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(A)

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CHEMICAL MANUFACTURERS ASSOCIATION

NINE ZINC DIALKYL DITHIOPHOSPHATES AND CALCIUM DIALKYL DITHIOPHOSPHATE, ZINC OLEATE, LOT NO. 34495-10 AND ZINC CHLORIDE, LOT NO. KTJY

Submitted by the Chemical Manufacturers Association to EPA in 1984

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MICROBIOLOGICAL ASSOCIATES

Microbiological Associates
A Unit of Whittaker Corporation
5221 River Road
Bethesda, Maryland 20816
(301) 654-3400
Telex No. 90-8793

Whittaker

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

Sponsor: Chemical Manufacturers Association
2501 M Street, N.W.
Washington, D.C. 20037

Testing Facility: Microbiological Associates
5221 River Road
Bethesda, Maryland 20816

Study No.'s: See Page I-2

Test Article I.D.'s: Group 1: T1851-T1857, T1866-T1867
Group 2: T1856, T2253-T2256
See Page I-2

Test Article Lot No.'s: Group 1: None Provided
Group 2: See Page I-2

Test Article Descriptions: See Page I-2

Storage Conditions: Group 1: Room Temperature
Group 2: See Page I-2

Dates Received: See Page I-2

Dates Studies Started: See Page I-2

Date Study Completed: 8/14/84

Report Date: 8/14/84

Study Coordinator: Carol R. Stack, Ph.D.
Chemical Manufacturers Association

Study Director: Andrea M. Rogers-Back, Ph.D.
Microbiological Associates

Andrea M. Rogers-Back, Ph.D. Date
Study Director

Jane J. Clarke Date
Group Leader

Janine J. Leffert Date
Group Leader

Dennis A. Sorrells Date
Biologist

QUALITY ASSURANCE STATEMENT

Study Title: L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay

Study Number: T1856.701006, T1866.701006, T1854.701006, T1857.701006, T1852.701006, T1855.701006, T1851.701006, T1867.701006, T1853.701006, T2253.701012, T2254.701012, T2255.701012 and T2256.701012

Study Director: Andrea M. Rogers-Back, Ph.D.

Initiation Date: See Page I-2

Review Completed Date:

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc. are examined in order to assure that the study is performed in accordance with the Good Laboratory Practice Regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATE OF INSPECTION	PHASE INSPECTED	REPORT SUBMITTED TO STUDY DIRECTOR	REPORT SUBMITTED TO MANAGEMENT
T1856.701006:			
12/8/82	Protocol review	12/8/82	12/8/82
1/12/83	Initial toxicity: Test article removal and culture rinsing	1/13/83	1/13/83
2/21/83	Counting the plates	2/21/83	2/22/83
2/23/83	Initial toxicity: Preparation of S-9 mix	2/23/83	2/23/83
3/1/83	Dilution of test article and positive controls	3/1/83	3/14/83
3/3/83	Cloning medium preparation	3/3/83	3/14/83
4/4/83	Raw data audit	4/4/84	4/20/83
6/7/83	Draft report	6/7/83	6/8/83
9/7/83	Preparation of cultures Treatment of cultures	9/7/83	9/7/83
10/5/83	Preparation of cultures	10/5/83	10/5/83
10/5/83	Preparation of S-9 mix	10/5/83	10/5/83

QUALITY ASSURANCE STATEMENT CONTINUED

DATE OF INSPECTION	PHASE INSPECTED	REPORT SUBMITTED TO STUDY DIRECTOR	REPORT SUBMITTED TO MANAGEMENT
T1866.701006:			
12/20/82	Protocol review	12/20/82	12/20/82
1/18/83	Initial toxicity: Preparation of cultures	1/18/83	1/19/83
1/25/83	Preparation of cultures Test article removal and culture rinsing	1/25/83	1/25/83
2/23/83	Initial toxicity: Preparation of S-9 mix	2/23/83	2/23/83
4/5/83	Raw data audit	4/5/83	4/20/83
6/7/83	Draft report	6/7/83	6/8/83
T1854.701006:			
12/8/82	Protocol review	12/8/82	12/8/82
1/11/83	Initial toxicity: Dilution of test article	1/11/83	1/11/83
1/28/83	Cloning medium preparation	1/28/83	1/28/83
2/10/83	Treatment of cultures	2/10/83	2/10/83
3/15/83	Daily cell counts and readjustments	3/15/83	3/15/83
4/15/83	Raw data audit	4/15/83	5/2/83
6/7/83	Draft report	6/7/83	6/8/83
T1857.701006:			
12/8/82	Protocol review	12/8/82	12/8/82
1/4/83	Initial toxicity: Dilution of test article	1/4/83	1/5/83
1/26/83	Test article removal and culture rinsing	1/26/83	1/26/83
1/28/83	Cloning medium preparation	1/28/83	1/28/83
2/10/83	Dilution of test article and positive control	2/10/83	2/10/83
3/1/83	Initial toxicity: Test article removal and culture rinsing	3/1/83	3/14/83
3/14/83	Preparation of S-9 mix	3/14/83	3/14/83
4/15/83	Raw data audit	4/15/83	4/18/83
6/7/83	Draft report	6/7/83	6/8/83

QUALITY ASSURANCE STATEMENT CONTINUED

DATE OF INSPECTION	PHASE INSPECTED	REPORT SUBMITTED TO STUDY DIRECTOR	REPORT SUBMITTED TO MANAGEMENT
T1852.701006:			
12/8/82	Protocol review	12/8/82	12/8/82
12/16/82	Initial toxicity: Preparation of cultures	12/16/82	12/17/82
1/18/83	Dilution of test article and positive controls	1/18/83	1/19/83
2/7/83	Initial toxicity: Preparation of S-9 mix	2/7/83	2/7/83
2/23/83	Preparation of S-9 mix	2/23/83	2/23/83
3/22/83	Dilution of test article and positive control Treatment of cultures	3/22/83	3/23/83
4/28/83	Raw data audit	4/28/83	5/4/83
6/7/83	Draft report	6/7/83	6/8/83
T1855.701006:			
12/8/82	Protocol review	12/8/82	12/8/82
1/17/83	Dilution of test article and positive control Treatment of cultures	1/17/83	1/17/83
1/19/83	Cell dilution and TFT addition to cloning flasks	1/19/83	1/20/83
3/22/83	Dilution of test article and positive control	3/22/83	3/23/83
4/15/83	Raw data audit	4/15/83	4/22/83
6/7/83	Draft report	6/7/83	6/8/83
T1851.701006:			
12/8/82	Protocol review	12/8/82	12/8/82
12/16/82	Initial toxicity: Preparation of cultures	12/16/82	12/17/82
12/17/82	Initial toxicity: Daily cell counts and readjustments	12/17/82	12/17/82
1/11/83	Dilution of test article and positive controls	1/11/83	1/11/83
2/9/83	Test article removal and culture rinsing	2/9/83	2/10/83
5/4/83	Raw data audit	5/4/83	5/11/83
6/7/83	Draft report	6/7/83	6/8/83

QUALITY ASSURANCE STATEMENT CONTINUED

DATE OF INSPECTION	PHASE INSPECTED	REPORT SUBMITTED TO STUDY DIRECTOR	REPORT SUBMITTED TO MANAGEMENT
T1867.701006:			
12/20/82	Protocol review	12/20/82	12/20/82
1/27/83	Cell preparation and addition to cloning flasks Cell dilution and TFT addition to cloning flasks	1/28/83	1/28/83
4/5/83	Treatment of cultures	4/5/83	4/5/83
5/5/83	Raw data audit	5/5/83	5/11/83
6/7/83	Draft report	6/7/83	6/8/83
T1853.701006:			
12/8/82	Protocol review	12/8/82	12/8/82
1/17/83	Test article removal and culture rinsing	1/17/83	1/17/83
1/19/83	Cloning medium preparation	1/19/83	1/19/83
2/9/83	Initial toxicity: Test article removal and culture rinsing	2/9/83	2/10/83
2/16/83	Preparation of S-9 mix	2/16/83	2/16/83
4/13/83	Raw data audit	4/13/83	4/15/83
6/7/83	Draft report	6/7/83	6/8/83
T2253.701012, T2254.701012, T2255.701012 and T2256.701012:			
1/16/84	Protocol review	1/16/84	1/16/84
T2253.701012:			
1/23/84	Initial toxicity: Test article removal and culture rinsing	1/24/84	1/26/84
2/7/84	Preparation of S-9 mix Preparation of cultures	2/7/84	2/7/84
3/28/84	Removal of test article and culture rinsing	3/29/84	3/29/84
T2254.701012:			
1/23/84	Initial toxicity: Test article removal and culture rinsing	1/24/84	1/27/84
2/7/84	Preparation of S-9 mix Preparation of cultures	2/7/84	2/7/84

QUALITY ASSURANCE STATEMENT CONTINUED

DATE OF INSPECTION	PHASE INSPECTED	REPORT SUBMITTED TO STUDY DIRECTOR	REPORT SUBMITTED TO MANAGEMENT
T2254.701012 Cont.:			
2/9/83	Cell preparation and addition to the cloning flasks	2/9/84	2/10/84
T2255.701012:			
2/8/84	Test article removal and culture rinsing	2/8/84	2/9/84
2/10/84	Cell preparation and addition to cloning flasks Cell dilution and TFT addition to the cloning flasks	2/10/84	2/10/84
T2256.701012:			
1/25/84	Initial toxicity: Cell counts and readjustments	1/25/84	1/26/84
2/8/84	Test article removal and culture rinsing	2/8/84	2/9/84
2/10/84	Cell preparation and addition to cloning flasks	2/10/84	2/10/84
T1856.701006, T2253.701012, T2254.701012, T2255.701012 and T2256.701012:			
5/22/84	Draft report	5/24/84	5/31/84
T1851.701006 - T1857.701006, T1866.701006 - T1867.701006, T2253.701012 - T2256.701012:			
8/13/84	Draft to final report	8/13/84	8/14/84

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Quality Assurance
RA/QA Department

TEST ARTICLE INFORMATION

GROUP 1

Study #	T1856.701006	T1966.701006	T1854.701006	T1857.701006
Test Article I.D.	CMA-102	CMA-105	CMA-106	CMA-107
Test Article Description	Clear, Slightly Viscous Liquid	Yellow, Slightly Viscous Liquid	Clear, Viscous Liquid	Clear Amber, Slightly Viscous Liquid
Date Received	11/24/82	12/10/82	11/24/82	11/24/82
Date Study Started	1/13/83	1/18/83	1/11/83	1/4/83

Study #	T1852.701006	T1855.701006	T1851.701006	T1867.701006	T1853.701006
Test Article I.D.	CMA-108	CMA-109	CMA-110	CMA-111	CMA-112
Test Article Description	Light Brown Viscous Liquid	Clear Amber, Slightly Viscous Liquid	Clear, Dark Amber, Viscous Liquid	Dark Amber, Slightly Viscous Liquid	Clear, Dark Amber, Viscous Liquid
Date Received	11/24/82	11/24/82	11/24/82	12/10/82	11/24/82
Date Study Started	12/16/82	12/20/82	12/16/82	1/18/83	12/20/82

GROUP 2

Test Article I.D.	Zinc Dialkyl Dithiophosphate (CMA-102 & CMA-102B)	Calcium Dialkyl Dithiophosphate	Zinc Oleate	Zinc Chloride
Test Article Lot No.	None Provided	None Provided	34495-10	KTJY
Study No.	T1856.701006 T2254.701012	T2253.701012	T2256.701013	T2255.701012
Test Article Description	T1856: Clear, Slightly Viscous Liquid T2254: Yellow, Slightly Viscous Liquid	Light Green Viscous Liquid	Off-white Solids	White Granules
Storage Conditions	Room Temperature Protected from Light	Room Temperature Protected from Light	Room Temperature with Desiccation Protected from Light	Room Temperature with Desiccation Protected from Light
Date Received	T1856: 11/24/82 T2254: 12/12/83	12/12/83	12/12/83	12/12/83
Date Study Started	T1856: 9/7/83 T2254: 1/23/84	1/23/84	1/24/84	1/24/84

Note: All test articles in Group I are Zinc Dialkyl Dithiophosphates

Summary

Chemical Manufacturers Association's test articles CMA-102 (MA #'s T1856 and T2254), CMA-105 (MA #T1866), CMA-106 (MA #T1854), CMA-107 (MA #T1857), CMA-108 (MA #T1852), CMA-109 (MA #T1855), CMA-110 (MA #T1851), CMA-111 (MA #T1867) and CMA-112 (MA #T1853) were tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. All the test articles were Zinc Dialkyl Dithiophosphates. In addition, Calcium Dialkyl Dithiophosphate (MA #T2253), Zinc Oleate, Lot No. 34495-10 (MA #T2256) and Zinc Chloride, Lot No. KTJY (MA #T2255) were also tested in the presence of Aroclor induced rat liver S-9. The results are summarized in Summary Table 1.

Detailed results for each test article are presented in separate sections distinguished by a blue tab with the test article I.D.

