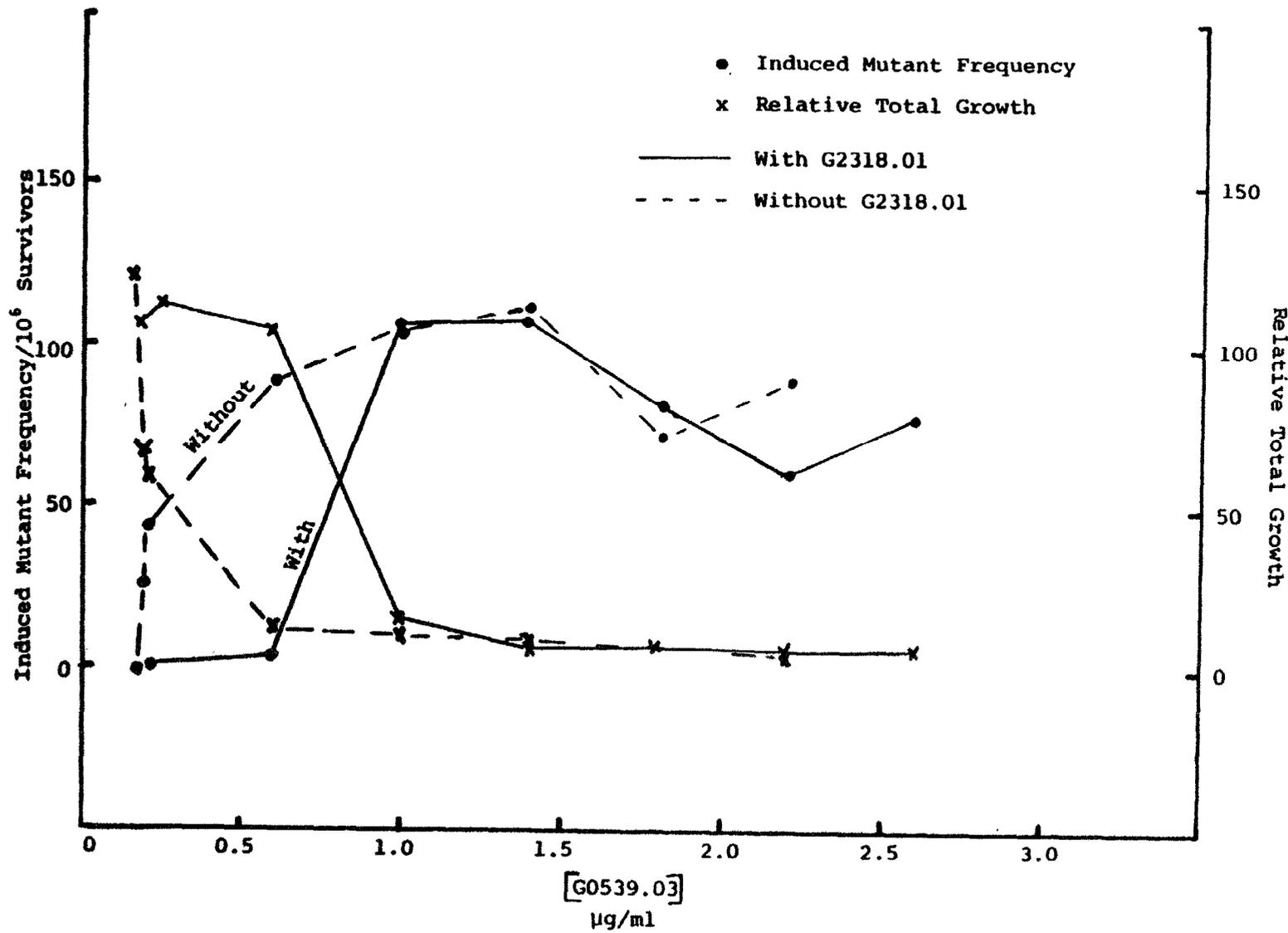


TABLE 6

LS178Y TK+/- ASSAY - CLONING DATA

WITH ACTIVATION WITHOUT G231B.01

STUDY DIRECTOR: Paul E. Kirby, Ph.D.

STUDY NUMBER: 0066-2400

EXPERIMENT NO: B-2

TEST DOSES IN: μ g/ml

TEST ARTICLE I.D.: SC-0066

SOLVENT: ETHANOL

TEST ARTICLE CONCENTRATION	COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM)			AVERAGE COLONIES/ RM PLATE	COLONIES PER VIABLE COUNT (VC) PLATE			AVERAGE COLONIES/ VC PLATE	CLONING EFFI- CIENCY	MUTANT FREQUENCY (MF)/10 ⁶	INDUCED MF/ 10 ⁶	RELATIVE TOTAL GROWTH
	1	2	3		1	2	3					
12 A	-2	-2	-2	NA	-2	-2	-2	NA	NA	NA	NA	NA
12 B	-2	-2	-2	NA	-2	-2	-2	NA	NA	NA	NA	NA
9.0 A	91	113	73	92	167	171	184	174	87%	106	47	7%
9.0 B	98	93	87	93	156	181	152	163	82%	114	55	6%
6.0 A	135	111	130	125	174	161	151	162	81%	154	95	11%
6.0 B	106	89	107	101	164	167	157	163	81%	124	65	9%
3.0 A	92	91	-1	92	182	168	161	170	85%	108	49	18%
3.0 B	93	78	75	82	174	182	154	170	85%	96	37	18%
1.0 A	70	72	75	72	260	227	246	244	122%	59	0	86%
1.0 B	84	76	62	74	230	247	241	239	120%	62	3	91%
0.5 A	87	80	-1	84	250	247	228	242	121%	69	10	89%
0.5 B	74	88	-1	81	273	243	260	259	129%	63	4	99%
SOLVENT 1	80	82	81	81	268	259	273	267	133%	61	AVE. SOL- VENT MF	
SOLVENT 2	75	73	68	72	263	246	241	250	125%	58	59	

-1=CULTURE LOST
-2=NOT CLONED

TABLE PREPARED BY:

Regina Brumby

DATE:

9/29/87

TABLE 7

L5178Y TK+/- ASSAY - CLONING DATA

WITH 89 ACTIVATION WITH 62318.01

STUDY DIRECTOR: Paul E. Kirby, Ph.D.

STUDY NUMBER: 0066-2400

EXPERIMENT NO: B-2

TEST DOSES IN: $\mu\text{g/ml}$

TEST ARTICLE I.D.: SC-0066

SOLVENT: ETHANOL

TEST ARTICLE CONCENTRATION	COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM)			AVERAGE COLONIES/RM PLATE	COLONIES PER VIABLE COUNT (VC) PLATE			AVERAGE COLONIES/VC PLATE	CLONING EFFICIENCY	MUTANT FREQUENCY (MF)/10 ⁴	INDUCED MF/10 ⁴	RELATIVE TOTAL GROWTH
	1	2	3		1	2	3					
12 A	-2	-2	-2	NA	-2	-2	-2	NA	NA	NA	NA	NA
12 B	-2	-2	-2	NA	-2	-2	-2	NA	NA	NA	NA	NA
9.0 A	98	82	79	86	113	142	110	122	61%	141	79	5%
9.0 B	87	94	88	90	120	144	-1	132	66%	136	74	4%
6.0 A	129	114	-1	122	179	137	145	154	77%	158	96	9%
6.0 B	123	116	110	116	174	165	164	168	84%	138	76	11%
3.0 A	91	94	85	90	181	190	176	182	91%	99	37	16%
3.0 B	87	74	80	80	161	152	146	153	77%	105	43	15%
1.0 A	87	66	95	83	208	212	228	216	108%	77	15	70%
1.0 B	76	95	-1	86	243	231	216	230	115%	75	13	77%
0.5 A	75	82	89	82	264	264	272	267	133%	61	-1	92%
0.5 B	78	75	69	74	263	253	235	250	125%	59	-3	90%
SOLVENT 1	69	78	80	76	241	269	273	261	131%	58		
SOLVENT 2	87	92	84	88	260	265	273	266	133%	66	62	

AVE. SOL-

VENT MF

-1=CULTURE LOST

-2=NOT CLONED

TABLE PREPARED BY:

Ray Brannigan

DATE:

9/28/80

TABLE 8

L5178Y TK+/- ASSAY - CLONING DATA

POSITIVE CONTROLS WITH AND WITHOUT G2318.01

STUDY DIRECTOR: Paul E. Kirby, Ph.D.