SUMMARY TABLE 1

	CMA #	MA #	Threshold Toxicity Level (µl/ml)		Mutation Assay Results	
			- S-9	+ S-9	- S-9	+ S-9
Zinc Dialkyl Dithio-phosphates	102 *	T1856	0.05	0.05	-	± (+) *
	102(B)	T2254	nt	0.05	nt	±
	105	T1866	0.05	0.05	-	±
	106	T1854	0.05	0.05	-	+
	107	T1857	0.1	0.1	-	+
	108	T1852	0.1	0.05	-	±
	109	T1855	0.1	0.05	-	-
	110	T1851	0.1	0.05	-	±
	111	T1867	0.05	0.1	-	±
	112	T1853	0.1	0.05	-	-
Calcium Dialkyl Dithiophosphate		T2253	nt	0.1	nt	-
Zinc Chloride		T2255	nt	50 µg/ml	nt	+
Zinc Oleate		T2256	nt	100 µg/ml	nt	-

nt = not tested

*Second series of experiments

Introduction

Ten Zinc Dialkyl Dithiophosphate test articles, CMA-102 (MA #T1856 and T2254), CMA-105 (MA #T1866), CMA-106 (MA #T1854), CMA-107 (MA #T1857), CMA-108 (MA #T1852), CMA-109 (MA #T1855), CMA-110 (MA #T1851), CMA-111 (MA #T1867) and CMA-112 (MA #T1853); were tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay with and without exogenous metabolic activation by Aroclor induced rat liver microsomes. In addition, Calcium Dialkyl Dithiophosphate (MA #T2253), Zinc Oleate, Lot No. 34495-10 (MA #T2256) and Zinc Chloride, Lot No. KTJY (MA #T2255) were tested in the presence of S-9.

Objective

To evaluate the mutagenic potential of the test article using the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay.

Materials and Methods

The experimental protocols (see Appendices) are based on that described by Clive, D. and Spector, J. F. S. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y Mouse Lymphoma cells. Mutation Research 31:17-29, 1975.

A. DETERMINATION OF TOXICITY

A preliminary toxicity test with and without S-9 activation was conducted. Tube cultures were initiated by seeding one Corning polypropylene centrifuge tube per dose level and two per solvent control with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells per ml. The solubility of the test articles was determined in Acetone, DMSO, Ethanol and H_2O, and Acetone was selected as the solvent of choice. Test articles CMA-102 and CMA-105 through CMA-112 were tested in the presence and absence of S-9. In the presence of S-9, test articles T1851 through T1867 were solubilized and diluted for testing at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 µl/ml. In the absence of S-9, test articles CMA-102, CMA-105, CMA-106 and CMA-111 were solubilized and diluted for testing at 100, 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 µl/ml. Test articles CMA-107, CMA-108, CMA-109, CMA-110 and CMA-112 were solubilized and diluted for testing at 100, 10, 1.0, 0.1, 0.01 and 0.001 µl/ml.

A preliminary toxicity test with S-9 activation was conducted on test articles T2253, T2254, T2255 and T2256. Acetone was selected as the solvent of choice for T2254, T2253 and T2256. Water was selected as the solvent for T2255. Test articles T2254 and T2253 were solubilized and diluted for testing at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 µl/ml. Test article T2255 was solubilized and diluted for testing at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 µg/ml. Test article T2256 was solubilized and diluted for testing at 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 µg/ml.

The test article was added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration was nontoxic to the cell suspension. Four ml of F_0P or S-9 activation mixture was added to each tube. The tube was gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at approximately 25 rpm for a 4-hour exposure period. The test solutions were prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article was removed by centrifuging the cells at 1000 rpm for 10 minutes and decanting the supernatant. The cells were washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Test article toxicity was determined by comparing the cell population growth at each dose level with that of the solvent controls. Cell population density was determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes, and counting the samples with an electronic cell counter.

All calculations were performed using a Texas Instruments TI-59 calculator and/or a Hewlett Packard 86 Minicomputer with programs labeled "Cell Culture Adjustment" and "Initial Toxicity".

B. TESTING FOR MUTAGENIC ACTIVITY

1. Cell Preparation

Prior to use in the assay, L5178Y cells which were actively growing in culture were cleansed as described by Clive, et al.[1] Three ml of THMG stock solution was added to a 100 ml cell suspension containing 0.1 x 10^6 cells per ml. The culture was gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours the THMG was removed by pelletizing the cells and decanting the supernatant. The cells were rinsed in 20 ml of F10P and reinstated in culture at 3 x 10^5 cells per ml in 100 ml of F10P plus 1 ml of THG stock solution.

The cell population density of the prepared cultures was determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0 x 10^6 cells per ml was prepared, and 6 ml aliquots were dispensed in Corning polypropylene centrifuges.

2. Test Compound Preparation

Based on the data derived from the toxicity test, the test article was prepared so that the highest concentration was 100% toxic. The test article was solubilized and 15 serial eighth log dilutions were carried out. This produced 16 dose levels decreasing approximately 75-fold from highest to lowest. In some experiments, 8 evenly spaced dose levels, which decreased approximately 10-fold from highest to lowest dose were used. The test article was added to duplicate cultures labelled with the T# and A or B. For T1856 and T2253 (Trial II), the test article was solubilized

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

and 13 serial dilutions were carried out. Fourteen dose levels were produced and cultures were treated in triplicate. The test article was added to each tube, labelled with the test article T No., test concentration and NA or S-9, where appropriate, in amounts at which the final solvent concentration was nontoxic to the cells. Four ml of S-9 activation mixture or 4 ml F_0P was added to the tubes. This yielded a final cell suspension of 0.6×10^6 cells per ml.

Two control tubes received solvent only and the positive controls were treated with 7,12-DMBA (7.5 and 5.0 µg/ml) or EMS (1.0 and 0.5 µl/ml). All tubes were gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article was carried out under amber lighting and the cells were incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells were washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells were resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

3. Expression Time

After the initial exposure to the test article, the cells were incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment was made by taking daily cell counts and then replacing a volume of cells with fresh medium which yielded a cell population density of 0.3×10^6 cells per ml.

4. Cloning

At the end of the expression period, the cells were placed in cloning medium (C.M.) containing 0.34% Noble agar. TFT at a final concentration of 3 µg/ml was used as the restrictive agent.

a. General Preparation

Two Florence flasks per culture to be cloned were labeled with the compound concentration and whether they received S-9

activation. For each pair of flasks one was labeled TFT and one was labeled V.C. (viable count). Each flask was prewarmed to 37°C, filled with 100 ml of C.M., and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per culture were labeled with the concentration, and the experiment number. Three of the six were labeled TFT and three were labeled V.C.

b. Cell Plating

Cell counts were made for each tube to determine the volume of each cell population which would yield 3 x 10^6 cells. This volume was removed, the remainder of the cells were discarded, and the 3 x 10^6 cells were replaced in the centrifuge tube. The cells were centrifuged at 1000 rpm for 10 minutes, and the supernatant, except for 2 ml, was removed by pipetting. The cells were resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article.

A 2 x 10^{-4} dilution was carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{12}P$, adding 1.0 ml of this to 9 ml of $F_{12}P$, and adding 1.0 ml of that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution 1 ml of stock solution of TFT was added to the TFT flask, and both this flask and the V.C. flask were placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks were removed one at a time, and 33 ml of the cell suspension was pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates were placed in cold storage (4°C) for 20 minutes. The plates were removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

5. Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates were scored for the total number of colonies per plate. Three counts per plate were made on an automatic colony counter, and the median count was recorded. The mutation frequency was determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. All mutant frequencies and toxicity data calculations were performed using either a Texas Instruments TI-59 calculator with programs labelled "Mutant Frequency" or "Total Compound Toxicity" or a Hewlett-Packard microcomputer with programs labelled "Final Report Generation".

Chemical information on control articles and solvents used for this study:

Control Article:

7,12-Dimethylbenz(a)anthracene	
CAS Registry Number	57-97-6
Assay (by UV-VIS)	95% min
Melting Range	2°C range including 123°C
Ethyl Methanesulfonate	$CH_3SO_3C_2H_5$
CAS Registry Number	62-50-0
Assay (by GLC)	98% min

Solvents:

Acetone	CH_3COCH_3
CAS Registry Number	67-64-1
Assay (CH_3COCH_3)	Not less than 99.5%
Isopropyl Alcohol [$(CH_3)_2CHOH$]	0.05%
Color (APHA)	10
Density (g/ml) at 25°C	Not above 0.7857
Boiling Range	Not more than 0.8°C
Boiling Point	56.1 ± 0.1°C
Residue after Evaporation	0.001%
Solubility in Water	Clear
Acidity (CH_3COOH)	0.002%
Alkalinity (as NH_3)	0.001%
Aldehyde (HCHO)	0.002%
Methanol (CH_3OH) (by G.C.)	0.05%
Subs Reducing $KMnO_4$ (Color)	Pass test
Water (H_2O)	0.5%

DMSO	$(CH_3)_2SO$
CAS Registry Number	67-68-5
Appearance	Clear, Colorless liquid
Density (gms./ml) at 25°C	1.095 min
Freezing Point	18.0°C min
Residue after Evaporation	0.01%
Color (APHA)	15
Ethanol	C_2H_5OH
Brand Name	Pharmco
Bottled by	Publicker Industries Co. Linfield, Pa.
Assay	190 Proof
Molecular Weight	46.07
Density (gms/ml) at 20°C	0.789
Boiling Point	78.5°C
Melting Point	-114.1°C
Solidifies below	-130°C
Flash Point	9-11°C

The following criteria were used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the scientist's evaluation must be the final endpoint.

Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

Note: 1. Some of the numbers generated by the test data, whether it is Toxicity, Mutant Frequency, etc., are computed using non-rounded numbers. This may, in some instances, cause what appear to be errors in calculation if only the rounded numbers are used when checking the data.

2. All of the raw data generated by the assay and the original final report will be maintained in Microbiological Associates' archives located in our Bethesda, Maryland facilities.

3. The stability of the test article under the actual experimental conditions used in this study was not determined by Microbiological Associates.

4. All test article stock solutions were freshly prepared immediately before their use in each procedure.

PROTOCOL AMENDMENT

Date: August 7, 1984

Sponsor: Chemical Manufacturers Association

Sponsor's Test Article Designation:
1) CMA-110
2) CMA-108
3) CMA-112
4) CMA-106
5) CMA-109
6) CMA-102
7) CMA-107
8) CMA-105
9) CMA-111

Study No.'s:
1) T1851.701006
2) T1852.701006
3) T1853.701006
4) T1854.701006
5) T1855.701006
6) T1856.701006
7) T1857.701006
8) T1866.701006
9) T1867.701006

Protocol No.: SPGT701006 1082

Protocol Title: L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay

1. Section 4.4 Study Director, should be changed to Andrea M. Rogers-Back, Ph.D.

Reason for Amendment:

After July 13, 1984, Paul E. Kirby, Ph.D. was no longer in the employ of Microbiological Associates.

APPROVAL:

Andrea M. Rogers-Back 8/14/84
Andrea M. Rogers-Back, Ph.D. Date
Study Director

Carol R. Stack 8/16/84
Carol R. Stack, Ph.D. Date
Study Coordinator

PROTOCOL AMENDMENT

Date: August 7, 1984

Sponsor: Chemical Manufacturers Association

Sponsor's Test Article Designation:
1) Calcium Dialkyl Dithiophosphate,
2) Zinc Dialkyl Dithiophosphate, CMA-102B
3) Zinc Chloride
4) Zinc Oleate

Study No.'s:
1) T2253.701012
2) T2254.701012
3) T2255.701012
4) T2256.701012

Protocol No.: SPGT701012 112383

Protocol Title: L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay (With S-9 Activation)

1. Section 4.4 Study Director, should be changed to Andrea M. Rogers-Back, Ph.D.

Reason for Amendment:

After July 13, 1984, Paul E. Kirby, Ph.D. was no longer in the employ of Microbiological Associates.

APPROVAL:

Andrea M. Rogers-Back, Ph.D. 8/16/84
Study Director Date

Carol R. Stack, Ph.D. 8/16/84
Study Coordinator Date

Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-102 (MA #T1856) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of concentrations which produced from 35% to 98% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 22% to 117% Total Growth.

The highest test article concentration cloned in the S-9 activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. None of the nonactivated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicated that, under the conditions of this test, test article CMA-102 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

Three assays were conducted on the test article in the presence of S-9 as follow-up studies to that reported above. In the first assay, the cultures that were cloned were treated with a range of test article concentrations which produced from 3% to 71% Total Growth. There was some contamination in this assay and complete results were obtained for eleven of the eighteen cultures that were cloned. However, all eleven cultures exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The assay was repeated due to the contamination and the erratic dose-response relationship in toxicity in the treated cultures. Previous studies with the test article had also demonstrated a precipitous toxic response. In the second experiment, the cultures that were cloned were treated with a range of concentrations which produced from 3% to 44% Total Growth. Seven of seven cultures that were cloned exhibited mutant frequencies

which were significantly greater than the mean mutant frequency of the solvent controls. In a third experiment, conducted concurrently with an assay on Calcium Dialkyl Dithiophosphate (MA #T2253) and with a second sample of test article (T2254), the cultures that were cloned were treated with a range of concentrations which produced from 27% to 96% Total Growth. None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. However, a dose-dependent response was noted.

Results

The Initial Toxicity Test (Table 1) performed on test article CMA-102 (MA #T1856) in the absence of S-9 indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The Cloning Data indicated a low cloning efficiency which was unacceptable. The data for this assay will not be reported here. A repeat assay was performed over the same dose range.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, 0.0012, 0.00089 or 0.00067 µl/ml test article. These concentrations produced a range in Suspension Growth of 37% to 91%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 35% to 98%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-102 (MA #T1856). The results indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.021, 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, or 0.0016 µl/ml. These concentrations produced a

range in Suspension Growth of 25% to 97%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2. One culture (0.021 µl/ml) that was cloned exhibited a mutant frequency which was 2.3 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 22%.

None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 91% to 117%.

Two mutagenesis assays in the presence of S-9 were conducted as follow-up studies to the original assays. In the first assay, the cultures were treated in triplicate with a range of test article concentrations from 0.030 µl/ml to 0.017 µl/ml. After a two day expression period, eighteen cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.022, 0.021, 0.020, 0.019, 0.018 or 0.017 µl/ml. These concentrations produced a range in Suspension Growth of 7% to 76%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 11 and 12. These data are also presented graphically in Figure 3. The Cloning Data and Total Compound Toxicity Data for the positive control are presented in Table 13.

All the cultures that were cloned for which complete results were obtained exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The increases in mutant frequency ranged from 9.4 to 2.2 times the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 3% to 71%.

The assay was repeated due to some contamination and an erratic toxic response. The repeat assay was conducted over the same dose range as described above. After a two day expression

period, seven cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.021, 0.018 or 0.017 µl/ml. These concentrations produced a range in Suspension Growth of 7% to 47%. The Cloning Data are presented in Tables 14 and 15 and the Total Compound Toxicity Data are presented in Tables 16 and 17. These data are also presented graphically in Figure 4.

All the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The increases in mutant frequency ranged from 11.3 to 3.1 times the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 3% to 44%.

A second sample of the test article was received. The Initial Toxicity Test (Table 18) indicated a complete toxicity in the presence of S-9 at 0.05 µl/ml.* Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.021, 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021 or 0.0016 µl/ml. These concentrations produced a range in Suspension Growth of 31% to 100%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 19 and 20. These data are also presented graphically in Figure 5. The data for the positive control are presented in Table 21.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 27% to 96%. However, a dose-dependent increase in mutant frequency was noted. The increase ranged from 1.0 to 1.9 times the mean mutant frequency of the solvent controls.

*Cultures treated with 10 µl/ml and 5 µl/ml appeared to have cell growth. Upon microscopic examination of the cultures, there were few viable cells present and a large quantity of compound precipitate and cell debris.

Study No. T1856.701006

Table 1

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1856.701006	Paul E. Kirby	100 µl/ml to 0.001 µl/ml
Study Number	Study Director	Dose Range
CMA-102 (T1856)		Acetone
Test Article Identity		Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation	100 µl/ml	2.519*	3.537*	0.0	0%
	10 µl/ml	1.094*	1.294*	0.0	0%
	5.0 µl/ml	0.204	0.284	0.0	0%
	1.0 µl/ml	0.006	0.007	0.0	0%
	0.5 µl/ml	0.000	0.000	0.0	0%
	0.1 µl/ml	0.000	0.000	0.0	0%
	0.05 µl/ml	0.002	0.001	0.0	0%
	0.01 µl/ml	0.577	0.714	4.6	30%
	0.005 µl/ml	1.102	1.247	15.3	100%
	0.001 µl/ml	1.129	1.262	15.8	104%
	Solvent 1	1.097	1.258	15.3 } 15.2	
	Solvent 2	1.104	1.232	15.1 }	
With S-9 Activation					
	Solvent 1				
	Solvent 2				

not applicable AMRB 1/14/83

+ Culture Lost * counts indicated debris, not cells JSC 1-14-83

Table Prepared By [signature] 1-14-83
Signature Date

Form No. ML-41
2/25/82

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1856 701006	Paul E Kirby	1856B2
Study Number	Study Director	Experiment Number
CMA 102 (T1856)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced Mutant * Frequency
0.0059 4/4	64	62	98	75	187	186	174	182	0.8	0.3
0.0067 4/4	62	55	65	61	164	186	225	192	0.6	0.1
0.0050 4/4	46	57	51	51	209	174	190	191	0.5	0.0
0.0038 4/4	+	+	46	46	190	186	200	192	0.5	0.0
0.0028 4/4	65	56	69	63	183	177	161	174	0.7	0.2
0.0021 4/4	+	44	49	47	199	+	215	182	0.5	0.0
0.0016 4/4	29	40	29	33	196	194	219	203	0.3	-0.2
0.0012 4/4	52	57	51	53	201	200	200	200	0.5	0.0
0.0089 4/4	64	53	52	56	154	179	179	171	0.7	0.2
0.0067 4/4	63	54	67	61	218	196	212	202	0.6	0.1
Solvent 1	52	48	47	49	189	187	177	184	0.57	0.5
Solvent 2	47	48	55	50	189	188	199	192	0.5	

Plates Counted By: Karen Joan Poms 2/21/83 (Signature & Date)

Calculations Performed By: Karen Joan Poms 2/21/83 (Signature & Date)

* Per 10^4 surviving cells + Culture Lost

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Study No. T1856 701006

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1856 701006	Paul E Kirby	1856B2
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	237	231	196	221	20	15	+	18	25.1	23.9
0.5µl/ml	310	305	311	309	136	123	116	125	4.9	3.7
SOLVENT 1	97	88	93	93	171	158	187	172	1.1	1.2
SOLVENT 2	98	89	98	95	173	164	151	163	1.2	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1										
SOLVENT 2										

Not Applicable for M Marshall 2-21-83

Plates Counted By: Karen Joan Pons 2/21/83 (Signature & Date)

Calculations Performed By: Karen Joan Pons 2/21/83 (Signature & Date)

*Per 10^4 surviving cells

+ Culture Lost

Study No. T1856 701006

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Form No. WL-128

Form No. MS-227
2/22/83

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1856.701006	Paul E Kirby	1856B2
Study Number	Study Director	Experiment Number
CMA 102 (T1856)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth *
	Day 1	Day 2	Total	% Control	Ave. V. C.	% Control	
0.016 µl/ml	0.061	0.070	0.0	0%	++	0.0 %	0%
0.012 µl/ml	0.036	0.030	0.0	0%	++	0.0 %	0 %
0.0089 µl/ml	0.608	0.921	6.2	37 %	182	97 %	35 %
0.0067 µl/ml	0.776	1.119	9.6	~~54~~ 57%	192	102 %	58 %
0.0050 µl/ml	0.836	1.201	11.2	66 %	191	100 %	67 %
0.0038 µl/ml	0.900	1.395	14.0	82 %	192	102 %	84 %
0.00284 µl/ml	0.992	1.333	14.7	86 %	174	93 %	80 %
0.0021 µl/ml	1.001	1.335	14.8	87 %	182	97 %	85 %
~~0.0016~~ 0.0016 µl/ml entry error KJP 2/22/83	0.990	1.406	15.5	91 %	203	108 %	98 %
0.0012 µl/ml	0.862	1.398	13.4	79 %	200	106 %	84 %
0.00089 µl/ml	0.959	1.440	15.3	90 %	171	91 %	82 %
0.00067 µl/ml	0.995	1.398	15.5	91 %	202	107 %	98 %
SOLVENT 1	0.989	1.576	17.3	17.0	184	188	
SOLVENT 2	1.061	1.410	16.6		192		

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost

++ Too Toxic to Clone

@ entry error KJP 2/22/83

Tables prepared and calculations performed by: Karen Jan Bond 2/22/83
(Signature & Date)

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Study No. 1856.701006

Form No. WI-229

Table 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1856.701006 (Study Number) | Paul E Kirby (Study Director) | 1856B2 (Experiment No.)

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml	0.603	0.701	4.7	33 %	18	11 %	3 %
0.5 µl/ml	0.860	1.155	11.0	77 %	125	74 %	57 %
SOLVENT 1	1.075	1.220	14.6	14.4	172	168	
SOLVENT 2	1.071	1.189	14.2		163		
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml							
5.0 µg/ml							
SOLVENT 1	Not Applicable		Karen J. Bonn		2/22/83		
SOLVENT 2							

Tables prepared and calculations performed by: Karen Joan Bonn 2/22/83
(Signature & Date)

\+ Culture Lost
++ Too Toxic to Clone

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Study No. T1856 701006

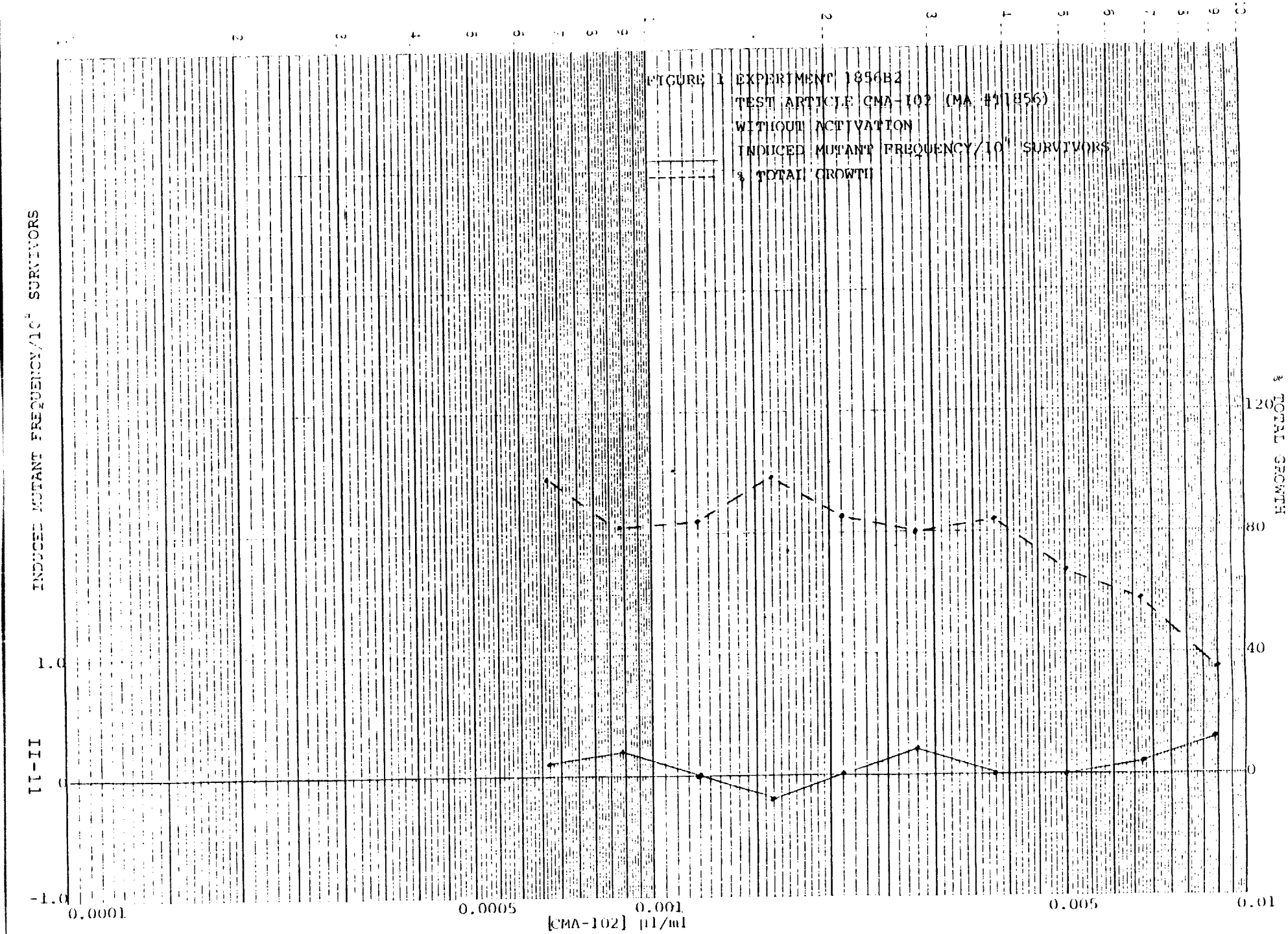
FIGURE 1 EXPERIMENT 1856B2
TEST ARTICLE CMA-102 (MA #11856)
WITHOUT ACTIVATION
INDUCED MUTANT FREQUENCY/10⁶ SURVIVORS
% TOTAL GROWTH
INDUCED MUTANT FREQUENCY/10⁶ SURVIVORS
% TOTAL GROWTH
1.0
0
-1.0
120
80
40
0
0.0001
0.0005
0.001
0.005
0.01
[CMA-102] µl/ml