STUDY NUMBER: 0066-2400

EXPERIMENT NO: B-2

TEST ARTICLE I.D.: SC-0066

SOLVENT: DMBA=ACETONE

DMBA DOSES IN µg/ml

TEST ARTICLE CONCENTRATION	COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM)			AVERAGE COLONIES/RM PLATE	COLONIES PER VIABLE COUNT (VC) PLATE			AVERAGE COLONIES/VC PLATE	CLONING EFFICIENCY	MUTANT FREQUENCY (MF)/10 ⁴	INDUCED MF/10 ⁴	RELATIVE TOTAL GROWTH
	1	2	3		1	2	3					
DMBA WITHOUT G2318.01												
7.5	211	201	206	206	165	177	162	168	84%	245	182	28%
5.0	182	163	175	173	202	183	177	187	94%	185	122	56%
											AVE. SOLVENT MF	
SOLVENT 1	92	93	70	85	263	239	247	250	125%	68		
SOLVENT 2	80	74	69	74	266	246	259	257	129%	58	63	
=====												
DMBA WITH G2318.01												
7.5	228	209	199	212	176	141	171	163	81%	262	199	17%
5.0	209	196	201	202	227	208	227	221	110%	184	121	54%
											AVE. SOLVENT MF	
SOLVENT 1	94	89	89	91	273	249	256	259	130%	70		
SOLVENT 2	84	70	68	74	264	240	283	262	131%	56	63	

DMBA=7,12-dimethylbenz(a)anthracene

-1=CULTURE LOST

-2=NOT CLONED

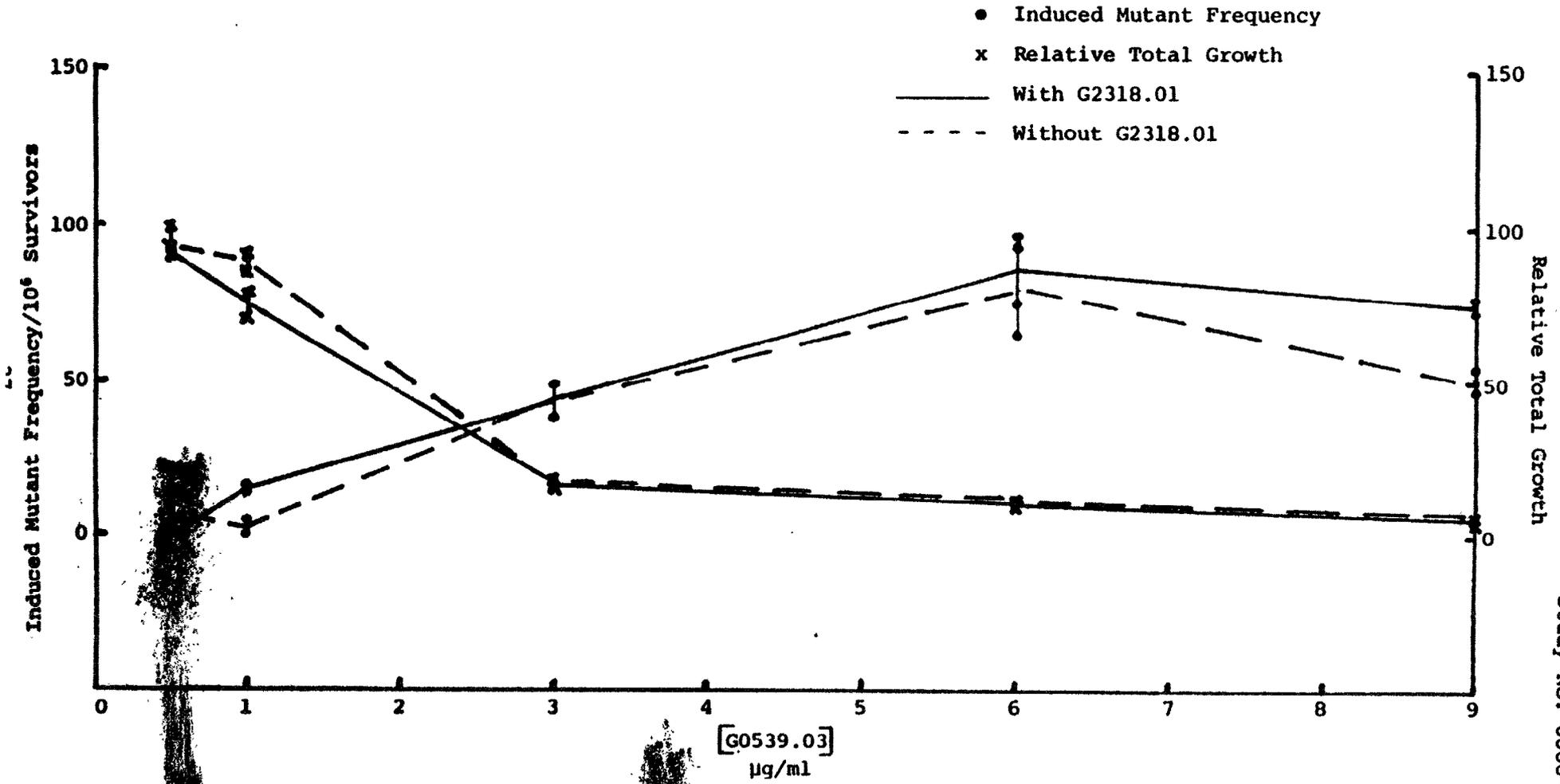
TABLE PREPARED BY:

Regis Brauning

DATE:

7/28/87

FIGURE 2



Study No. 0066-2400

CONCLUSIONS

The Procter & Gamble Company's test article G0539.03 was tested in the L5178Y TK+/- Mouse Lymphoma Mutation Assay both in the presence and absence of G2318.01. Assays were conducted without exogenous activation and with Aroclor-induced rat liver S-9.

The results of the assays performed with this test article indicate that, under the test conditions, the test article did cause significant increases in the mutant frequency of cultures treated at highly toxic doses as compared to the corresponding solvent controls' mutant frequencies. The presence of G2318.01 with S-9 activation did not have any detectable effect on the mutant frequency or toxic response. However, cultures treated in the absence of exogenous activation and in the presence of G2318.01 had a reduced response at specific doses as compared to cultures treated in the absence of G2318.01.

REFERENCES

1. Clive, D. and J. Spector. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mut. Res. 31:17-29, 1975.

APPENDIX I
PROTOCOL

PROTOCOL NO. C29

Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺/⁻ Mouse Lymphoma Assay

Issue Date: August 29, 1985
Supersedes Issue Dated: March 1, 1982

Test Substance Identification Number (TSIN) # G 0539.03

Divisional Request Document Number (DRD) # BY0708

Sponsor: The Procter & Gamble Company
Cincinnati, Ohio

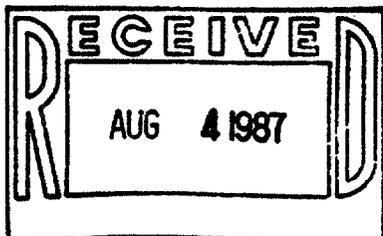
Testing Facility: SITEK RESEARCH LABORATORIES
(To be filled in by Operations Section) ROCKVILLE, MARYLAND 20852
Study # 0066-2400
(To be filled in by Testing Facility)

Purpose: To determine the potential of a chemical compound to induce mutations at the thymidine kinase (TK) locus of cultured L5178Y TK⁺/⁻ mouse lymphoma cells. 1, 2, 3

Justification for Selection of Test System: The L5178Y/TK⁺/⁻ mouse lymphoma cells clone 3.7.2C is the system of choice due to the amount of background data available.

Route of Administration of Test Substance and Reason for Choice: IN VITRO with and without metabolic activation.
Route specified by test procedure.

Records to be Maintained: All records that would be required to reconstruct the study and demonstrate adherence to the protocol.



Approved by
QAU PP
8/10/87

PROTOCOL NO. C29 (Cont'd)Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay

Issue Date: August 29, 1985

<u>Test Substance(s)</u>		<u>Description</u>		<u>Expiration</u>
<u>TSIN #</u>	<u>DRD Number</u>	<u>Color</u>	<u>Physical Form</u>	<u>Date</u>
G 0539.03	B4 0708	off-white	powder	11/87

Storage Conditions: (Check one)

Room temperature Refrigerator Freezer
 Other

Hazards: (Check one)

None known. Take ordinary precautions in handling.
 As follows: Irritant

Special Instructions: (Check one)

None
 As follows: Avoid undue skin and eye exposure. Flush with water. - See Attached protocol amendment for special medium preparation. EWT 7/25/87

Dose Preparation:

Vehicles in order of preference

F₀P₈
 DMSO
 EtOH
 Acetone
 Other

Solubility > 10% w ethanol

Unless the solubility properties of the test substance are provided by the Sponsor or the solubility properties are available from another source, a suitable solvent must be found for the test substance prior to testing using the Standard Operating Procedures of the Test Facility.

*See Appendix 1 for abbreviations and glossary of terms.