Table 6

Study No. T1856 701006

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1856.701006
Study Number

Paul E. Kirby
Study Director

10.0 ug/ml to 0.001 ug/ml
Dose Range

CMA-102 (T1856)
Test Article Identity

Acetone
Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation					
	Solvent 1				
	Solvent 2				
With S-9 Activation	10 ug/ml	0.491	0.806	*0.0	0%
	5.0 ug/ml	0.199	0.285	0.0	0%
	1.0 ug/ml	0.010	0.015	0.0	0%
	0.5 ug/ml	0.007	0.025	0.0	0%
	0.1 ug/ml	0.012	0.070	0.0	0%
	0.05 ug/ml	0.021	0.017	0.0	0%
	0.01 ug/ml	0.886	1.440	14.7	99%
	0.005 ug/ml	0.906	1.400	14.1	99%
	0.001 ug/ml	0.957	1.437	15.3	1[illegible]%
	Solvent 1	0.847	1.446	13.6 } 14.3	
	Solvent 2	0.927	1.451	14.9	

Not Applicable - Eleanor M. Cory 2-25-83

+ Culture Lost

* counts indicated debris, not cells 2-25-83

Table Prepared By Leah Griswold 2/25/83
Signature Date

Form No. MA-127
2/16/83

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Table 7

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1856 701006	Paul E Kirby	1856 B3
Study Number	Study Director	Experiment Number
CMA 102 (T1856)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced Mutant Frequency *
0.021 ug/ml	137	+	+	137	153	176	187	172	1.6	0.9
0.016 ug/ml	73	+	+	73	235	219	231	228	0.6	-0.1
0.012 ug/ml	103	97	+	100	~~231~~ ⊗ 226	239	211	225	0.9	0.2
0.0089 ug/ml	79	+	+	79	208	202	+	205	0.8	0.1
0.0067 ug/ml	66	+	+	66	218	234	211	221	0.6	-0.1
0.0050 ug/ml	76	66	73	72	226	214	194	211	0.7	0.0
0.0038 ug/ml	67	65	+	66	218	195	+	207	0.6	-0.1
0.0028 ug/ml	73	+	+	73	200	183	220	201	0.7	0.0
0.0021 ug/ml	78	63	74	72	235	218	183	212	0.7	0.0
0.0016 ug/ml	86	54	+	70	233	221	+	227	0.6	-0.1
					~~191~~ ⊗					
Solvent 1	65	54	74	64	196	176	201	191	0.7 } 0.7	~~0.7~~ ⊗
Solvent 2	79	55	62	65	195	196	170	187	0.7	

Plates Counted By: [signature] 3/15/83 (Signature & Date)

Calculations Performed By: Karen J. Poms 3/15/83 (Signature & Date)

Recorded by Karen J Poms

⊗ entry error KJP 3/15/83

Study No. T1856 701005

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TABLE 8

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1856.701006	Paul E Kirby	1856B3
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced Mutant Frequency*
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml					KJP	3/15/83				
0.5µl/ml										
SOLVENT 1	Not Applicable									
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	287	317	⊛ ~~2~~ +	302	127	113	136	125	4.8	4.2
5.0 µg/ml	+	+	+		238	197	+	218		
SOLVENT 1	41	46	49	45	210	258	225	231	0.4	0.6
SOLVENT 2	56	53	57	56	153	149	+	151	0.7	

Plates Counted By: [signature] 3-15-83 (Signature & Date)

Calculations Performed By: Andrea N Rogers-Bank (Signature & Date) 3/15/83

Recorded by Karen J Ferns 3/15/83

⊛ entry error KJP 3/15/83

*Per 10^4 surviving cells

+ Culture Lost

Study No. T1856 701006

Form No. MA-228 2/16/83

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Table 9

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1856.701006	Paul E Kirby	1856B3
Study Number	Study Director	Experiment Number
CMA102 (T1856)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^5 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.038 ul/ml	0.008	0.023	0.0	0%	++		
0.028 ul/ml	0.019	0.044	0.0	0%	++		
0.021 ul/ml	0.379	1.026	4.3	25%	172	91%	22%
0.016 ul/ml	0.754	1.633	13.7	78%	228	121%	94%
0.012 ul/ml	0.782	1.604	13.9	79%	225	119%	94%
0.0089 ul/ml	0.818	1.644	14.9	85%	205	108%	92%
0.0067 ul/ml	0.901	1.568	15.7	89%	221	117%	104%
0.0050 ul/ml	0.880	1.646	16.1	91%	211	112%	102%
0.0038 ul/ml	0.885	1.666	16.4	93%	207	110%	102%
0.0028 ul/ml	0.832	1.636	15.1	86%	201	106%	91%
0.0021 ul/ml	0.866	1.718	16.5	94%	212	112%	105%
0.0016 ul/ml	0.916	1.683	17.1	97%	227	120%	117%
SOLVENT 1	0.959	1.682	17.9	17.6	191	89	
SOLVENT 2	0.942	1.641	17.2		187		

* % Total Growth = $\frac{(\%\text{ Suspension Growth})\ (\%\text{ Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: Anita M Fuyes-Brooks
(Signature & Date) 3/16/83

Study No. T1856.701006

Form No. MA-229
2/16/83

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Table 10

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1856 701006
Study Number

Paul E Kirby
Study Director

T1856B3
Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V.C.	% Control	% Total Growth
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml	not applicable MRB 2/16/83						
0.5 µl/ml							
SOLVENT 1							
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.700	1.667	13.0	74%	125	65%	48%
5.0 µg/ml	0.762	1.473	12.5	71%	218	114%	81%
SOLVENT 1	1.018	1.585	17.9 } 17.5		231 } 191		
SOLVENT 2	0.944	1.630	17.1 }		151 }		

Tables prepared and calculations performed by: Andrea N Rogers-Brown

(Signature & Date) 3/16/83

Study No. T1856 701006

\+ Culture Lost

++ Too Toxic to Clone

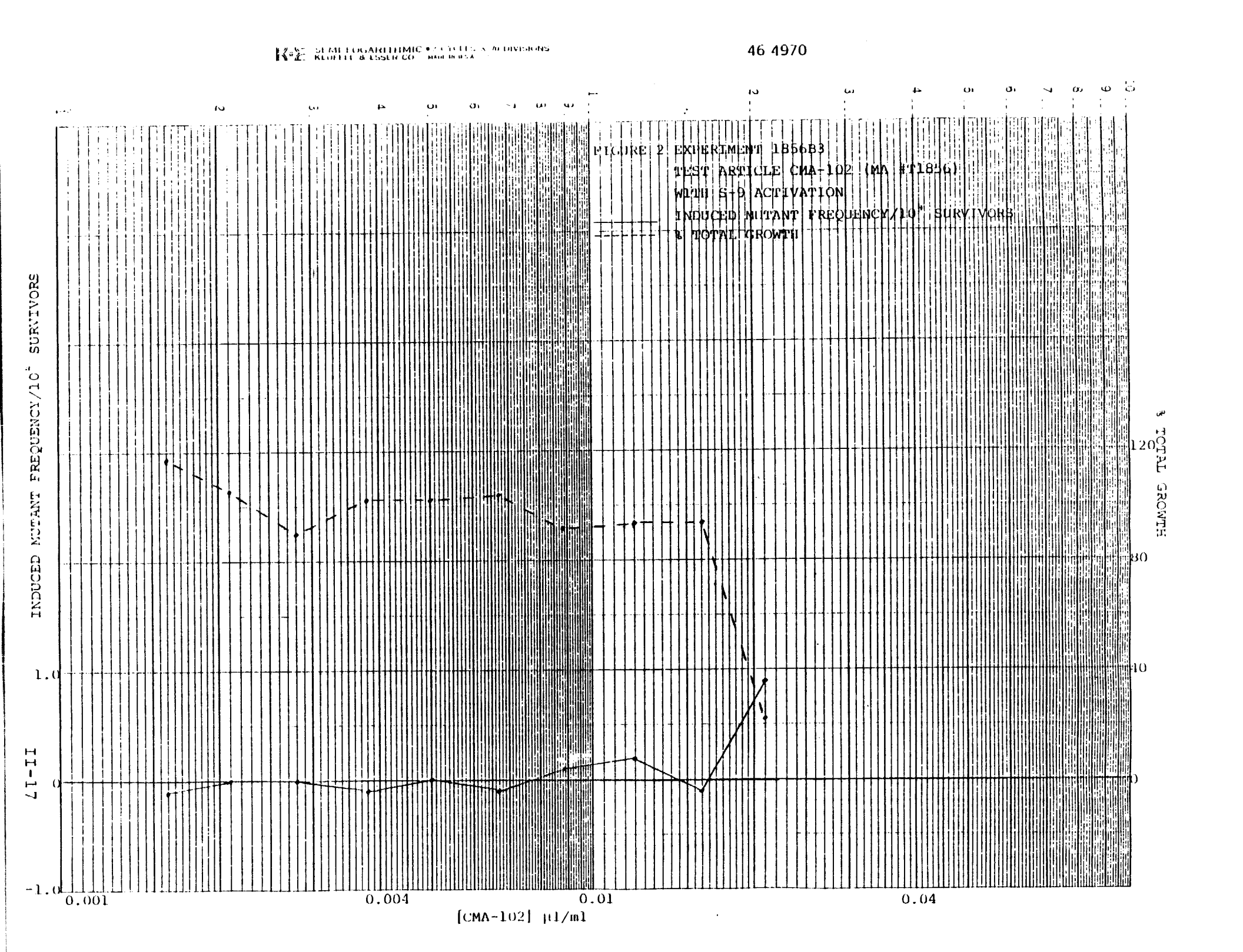
FIGURE 2 EXPERIMENT 1856B3
TEST ARTICLE CMA-102 (MA #T1856)
WITH S+9 ACTIVATION
INDUCED MUTANT FREQUENCY/10⁵ SURVIVORS
% TOTAL GROWTH
INDUCED MUTANT FREQUENCY/10⁵ SURVIVORS
% TOTAL GROWTH
[CMA-102] µl/ml
0.001
0.004
0.01
0.04
1.0
0
-1.0
120
80
40
0

TABLE 11

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

Study Number : T1856.701006
Test Article : CMA-102
Activation : Induced Rat Liver S-9
Trial Number : 4
Solvent : Acetone
Operator : Janine L. Johnson
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 09/30/83 2:58 pm

Test Art Concentr (µl/ml)		Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
.022	A	136	± 5	169	± 8	1.6	1.1	29
.022	B	230	±16	97	±12	4.7	4.2	3
.022	C	175	± 4	122	± 7	2.9	2.4	5
.021	A	112	± 9	179	±14	1.3	0.8	50
.021	B	+		134	± 0			
.021	C	+		+				
.02	A	128	± 3	194	± 7	1.3	0.8	46
.02	B	115	± 9	190	± 3	1.2	0.7	58
.02	C	150	±12	173	±10	1.7	1.2	29
.019	A	+		+				
.019	B	+		+				
.019	C	106	± 2	195	± 0	1.1	0.6	61
.018	A	+		+				
.018	B	141	±17	190	± 9	1.5	1.0	34
.018	C	+		178	±10			
.017	A	108	± 4	194	± 9	1.1	0.6	71
.017	B	+		+				
.017	C	135	± 1	191	±15	1.4	0.9	37
Solvent 1		53	± 5	204	±11	0.5		
Solvent 2		53	± 9	212	±11	0.5		
Solvent 3		+		+				

+ Culture Lost * Per 10^4 surviving cells

Table Prepared By: Janine L. Johnson 9-30-83
Signature Date

Workbook No. 119

TABLE 12

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

Study Number : T1856.701006
Test Article : CMA-102
Activation : Induced Rat Liver S-9
Trial Number : 4
Solvent : Acetone
Operator : Janine L. Johnson
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 09/30/83 3:00 pm

Test Art Concentr (µl/ml)		Cell Concentr (X 10⁵) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
.024	A	0.039	0.021	0.0	0	++		
.024	B	0.033	0.009	0.0	0	++		
.024	C	0.021	0.008	0.0	0	++		
.023	A	0.053	0.103	0.0	0	++		
.023	B	0.044	0.040	0.0	0	++		
.023	C	0.063	0.167	0.0	0	++		
.022	A	0.417	1.390	6.4	36	169	81	29
.022	B	0.116	0.364	1.2	7	97	47	3
.022	C	0.131	0.488	1.6	9	122	59	5
.021	A	0.558	1.661	10.3	58	179	86	50
.021	B	0.197	0.761	2.5	14	134	64	9
.021	C	0.178	0.666	2.2	12	+		
.02	A	0.512	1.555	8.8	49	194	93	46
.02	B	0.610	1.680	11.4	64	190	91	58
.02	C	0.416	1.322	6.1	34	173	83	28
.019	A	0.684	1.715	13.0	73	+		
.019	B	0.684	1.434	10.9	61	+		
.019	C	0.631	1.647	11.5	65	195	94	61
.018	A	0.543	1.716	10.4	58	+		
.018	B	0.403	1.444	6.5	37	190	91	34
.018	C	0.424	1.558	7.3	41	178	86	35
.017	A	0.728	1.676	13.6	76	194	93	71
.017	B	0.407	1.480	6.7	38	+		
.017	C	0.447	1.430	7.1	40	191	92	37
Solvent 1		.997	1.612	17.9		204		
Solvent 2		.959	1.662	17.7		212		
Solvent 3		.991	1.619	17.8		+		

+ Culture Lost ++ Too Toxic To Clone

Table Prepared By: Janine L. Johnson 9-30-83
Signature Date

Workbook No. 120 Report Page No. II-19

TABLE 13

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
POSITIVE CONTROL - WITH ACTIVATION

Study Number : T1856.701006
Test Article : 7,12 Dimethylbenz(a)anthracene
Activation : With activation
Trial Number : 4
Solvent : Acetone
Operator : Janine L. Johnson
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 09/30/83 3:02 pm

CLONING DATA

Test Art Concentr (µl/ml)	Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
7.5	305	± 5	81	± 0	7.5	6.9	10
5	290	± 5	142	±10	4.1	3.5	47
Solvent 1	65	± 2	189	± 5	0.7		
Solvent 2	52	± 6	192	±12	0.5		

TOTAL COMPOUND TOXICITY DATA

Test Art Concentr (µl/ml)	Cell Concentr (X 10⁴) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
7.5	0.472	0.835	4.4	23	81	43	10
5	0.743	1.435	11.8	63	142	75	47
Solvent 1	1.024	1.635	18.6		189		
Solvent 2	1.007	1.688	18.9		192		

* Culture Lost ‡ Per 10^4 surviving cells

Table Prepared By: Janine L. Johnson 9-30-83
Signature Date

Workbook No. 121 Report Page No. II-20

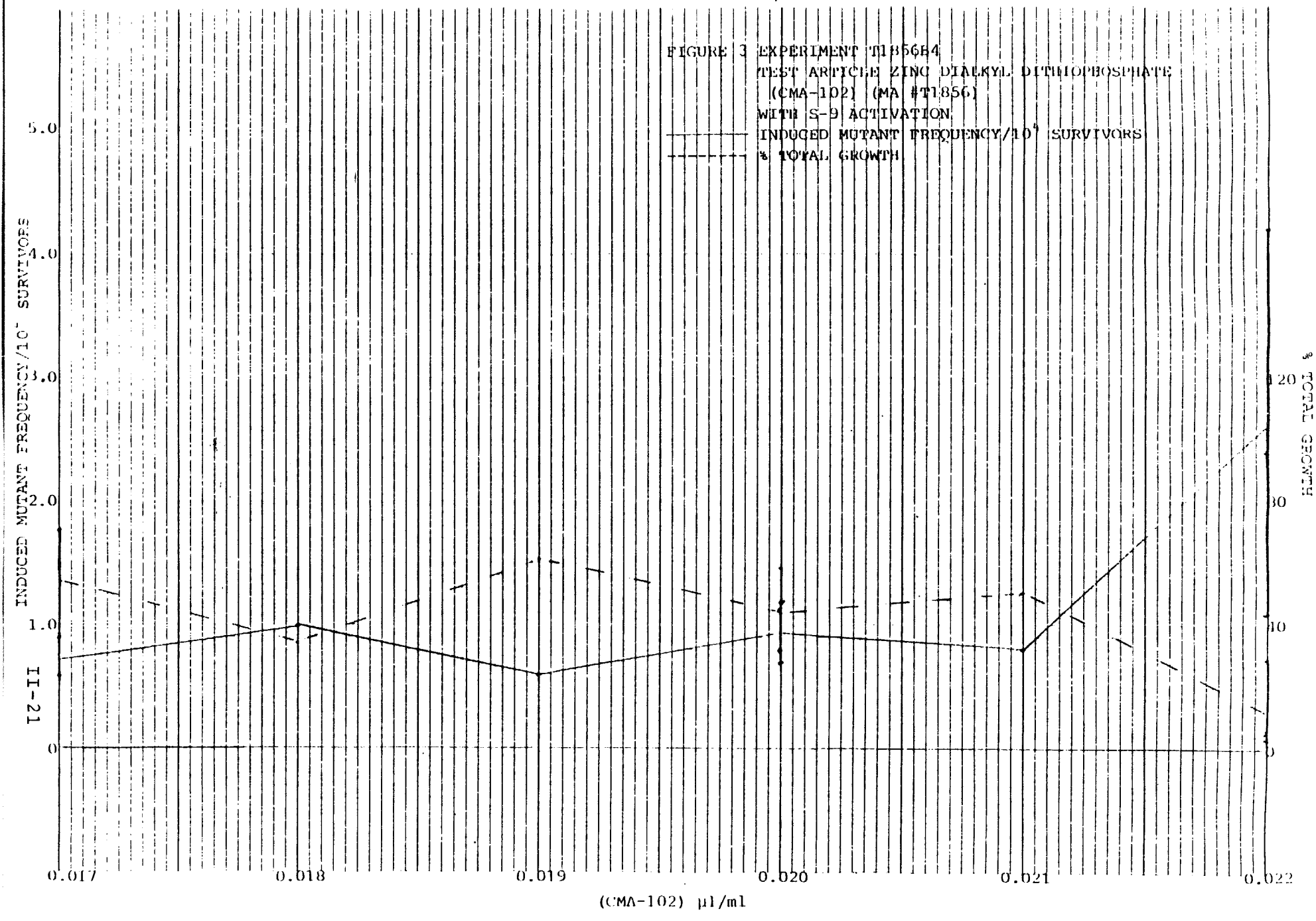
FIGURE 3 EXPERIMENT T1856B4
TEST ARTICLE ZINC DIALKYL DITHIOPHOSPHATE
(CMA-102) (MA #T1856)
WITH S-9 ACTIVATION
INDUCED MUTANT FREQUENCY/10⁴ SURVIVORS
% TOTAL GROWTH
INDUCED MUTANT FREQUENCY/10⁴ SURVIVORS
% TOTAL GROWTH
5.0
4.0
3.0
2.0
1.0
0
120
110
100
90
0.017
0.018
0.019
0.020
0.021
0.022
(CMA-102) µl/ml

Form No. MA-127
2/16/83

Table 14

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

1856.701006	Paul E. Kisby	1856B5
Study Number	Study Director	Experiment Number
CMA-102 (T1856)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced Mutant* Frequency
0.021B ml/ml	318	282	304	301	74	75	81	77	7.9	7.2
0.018A ml/ml	238	232	263	244	128	117	127	124	3.9	3.2
0.018B ml/ml	197	207	163	189	171	172	183	175	2.2	1.5
0.018C ml/ml	231	258	224	238	152	170	191	171	2.8	2.1
0.017A ml/ml	226	+	+	226	171	190	+	181	2.5	1.8
0.017B ml/ml	227	+	+	227	179	163	+	171	2.7	2.0
0.017C ml/ml	239	253	230	241	159	153	153	155	3.1	2.4
Solvent 3	~~72~~ 68	72	81	74	171	185	174	177	0.8	
Solvent 1	64	52	57	58	222	219	187	209	0.6 0.7	
Solvent 2	53	78	+	66	187	206	+	197	0.7	

Plates Counted By: Terence R O'Keefe 10/18/83 (Signature & Date)

Calculations Performed By: Terence R. O'Keefe 10/18/83 (Signature & Date)

* Per 10^4 surviving cells

\+ Culture Lost

* entry error TRK 10/18/83

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Study No. T1856.701006

Form No. MA-128 2/16/83

Workbook Page No. 153

Report Page No. II-23

Table 15

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

1856.701006 — Study Number

Paul E. Kirby — Study Director

1856B5 — Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1										
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	244	262	239	248	26	12	31	23	21.6	20.5
5.0 µg/ml	483	463	+	473	103	114	123	113	8.3	7.2
SOLVENT 1	89	93	104	95	151	164	142	152	1.3) 1.1	
SOLVENT 2	91	87	86	88	199	184	174	186	0.9)	

not applicable dmKB 10/20/83

Plates Counted By: As copied from audit trail TAK 10/10/83 (Signature & Date)

Calculations Performed By: Terrence P O'Kelly 10/18/83 (Signature & Date)

*Per 10^4 surviving cells

+ Culture lost

Form No. MA-228
2/16/83

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Table 16

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

1856.701.006	Paul E. Kirby	185685
Study Number	Study Director	Experiment Number
CMA-102(T1856)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^5 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.022A ul/ml	0.082	0.039	++				
0.022B ul/ml	0.068	0.033	++				
0.022C ul/ml	0.082	0.060	++				
0.021A ul/ml	0.095	0.129	++	7%**			3%**
0.021B ul/ml	0.1350** 098	0.321	2.41**.07	15%	74	38%	5.7%
0.021C ul/ml	0.088** 0.135	0.083	++				
0.020A ul/ml	0.088	0.079	++				
0.020B ul/ml	0.088	0.123	++				
0.020C ul/ml	0.082	0.057	++				
0.019A ul/ml	0.144	0.264	++				
0.019B ul/ml	0.138	0.296	++				
0.019C ul/ml	0.114	0.126	++				
SOLVENT 1							
SOLVENT 2							

Study No. T1856.701.006

* % Total Growth = (% Suspension Growth) (% Cloning Growth) / 100

\+ Culture Lost
++ Too Toxic to Clone

* rounding error dmkb 10/20/83

** entry error dmkb 5/25/84

Tables prepared and calculations performed by: Terrence R. O'Keefe 10/19/83
(Signature & Date)

Table 16 cont.

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

1856.701006	Paul E. Kirby	1856B5
Study Number	Study Director	Experiment Number
CMA-102 (T1856	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.018A ml/ml	0.290	0.785	2.7	17%	124	64%	11%
0.018B ml/ml	0.413	0.886	4.1	26%	175	90%	24%
0.018C ml/ml	0.442	1.043	5.1	33%	171	88%	29%
0.017A ml/ml	0.546	1.202	~~0.8~~ 7.3	47%	181	93%	44%
0.017B ml/ml	0.493	1.053	5.8	37%	171	88%	33%
0.017C ml/ml	0.292	0.818	2.8	18%	155	80%	14%
SOLVENT 3	0.966	1.486	15.9		177		
SOLVENT 1	0.884	1.482	14.6 } 15.5		209 } 194		
SOLVENT 2	0.956	1.514	16.1		197		

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost

++ Too Toxic to Clone

Tables prepared and calculations performed by: Terrence R. O'Keefe 10/19/83

(Signature & Date)

Study No. T1856.701006

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Form No. MA-228
2/16/83

Form No. MA-229

Table 17

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

1856.701006 Study Number | Paul E. Kirby Study Director | 185613S Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V.C.	% Control	% Total Growth
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml	not applicable due to		5/8/84				
0.5 µl/ml							
SOLVENT 1							
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.378	0.431	1.8	12%	23	14%	2%
5.0 µg/ml	0.642	1.030	2.3	48%	113	67%	32%
SOLVENT 1	0.993	1.443	15.9)15.4		152)169		
SOLVENT 2	0.970	1.377	14.8)		186)		

Tables prepared and calculations performed by: Terrence P. O'Kelly 10/2/84
(Signature & Date)