PROTOCOL NO. C29 (Cont'd)Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay

Issue Date: August 29, 1985

Dose Preparation
(Cont'd):

When possible dissolve the test substance in F₀P. A 100X concentrated solution is preferable, but if the material is not soluble at that concentration, less concentrated solutions may be prepared. If the pH of the F₀P changes, adjust to neutrality before proceeding. Do not test a suspension unless agreed to by the Sponsor. Up to 3.0 ml of the F₀P solution may be added to the final dosing solution. Since 6×10^6 cells must be present in the final dosing solution, the cells will have to be concentrated such that the test substance solution (in F₀P) and the cells make a total volume of 6.0 ml e.g. If 3.0 ml of the concentrated test substance (in F₀P) were added to the final dosing solution, then 3.0 ml of cells with 2×10^6 cells/ml would also be added. The remaining 4.0 ml will either be S-9 mix or F₀P. Final volume of the dosing solution is 10 ml. The order of addition should be test substance, S-9 mix or F₀P, then cells.

If the test substance is not water soluble, then 100X concentrated solutions should be prepared in a suitable solvent. Up to 100 μ l of these solvents may be added to the final dosing solutions. The order of addition should be the same as above. If the S-9 mix becomes acidic, discard the tube and adjust the pH of the test substance solution. The preferred solvents, in order of preference, are dimethyl-sulfoxide, ethanol, and acetone. Any other solvent which shows no toxic effect to the L5178Y cells and no significant increase in background mutation frequency at the levels used is acceptable subject to approval by the Sponsor.

Chemicals:

Positive controls and other chemicals to be used for testing will be purchased from a commercial source or obtained from the Sponsor. Chemicals are stored according to the recommendations of the commercial supplier or Sponsor. After completion of the assay, unused commercially obtained chemicals may be saved for future use. Excess chemicals obtained from a Sponsor, however, will be either returned or discarded at the discretion of the Sponsor.

Dosage Level:

All solutions of the test substance are prepared on the day of the test. Doses are chosen on the basis of the toxicity test described in the Toxicity Test Section. A complete mutagenicity assay consists of at least: 1) five cloned doses of the test substance (see

PROTOCOL NO. C29 (Cont'd)Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK^{+/+} Mouse Lymphoma Assay

Issue Date: August 29, 1985

Dosage Level (Cont'd): mutagenicity test section for criteria used for selection of doses to be cloned) 2) a solvent control, (both 1 & 2 are tested with and without activation) 3) a positive control of ethyl methanesulfonate (EMS), (a mutagen that does not require activation) and 4) a positive control either of 2-acetylaminofluorene (2-AAF), 7,12 dimethyl benzanthracene (DMBA), benzo(a)pyrene (Bap), or Dimethyl nitrosamine (DMN) (mutagens that require metabolic activation by an S-9 fraction obtained from the livers of rodents induced with a chemical such as Aroclor). In some special cases the S-9 fraction used will be obtained from the livers of uninduced rodents. In these cases, the positive control used will be dimethylnitrosamine (DMN).

[] Other, specify:

Note

A concentration analysis of the test substance - vehicle mixture(s) will []; will not be required.

If a concentration analysis is required:

[] Prepare a sufficient quantity of the most concentrated test substance - vehicle mixture(s) so that a portion can be returned to the Sponsor's Divisional Toxicologist.

Shipping Instructions

Send approximately _____ ml. Send [] frozen; [] under ambient conditions; [] other _____

[] Analyze the test substance - vehicle mixture(s) for test substance concentration using the analytical method in Appendix _____.

Test System
Identification:

Individual cultures and cloning plates are to be identified according to the Standard Operating Procedures of the Test Facility.

Test System:

L5178Y/TK^{+/+}, clone 3.7.2C mouse lymphoma cells were obtained from D. Clive, Research Triangle Park, N.C., Burroughs-Wellcome Co.

Test System Storage:

Frozen stocks of the L5178Y clone 3.7.2C cells are prepared and maintained in a liquid nitrogen freezer according to the Standard Operating Procedures of the Test Facility.

PROTOCOL NO. G29 (Cont'd)Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺/⁻ Mouse Lymphoma Assay

Issue Date: August 29, 1985

Methods:Cell Line

The TK⁺/⁻ clone 3.7.2C L5178Y cell line is maintained as growing suspension cultures according to the Standard Operating Procedures of the Test Facility. The medium used is Fischer's Medium for Leukemic Cells of Mice containing approximately 10% (v/v) horse serum and supplemented according to the Standard Operating Procedures of the Test Facility. Medium may be obtained from a suitable commercial supplier as a powder, or 1X or 10X liquids.