\+ Culture Lost
++ Too Toxic to Clone

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Study No. 1856.701006

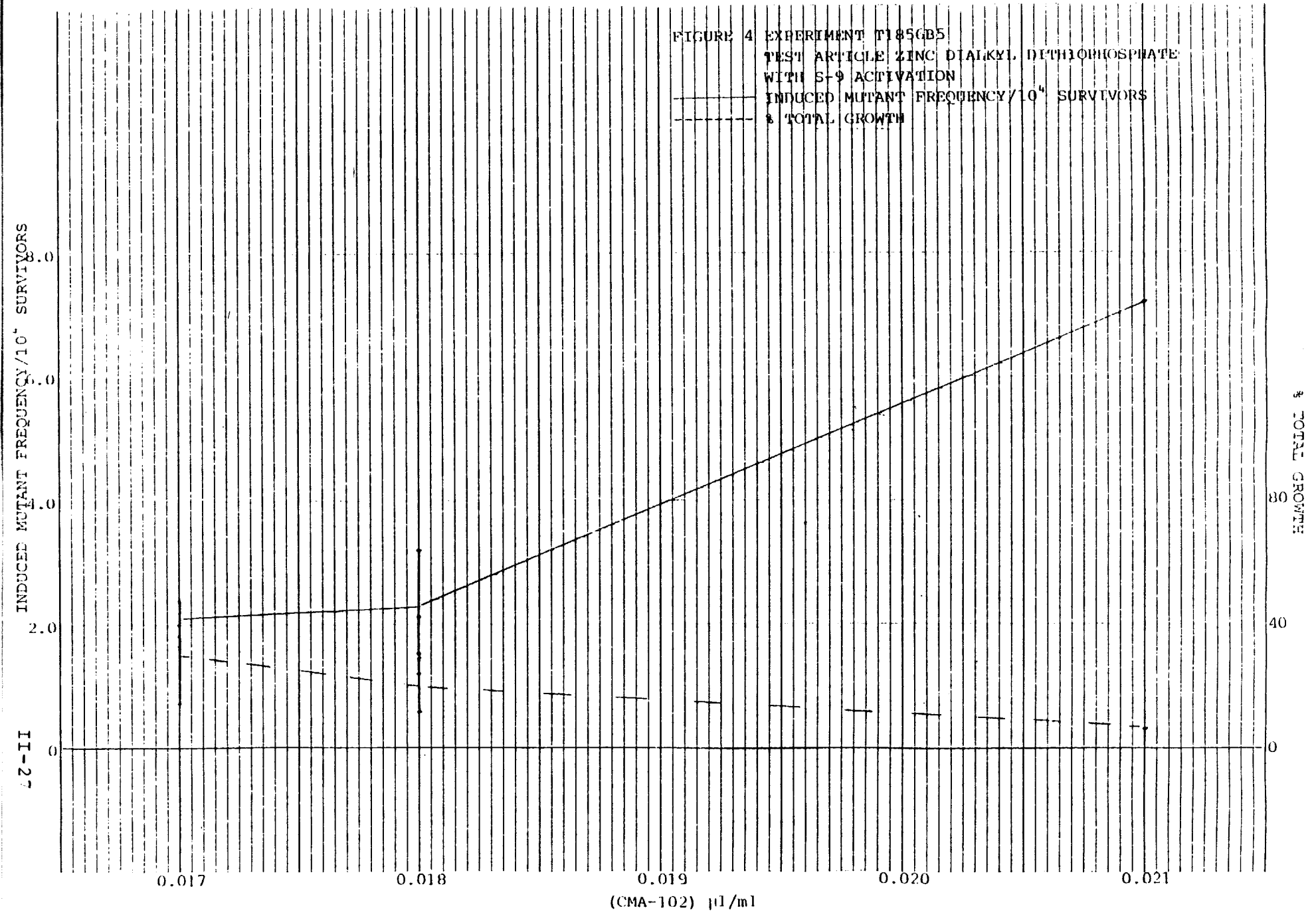


FIGURE 4 EXPERIMENT T185GB5
TEST ARTICLE ZINC DIALKYL DITHIOPHOSPHATE
WITH S-9 ACTIVATION
INDUCED MUTANT FREQUENCY/10⁴ SURVIVORS
% TOTAL GROWTH
INDUCED MUTANT FREQUENCY/10⁴ SURVIVORS
8.0
6.0
4.0
2.0
0
% TOTAL GROWTH
80
40
0
0.017
0.018
0.019
0.020
0.021
(CMA-102) µl/ml

TABLE 18

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL COMPOUND TOXICITY TEST

Study Number : T2254.701012
Test Article : CMA 1028
Activation : Induced Rat Liver S-9
Trial Number : 1
Dose Range : 10 - 0.001 microliters/ml
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 01/27/84 1:58 pm

Test Article Concentration (µl/ml)	Cell Concentration (X 10⁴) Day 1	Day 2	Suspension Growth Total	% of Control
WITH S-9 ACTIVATION				
10	0.436	0.375	1.8	12
5	0.257	0.345	1.2	8
1	0.022	0.023	0.0	0
.5	0.015	0.023	0.0	0
.1	0.040	0.033	0.0	0
.05	0.056	0.028	0.0	0
.01	0.812	1.417	12.8	83
.005	0.922	1.426	14.6	95
.001	1.031	1.361	15.6	101
Solvent 1	0.979	1.457	15.8	
Solvent 2	1.010	1.334	15.0	

+ Culture Lost

Table Prepared By: Terrence R O'Keefe 1/27/84
Signature Date

Workbook No. 15 Report Page No. II-28

TABLE 19

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

Study Number : T2254.701012
Test Article : CMA 102B
Activation : Induced Rat Liver S-9
Trial Number : 1
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 12:14 am

Test Art Concentr (µl/ml)	Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
.021	130	±40	134	±15	1.9	0.9	27
.016	123	± 7	136	± 3	1.8	0.8	61
.012	82	± 8	138	± 5	1.2	0.2	77
.0089	77	± 4	133	±12	1.2	0.2	77
.0067	69	± 6	132	± 5	1.0	0.0	62
.005	77	± 7	151	±13	1.0	0.0	91
.0038	100	± 6	158	±13	1.3	0.3	96
.0028	79	± 7	138	± 3	1.1	0.1	90
.0021	68	± 5	143	±12	1.0	0.0	89
.0016	72	± 5	145	± 2	1.0	0.0	91
Solvent 1	72	± 5	159	± 4	0.9		
Solvent 2	79	± 3	149	± 1	1.1		

+ Culture Lost * Per 10^4 surviving cells

Table Prepared By: Terrence R. O'Keefe 2/21/84
Signature Date

TABLE 20

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

Study Number : T2254.701012
Test Article : CMA 102B
Activation : Induced Rat Liver S-9
Trial Number : 1
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 12:16 am

Test Art Concentr (µl/ml)	Cell Concentr (X 10^4) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
.038	0.028	0.036	0.0	0	++		
.028	0.131	0.188	0.0	0	++		
.021	0.356	1.032	4.1	31	134	87	27
.016	0.610	1.354	9.2	69	136	88	61
.012	0.721	1.433	11.5	86	138	90	77
.0089	0.752	1.429	11.9	89	133	86	77
.0067	0.626	1.377	9.6	72	132	86	62
.005	0.793	1.408	12.4	93	151	98	91
.0038	0.809	1.391	12.5	93	158	103	96
.0028	0.814	1.484	13.4	100	138	90	90
.0021	0.808	1.433	12.9	96	143	93	89
.0016	0.828	1.411	13.0	97	145	94	91
Solvent 1	.846	1.389	13.1		159		
Solvent 2	.842	1.457	13.6		149		

+ Culture Lost ++ Too Toxic To Clone

Table Prepared By: Terrence R. O'Keefe 2/21/84
Signature Date

Workbook No. 45 Report Page No. II-30

Table 21

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
POSITIVE CONTROL - WITH ACTIVATION

Study Number : T2254.701012
Test Article : 7,12 Dimethylbenz(a)anthracene
Activation : With activation
Trial Number : 1
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 12:18 am

CLONING DATA

Test Art Concentr (µg/ml)	Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
7.5	303	±12	81	±11	7.5	6.5	22
5	259	±16	93	± 6	5.6	4.6	45
Solvent 1	58	± 1	130	± 6	0.9		
Solvent 2	71	± 5	133	± 5	1.1		

TOTAL COMPOUND TOXICITY DATA

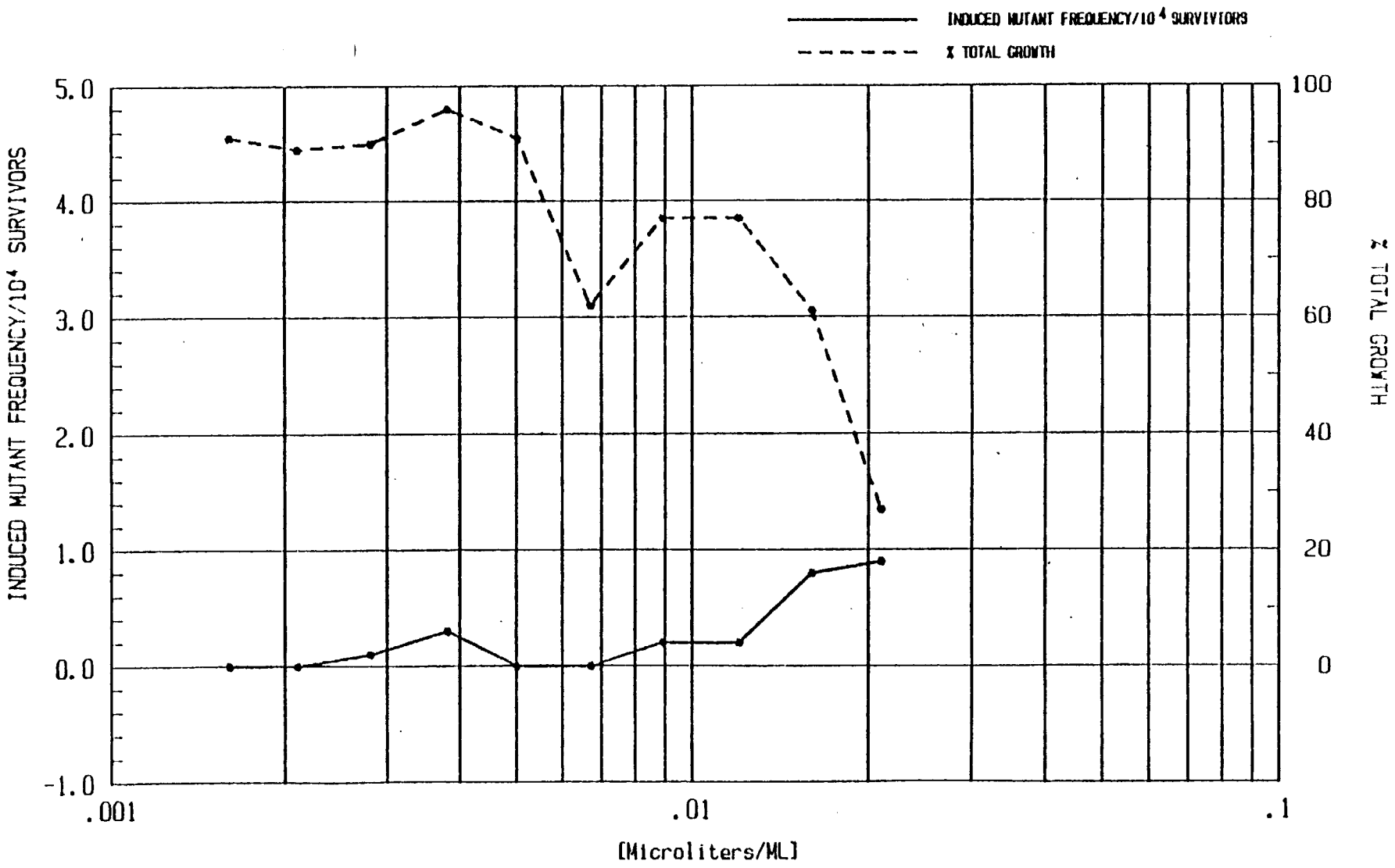
Test Art Concentr (µg/ml)	Cell Concentr (X 10*) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
7.5	0.481	0.918	4.9	36	81	62	22
5	0.636	1.205	8.5	63	93	71	45
Solvent 1	.857	1.342	12.8		130		
Solvent 2	.893	1.422	14.1		133		

* Culture Lost # Per 10^4 surviving cells

Table Prepared By: Terrence R O'Keefe 2/21/84
Signature Date

Workbook No. 46 Report Page No. II-31

FIGURE 5
Study No. T2254.701012 (Trial 1)
Test Article CMA 102B
With S-9 Activation



Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-102 (MA #T1856) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest test article concentration cloned in the S-9 activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. None of the nonactivated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-102 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of exogenous metabolic activation.

Three follow-up assays were conducted on test article Zinc Dialkyl Dithiophosphate (CMA-102 & CMA-102B) (MA #'s T1856 and T2254). In the first assay, eleven of eighteen cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The assay was repeated due to some contamination and an erratic dose-response in toxicity in the treated cultures. All the cultures that were cloned exhibited mutant frequencies which were more than twice the mean mutant frequency of the solvent controls. A second sample (MA #T2254) of the test article was tested concurrently with Calcium Dialkyl Dithiophosphate (MA #T2253). None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. However, a dose-related response was noted.

The results of all assays indicate that Zinc Dialkyl Dithiophosphate (MA #'s T1856 and T2254) produced a positive response in the presence of exogenous metabolic activation and a negative response in the absence of metabolic activation.

APPENDIX

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PROTOCOL AMENDMENT

Date: October 6, 1983

Sponsor: Chemical Manufacturers Association

Test Article Designation: CMA-102

Study No.: T1856.701006

Protocol No.: SPGT701006

Protocol Title: L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay

1. Section 6.1 Dosages, sentence 5 should read, "The test article will be solubilized and 14 doses in triplicate will be selected for treatment".

Reason for Amendment:

A previous study had shown a broad range in toxicity. Fourteen doses in triplicate were chosen to narrow the range.

APPROVAL:

10/6/83
Paul E. Kirby, Ph.D. Date
Study Director

10/13/83
Carol R. Stack, Ph.D. Date
Study Coordinator

Rec'd by
RA/QA 12/8/82.
N.K/jks

RECEIVED OCT 0 5 1982

L5178Y TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-102

.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source
The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:
$2,650

Total charge for assay with metabolic activation:
$2,650

6.1 Dosages
For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intra-peritoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-amino-anthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_2O, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0×10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F_0P will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3×10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3×10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2×10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of

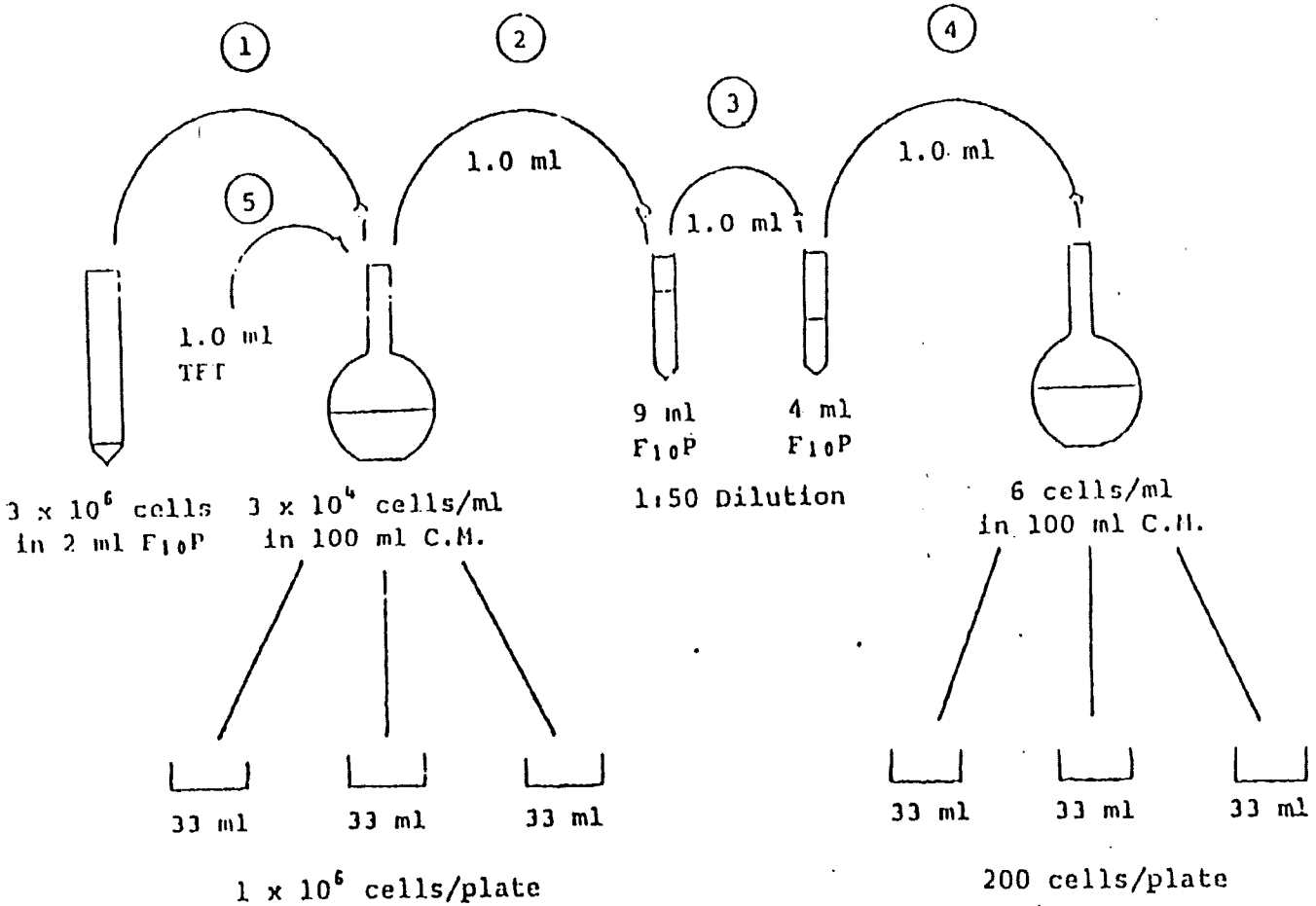

Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

MICROBIOLOGICAL ASSOCIATES

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency?_____
If so, to which agency or agencies? _____

Does the sponsor request that samples of the Test Article dosing solutions be returned?_____

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: ~~11/21/82~~ 11/21/82 pk 3/22/83 pk 4/6/83

13.2 Proposed Initiation Date: ~~December 28, 1982~~ 1/12/83 pk 4/6/83

13.3 Scheduled Completion Date: February 18, 1983

13.4 Report Submission to Sponsor Date: February 18, 1983

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

Carol Stark
SPONSOR'S AUTHORIZED REPRESENTATIVE

11/22/82
DATE PROTOCOL APPROVED BY SPONSOR

Paul E. Kinley — 11/24/82
STUDY DIRECTOR — DATE

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136) and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \text{Average Counts (corrected for coincidence)} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{cells/ml})\ (20\ \text{ml})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = 20\ \text{ml} - \text{Volume of Cells Retained}$$

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2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

---

3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138) and L5178Y TK+/- Assay - Daily Counts (Positive Controls) (Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^{5} = \text{Average Counts (corrected for coincidence)} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{Cells/ml})\ (20\ \text{ml}^{*})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20\ \text{ml}^{*}) - (\text{Volume of Cells Retained})$$

* The final volume is adjusted to 10 ml on the third day of expression.

---

4. Tables 2, 3, and 4 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^{6} \text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left(\text{Mutant Frequency of Treated Cultures}\right) - \left(\text{Average Mutant Frequency of Solvent Controls}\right)$$

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5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than 0.3×10^{5} cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

$$\text{\% Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\text{\% Growth} = \frac{(\text{\% Suspension Growth})\ (\text{\% Cloning Growth})}{100}$$

Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-105 (MA #T1866) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 11% to 98% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 59% to 101% Total Growth.

The highest test article concentration cloned in the S-9 activated cultures exhibited a mutant frequency which was twice the mean mutant frequency of the solvent controls. None of the nonactivated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that under the conditions of this test, the test article CMA-105 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

Results

The Initial Toxicity Test (Table 1) performed on test article CMA-105 (MA #T1866) in the absence of S-9 indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, 0.0012, 0.00089 or 0.00067 µl/ml test article. These concentrations produced a range in Suspension Growth of 21% to 98%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 11% to 98%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-105 (MA #T1866). The results indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.021, 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021 or 0.0016 µl/ml. These concentrations produced a range in Suspension Growth of 68% to 97%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. The Cloning

Data and Total Compound Toxicity Data are also presented graphically in Figure 2. One culture (0.021 µl/ml) that was cloned exhibited a mutant frequency which was 2.0 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 59%.

None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 78% to 101%.

Study No. T1866 701 006

Table 1

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1866.701006	Paul E Kirby	100 µl/ml to 0.001 µl/ml
Study Number	Study Director	Dose Range
CMA-105 (T1866)		Acetone
Test Article Identity		Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation	100 µl/ml	0.807 *	0.191	0.0	0%
	10 µl/ml	0.440 *	0.093	0.0	0%
	5.0 µl/ml	0.132	0.161	0.0	0%
	1.0 µl/ml	0.009	0.009	0.0	0%
	0.5 µl/ml	0.009	-0.001	0.0	0%
	0.1 µl/ml	0.011	0.016	0.0	0%
	0.05 µl/ml	0.030	0.028	0.0	0%
	0.01 µl/ml	0.631	0.915	6.4	45%
	0.005 µl/ml	0.989	1.169	12.8	91%
	0.001 µl/ml	0.957	1.312	14.0	99%
	Solvent 1	0.962	1.283	13.7 } 14.1	
	Solvent 2	1.004	1.289	14.4 }	
With S-9 Activation					
	Solvent 1				
	Solvent 2				

Not applicable JC 1-21-83

+ Culture Lost * observation showed no cells present JC 1-21-83

Table Prepared By [signature] 1-21-83

++ entry error JC 1-21-83

Signature Date

Form No. WL-233 Workbook Page No. 13 Report Page No. III-4

Form No. MZ-41
2/25/82

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1866-701006	Paul E. Kirby	1866B1
Study Number	Study Director	Experiment Number
CMA-105 (T1866)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
0.0089ul/ml	41	35	36	37	81	111	123	105	0.7	0.3
0.007ul/ml	36	44	29	36	177	160	198	178	0.4	0.0
0.0050ul/ml	40	34	41	38	194	172	176	181	0.4	0.0
0.0038ul/ml	29	25	37	30	177	162	170	170	0.4	0.0
0.0028ul/ml	36	46	44	42	199	195	188	194	0.4	0.0
0.0021ul/ml	35	33	33	34	195	218	219	211	0.3	-0.1
0.0016ul/ml	34	46	35	38	219	194	198	204	0.4	0.0
0.0012ul/ml	39	38	37	38	170	170	195	178	0.4	0.0
0.00089ul/ml	28	24	39	34	209	203	201	204	0.3	-0.1
0.0006ul/ml	30	49	45	41	188	215	200	201	0.4	0.0
Solvent 1	55	42	36	44	207	226	215	216	0.4	0.0
Solvent 2	38	38	36	37	198	169	212	191	0.4	

Plates Counted By: [signature] 2-7-83 (Signature & Date)

+ Calculations Performed By: [signature] 2-7-83 (Signature & Date)

* Per 10^4 surviving cells

\+ Culture Lost

Workbook Page No. 30

Report Page No. III-5

Study No. 1866

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1866.701006	Paul E. Kirby	1866B1
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	183	167	189	180	13	14	13	13	27.0	26.6
0.5µl/ml	417	403	406	409	81	87	88	85	9.6	9.2
SOLVENT 1	38	40	30	36	184	219	217	207	0.3	0.4
SOLVENT 2	48	48	42	46	219	210	191	207	0.4	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1										
SOLVENT 2										

Not applicable ff 2-7-83

Plates Counted By: Janine R. Johnson 2-7-83 (Signature & Date)

Calculations Performed By: Janine R. Johnson 2-7-83 (Signature & Date)

*Per 10^4 surviving cells

+ Culture Lost

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Study No. T1866 701006

Form No. WL-227
2/22/82

Workbook Page No. 32 Report Page No. III-7

Study No. T1866 701006

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1866.701006 — Study Number
Paul E. Kirby — Study Director
1866B1 — Experiment Number
CMA-105 (T1866) — Test Article Identity
Acetone — Solvent
None — Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.016 ul/ml	0.094	0.029			++		
0.012 ul/ml	0.169	0.093			++		
0.0089 ul/ml	0.479	0.749	4.0	21%	105	51%	11%
0.0067 ul/ml	0.695	1.072	7.7	40%	178	87%	35%
0.0050 ul/ml	0.815	1.332	12.1	62%	181	89%	55%
0.0038 ul/ml	0.957	1.516	16.1	84%	170	83%	70%
0.0028 ul/ml	1.027	1.466	16.7	87%	194	95%	82%
0.0021 ul/ml	1.030	1.603	18.3	95%	211	103%	98%
0.0016 ul/ml	1.130	1.505	18.9	98%	204	100%	98%
0.0013 ul/ml	1.096	1.543	18.8	97%	178	87%	85%
0.0008 ul/ml	1.103	1.534	18.8	97%	204	100%	97%
0.0007 ul/ml	1.011	1.638	18.9	95%	201	99%	94%
SOLVENT 1	1.029	1.630	18.6 / 19.3		216 / 204		
SOLVENT 2	1.146	1.572	20.0		191		

* % Total Growth = $\frac{(\%\text{ Suspension Growth})\ (\%\text{ Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: James D. Johnson 2-8-83
(Signature & Date)

Form No. WL-229

Table 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

71866.701006 Study Number	Paul E. Kirby Study Director	186631 Experiment No.

Test Article Concentration	Cell Concentration x 10⁻⁴/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V.C.	% Control	% Total Growth
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml	0.544	0.757	4.6	23%	13	6%	1%
0.5 µl/ml	0.815	1.357	12.3	61%	85	41%	25%
SOLVENT 1	1.141	1.637	20.8 } 20.1		207 } 207		
SOLVENT 2	1.097	1.581	19.3 }		207 }		
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml							
5.0 µg/ml							
SOLVENT 1							
SOLVENT 2							

Not applicable off d-8-83

Tables prepared and calculations performed by Janine L. Johnson 2-8-83 (Signature & Date)