Cells are periodically cleansed free of spontaneous TK⁻/⁻ mutants by treatment of stock cultures with THMG according to the Standard Operating Procedures of the Test Facility. Cultures used for the assay are cleansed within the two week period prior to initiation of the study.

Preparation of the Microsomal Enzyme (S-9)
Metabolic Activation SystemNon-Induced S-9 Fraction

A liver microsomal enzyme (S-9) activation system is employed in this assay to detect promutagens.^{2,4} S-9 is prepared by the homogenization of minced livers from commercially obtained male, Sprague-Dawley rats (200-~~grams~~). S-9 may be purchased or prepared according to the Standard Operating Procedures of the Test Facility.

Aliquots of the S-9 are stored frozen below -70°C until used.

Induced S-9 Fraction

Induced S-9 fraction is prepared from rats given a single intraperitoneal injection of a polychlorinated biphenyl (Aroclor) in corn oil five days prior to sacrifice. The standard dose of Aroclor is 500 mg/kg body weight. The Aroclor used for injection may be either a 2:1 mixture of Aroclor 1242:Aroclor 1254 or Aroclor 1254 alone according to the Standard Operating Procedures of the Test Facility.

PROTOCOL NO. C29 (Cont'd)Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay

Issue Date: August 29, 1985

Toxicity Test:

In addition to limitations imposed by the solubility of a substance, the levels at which it can be tested for mutagenicity are determined by its toxic effect on L5178Y cells. As a result the toxicity of a compound is first tested over a wide range of concentrations.

Toxicity is measured by the ability of a given dose of test substance to inhibit the suspension growth of treated cultures. The method and length of exposure of cells to chemical and incubation conditions are similar to those used in the Mutagenicity Test Section. The exact procedure is conducted according to the Standard Operating Procedures of the Test Facility.

The doses of test substance are determined from the information obtained in the toxicity test. From these results, the highest dose of test substance to be used in the mutagenicity test is chosen to give substantial or complete toxicity relative to the solvent control. Within the limits of predictability of the toxicity test, subsequent doses are chosen to span the range of relative toxicity to a level where little or no relative toxic effect is observed.

Mutagenicity Test:S-9 Mix (Metabolic Activation System)

Prior to dosing the cells, S-9 mix will be prepared by combining S-9 fraction with a neutralized solution of NADP and sodium isocitrate. The final concentrations of each component in the cultures during treatment are 100 μ l/ml S-9, 2.4 mg/ml NADP, and 4.5 mg/ml sodium isocitrate in F₀P. The S-9 mix will be prepared shortly before use from freshly thawed S-9 fraction. Unused portions should be discarded at the end of the day.

Dosing, Expression Growth and Cloning of Cells

Each sample will be prepared by combining the test substance, 4.0 ml of S-9 mix or 4.0 ml F₀P and then adding 6×10^6 L5178Y TK⁺ cells to a labeled, sterile 50 ml centrifuge. The volume of cells will depend on the concentration of the test substance. Final volume of the test substance and cells should be 6.0 ml. (See Dose Preparation Section)

PROTOCOL NO. C29 (Cont'd)Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK^{+/−} Mouse Lymphoma Assay

Issue Date: August 29, 1985

Mutagenicity Test
(Cont'd):Dosing, Expression Growth and Cloning of Cells (Cont'd)

Each sample vessel is then gassed with 5% CO₂-in-air, sealed and incubated at 37 ± 2 °C on a roller drum for four hours. The cell samples are then centrifuged (approximately 200 X g), the supernatant discarded, and the cells washed twice with fresh F₁₀P. The cells are then resuspended in F₁₀P at a concentration of approximately 3 x 10⁵ cells/ml, based on the original cell number of 6 x 10⁶ cells per culture prior to treatment with chemical, and all samples incubated as described above for a two or three day expression period according to the Standard Operating Procedures of the Test Facility. During the expression period, the cell concentration is determined daily and all cultures are diluted to 3 x 10⁵ cells/ml if necessary, in order to keep the cells in an active state of growth.

At the end of the expression period, doses are chosen for cloning based on the relative toxicity shown during the expression period. In general, dose levels which exhibit from 10 to 90% relative growth inhibition during the expression period are chosen for cloning. However, if that level of toxicity is not achieved within the solubility limits of the compound, then dose(s) showing less than 10% inhibition may be cloned. Dose levels showing greater than 90% growth inhibition will not be cloned. A portion of each culture is centrifuged and resuspended in F₁₀P. The appropriate dilutions are then made and a portion of each sample is plated on Petri dishes in soft agar medium with and without the selective agent (TFT) according to the Standard Operating Procedures of the Test Facility. Three dishes for each sample at 1 x 10⁶ cells/plate are prepared in TFT medium. Three dishes for each sample at an estimated cell number from 100–200 cells/plate are prepared in cloning medium without selective agent. All petri dishes are then incubated at 37 ± 2 °C for 10–14 days to allow colonies to form from individual cells. At the end of this time, the number of colonies on each plate is counted. The number of viable cells (survivors) originally placed on the plates containing the TFT medium is determined from the number of colonies in dishes containing the non-selective medium. The number of TK^{+/−} mutants is determined from the number of colonies in dishes containing the TFT medium.

PROTOCOL NO. C29 (Cont'd)Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the LS178Y TK⁺ Mouse Lymphoma Assay

Issue Date: August 29, 1985

Protocol Changes:

If it becomes necessary to change the approved protocol, verbal agreement to make this change should be made between the Study Director and the Sponsor. As soon as practical, this change and the reasons for it should be put in writing and signed by both the Study Director and the Sponsor's Divisional Toxicologist. This document is then attached to the protocol as an amendment.

Results:

Results of each test are considered independently, but in order to be considered a valid test, the spontaneous mutation frequencies observed for the negative controls should be no higher than 150 mutants per 10^6 survivors. In addition, the mutation frequencies observed for the positive controls must exceed the negative control mutation frequencies by at least 3 fold.

Report:Final Report

A report of the results will be prepared for this study by the contract laboratory within 30 days from the completion of the study. The report will include, but not be limited to, the following:

The raw data are reported for each negative and positive control and each dose of substance. Raw data consist of dose preparation information, the daily cell concentrations, the number of viable, colony-forming cells on each petri dish containing non-selective medium, and the number of TFI-resistant colony-forming cells on each dish. A mutation frequency (the number of TFI-resistant colony-forming cells per unit survivor) and the fold increase in mutation frequency relative to the solvent control is determined for each sample. The induced mutation frequency, the mutation frequency of each sample minus the spontaneous mutation frequency shown in the solvent controls may also be determined. In addition to the mutation frequencies, the percent survival relative to the control is reported for each sample for both the expression period growth in suspension and the overall growth (the relative suspension growth corrected for viability as determined by the plating efficiency in non-selective medium).

PROTOCOL - APPENDIX 1

Abbreviations and Glossary of Terms:

1. 2-AAF - 2-acetylaminofluorene
2. TFT - Triflurothymidine
3. Cloning medium- Fischer's Medium for Leukemic Cells of Mice supplemented as described below for F₀P and with approximately 20% (v/v) horse serum and 0.32-0.37% noble agar according to the Standard Operating Procedures of the Test Facility
4. DMN - dimethylnitrosamine
5. DMSO - dimethylsulfoxide
6. EMS - ethyl methanesulfonate
7. F₀P - Fischer's Medium for Leukemic Cells of Mice supplemented with sodium pyruvate, Pluronic F68, and penicillin-streptomycin according to the Standard Operating Procedures of the Test Facility
8. F₁₀P - F₀P plus approximately 10% (v/v) horse serum
9. Gassing - Replacement of the air in a culture vessel with 5% CO₂-in-air by purging with CO₂-air mixture
10. NADP - β-nicotinamide adenine dinucleotide phosphate
11. Selective cloning medium - Cloning medium containing TFT according to the Standard Operating Procedures of the Test Facility
12. S-9 - The supernatant obtained by centrifugation of a homogenate of liver at 9000 X g.
13. TK - thymidine kinase

REFERENCES

- ¹Clive, D. and J. F. S. Spector. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. *Mutation Res.*, 31: 17-29 (1975).
- ²Clive, D., K. O. Johnson, J. F. S. Spector, A. G. Batson, and M. M. M. Brown. Validation and characterization of the L5178Y/TK⁺/⁻ mouse lymphoma mutagen assay system. *Mutation Res.* 59: 61-108 (1979).
- ³Clive, D., W. G. Flamm, and J. B. Patterson. Specific locus mutational assay systems for mouse lymphoma cells. In A. Hollaender (ed.), *Chemical Mutagens: Principles and Methods for their Detection*. Volume 3, Plenum Press, New York, 1973, pp. 79-103.
- ⁴Ames, B. N., W. E. Durston, E. Yamasaki, and F. D. Lee. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Nat. Acad. Sci. USA* 70: 2281-2285 (1973).

Amendment to Protocol

The study will consist of two complete mouse lymphoma assays modified as described below. Preliminary toxicity assays will not be required. The highest dose without S-9 is to be 3.0 µg/ml. the highest dose with S-9 is to be 15 µg/ml.

Modification to the Protocol

1. On the day before the assay, collect cells from 100 ml of culture by centrifugation. Discard the cells.
2. Mix the supernatant, from step 1) above, with F₀P to make F₃P as per the protocol. Divide the F₃P into two equal portions.
3. To one portion, add a sufficient amount of G2318.01 such that the final concentration is 1000 µg/ml. Adjust the pH if necessary.
4. Do nothing to the other portion of F₃P.
5. Filter sterilize both solutions of F₃P and incubate them overnight in the CO₂ incubator.
6. On the day of the assay, prepare test substance G0539.03 as described in the protocol, and add appropriate amounts to the F₃P solutions prepared earlier. Use these solutions for the exposure.
7. Collect the appropriate number of cells for the assay by centrifugation, and discard the supernatant. Add the cells, and if appropriate the S-9, to the test solutions prepared in step 6 above, and proceed with the exposure portion of the protocol.
8. Perform the remainder of the assay as usual.
9. If it is not possible to perform all four segments of the assay on the same day, (1) G0539.03 without S-9; 2) G0539.03 plus G2318.01 without S-9; 3) G0539.03 with S-9; 4) G0539.03 plus G 2318.01 plus S-9), then perform both assays without S-9 together and both assays with S-9 together.

E D Thompson

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7/21/87

PROTOCOL DEVIATIONS

Deviation Nos.: 1, 2 and 3

Sponsor: The Procter & Gamble Company
P.O. Box 39175
Cincinnati, Ohio 45247

Study No.: 0066-2400

Sponsor's DRD No.: BY0708

Test Substance I.D.: G0539.03

Protocol Title: Test for Chemical Induction of Mutation in
Mammalian Cells in Culture - the L5178Y TK+/-
Mouse Lymphoma Assay

Deviation No. 1: Protocol Page No. 3, Dose Preparation (Cont'd) -
The second to last sentence of the first paragraph indicates that
the final volume of the dosing solution is 10 ml. For this study,
the culture volume was 10 ml, and then 100 ul amounts of test
article solutions were added directly to the cultures mixed in
ethanol.

Reason for Deviation: The treatment was performed according to
SITEK Research Laboratories Standard Operating Procedures, Section
24.6.3. The number of cells per culture was maintained at 6×10^6
cells, however, the increase in volume would reduce the concen-
tration of cells per ml. This variation in treatment regimen
would have no significant effect on the outcome of the study.

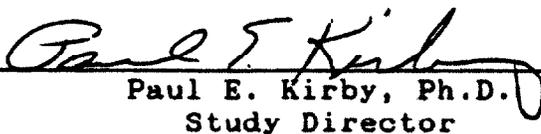
Deviation No. 2: Protocol Page No. 3, Dose Preparation (Cont'd) -
The last sentence of the first paragraph indicates that the order
of addition for dosing should be test article, S-9 mix or F₂P, then
cells. The order of addition used at SITEK was cells, S-9 mix or
F₂P, then test article.

Reason for Deviation: The addition of cells, S-9 mix or F₂P, then
test article is the method routinely used by SITEK and is
according to SITEK's Standard Operating Procedures. Combining the
cells and S-9 mix first creates the correct S-9 mix concentration
in the culture before the test article is added. The method
described in the protocol, adding test article and then S-9 mix,
would expose the test article to a much higher than normal concen-
tration of homogenate and cofactors.

Deviation No. 3: Protocol Page No. 10, Item No. 7 - The protocol indicates that F₀P is Fischer's Medium for Leukemic Cells of Mice supplemented with sodium pyruvate, Pluronic F68, and penicillin-streptomycin. SITEK's F₀P is not supplemented with sodium pyruvate or penicillin-streptomycin.

Reason for Deviation: The F₀P was prepared according to SITEK's Standard Operating Procedures. Sodium pyruvate was added to the cloning medium when it was prepared.

APPROVAL:



Paul E. Kirby, Ph.D.
Study Director

9-30-87

Date

Kenneth L. Hintze, Ph.D.
Divisional Toxicologist

Date