\+ Culture Lost

++ Too Toxic to Clone

Workbook Page No. 33 Report Page No. III-8

Study No. T 1866 7/+/-/3

K&E SEMI LOGARITHMIC • 2 CYCLES X 70 DIVISIONS
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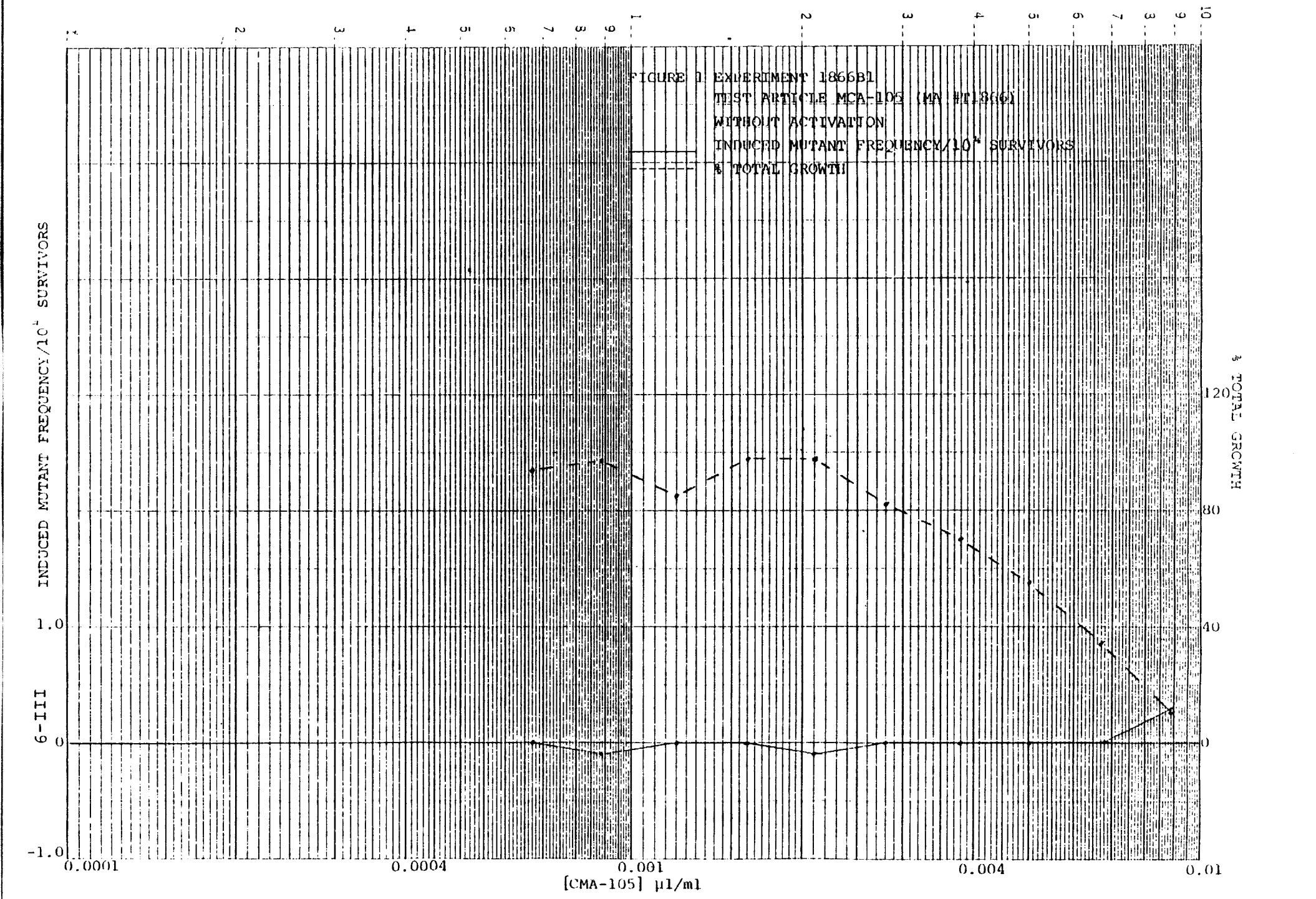

6-III

Study No. 1866 701006

Stamping error
gjd 2/28/83

Table 6

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

INITIAL TOXICITY TEST

T1866.701006	Paul E. Kirby	10 ug/ml to 0.001 ug/ml
Study Number	Study Director	Dose Range
CMA-105 (T1866)		Acetone
Test Article Identity		Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation					
	Solvent 1				
	Solvent 2				
With S-9 Activation	10 ug/ml	0.434*	0.888*	0.0	0%
	5.0 ug/ml	0.152	0.154	0.0	0%
	1.0 ug/ml	0.019	0.035	0.0	0%
	0.5 ug/ml	0.027	0.036	0.0	0%
	0.1 ug/ml	0.027	0.024	0.0	0%
	0.05 ug/ml	0.061	0.033	0.0	0%
	0.01 ug/ml	0.865	1.225	11.8	90%
	0.005 ug/ml	0.924	1.271	13.0	100%
	0.001 ug/ml	0.929	1.288	13.3	101%
	Solvent 1	0.945	1.314	13.8 13.1	
	Solvent 2	0.916	1.214	12.4	

Not applicable 2/25/83 LCG

+ Culture Lost

* counts indicated debris, not cells LCG

Table Prepared By Leah Griswold 2/25/83

Signature Date

Form No. MA-127
2/16/83

Workbook Page No. 60

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Study No. 1866 701006

TABLE 7
L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1866.701006
Study Number

Paul E. Kaiby
Study Director

1866B2
Experiment Number

CMA-105 (T1866)
Test Article Identity

Acetone
Solvent

Induced Rat Liver S-9
Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
0.021 ug/ml	78	85	80	81	197	174	206	192	0.8	0.4
0.026 ug/ml	56	72	52	60	200	187	197	195	0.6	0.2
0.017 ug/ml	60	57	61	59	209	214	189	198	0.6	0.2
0.0089 ug/ml	57	63	+	60	206	210	178	198	0.6	0.2
0.0067 ug/ml	52	44	51	49	191	225	+	208	0.5	0.1
0.0050 ug/ml	42	46	38	42	195	207	224	209	0.4	0.0
0.0038 ug/ml	36	49	47	44	191	201	214	202	0.4	0.0
0.0025 ug/ml	61	51	73	62	245	219	235	233	0.5	0.1
0.0021 ug/ml	55	57	49	54	223	211	219	218	0.5	0.1
0.0016 ug/ml	37	47	36	40	221	200	209	210	0.4	0.1
Solvent 1	45	49	40	45	233	244	223	233	0.4	0.4
Solvent 2	47	35	40	41	207	210	210	209	0.4	

Plates Counted By: Leah Griswold 3/14/85
(Signature & Date)

Calculations Performed By: Leah Griswold 5/14/85
(Signature & Date)

* Per 10^6 surviving cells

\+ Culture Lost

TABLE 8

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1866.701006 — Study Number

Paul E. Kirby — Study Director

1866B2 — Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1										
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	172	186	206	188	64	62	+	63	6.0	5.5
5.0 µg/ml	297	269	305	290	165	162	170	166	3.5	3.0
SOLVENT 1	48	44	49	47	212	260	244	239	0.4	0.5
SOLVENT 2	52	44	60	52	200	224	239	221	0.5	

Not Applicable AEF 3-14-83

Plates Counted By: Leah Ghuswald 3/14/83 (Signature & Date)

Calculations Performed By: Leah Ghuswald 3/14/83 (Signature & Date)

*Per 10^6 surviving cells

+ Culture Lost

Workbook Page No. 61

Report Page No. III-12

1866.701006

Form No. MA-226
2/16/83

Table 9

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T/866701006	Paul E. Kirby	1866B2
Study Number	Study Director	Experiment Number
CMA-105 (T1866)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.038 ul/ml	0.032	0.018	0.0	0%	++		
0.025 ul/ml	0.113	0.135	0.0	0%	++		
0.021 ul/ml	0.655	1.654	12.0	68%	192	87%	59%
0.016 ul/ml	0.832	1.747	16.2	91%	195	88%	80%
0.012 ul/ml	0.844	1.652	15.5	87%	198	90%	78%
0.0089 ul/ml	0.869	1.716	16.6	93%	198	90%	83%
0.0067 ul/ml	0.855	1.763	16.7	94%	208	94%	89%
0.005 ul/ml	0.918	1.604	16.4	92%	209	95%	87%
0.0038 ul/ml	0.901	1.637	16.4	92%	202	91%	84%
0.0028 ul/ml	0.885	1.734	17.1	96%	233	105%	101%
0.0021 ul/ml	0.895	1.635	16.3	91%	218	99%	90%
0.0016 ul/ml	0.915	1.703	17.3	97%	210	95%	92%
SOLVENT 1	0.969	1.665	17.9 } 17.8		233 } 221		
SOLVENT 2	0.948	1.680	17.7 }		209 }		

* % Total Growth = $\frac{(\%\text{ Suspension Growth})\ (\%\text{ Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: Leah Linowold 3/25/83
(Signature & Date)

Workbook Page No. 62 Report Page No. III-13

Study No. T1866.701006

Table 10

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1866.701006 — Study Number
Paul E. Kirby — Study Director
1866B2 — Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V.C.	% Control	% Total Growth
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml	Not Applicable 3/14/83 RLG						
0.5 µl/ml							
SOLVENT 1							
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.427	0.714	3.4	19%	63	27%	5%
5.0 µg/ml	0.898	1.042	10.4	57%	166	72%	41%
SOLVENT 1	0.985	1.647	18.0 / 18.1		239 } 230		
SOLVENT 2	0.971	1.686	18.2		221		

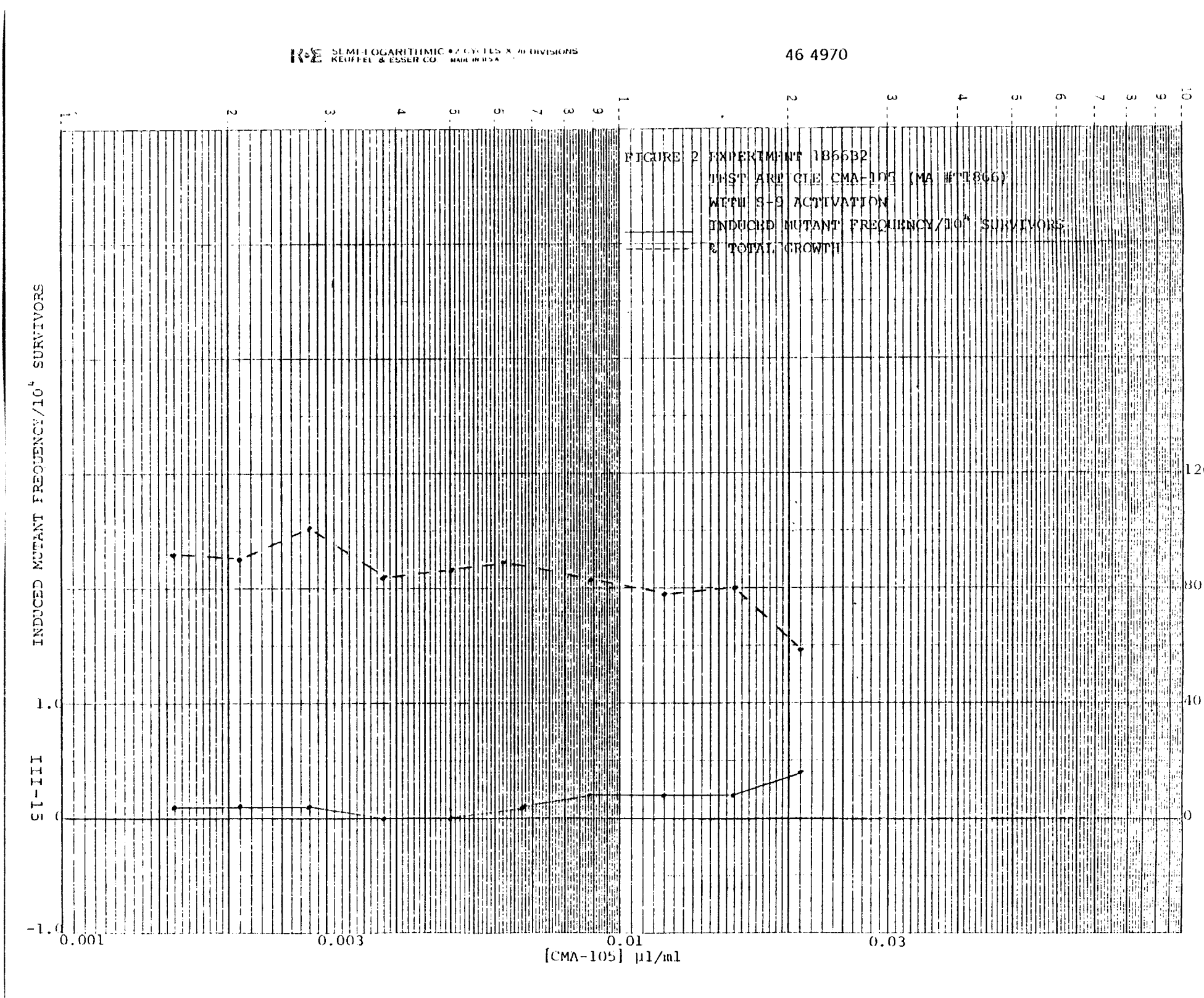
Tables prepared and calculations performed by Leah Griswold 3/14/83
(Signature & Date)

\+ Culture Lost
++ Too Toxic to Clone

Workbook Page No. 62 Report Page No. III-14

Study No. T1866.70.005

K&E SEMILOGARITHMIC 2 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO MADE IN U.S.A.



III-13

Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-105 (MA #T1866) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest concentration cloned in the S-9 activated cultures exhibited a mutant frequency which was twice the mean mutant frequency of the solvent controls. None of the nonactivated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-105 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of exogenous metabolic activation.

APPENDIX

Rec'd. by
RA/QA 12/15/82.
N.K /jkc

RECEIVED OCT 0 5 1982

L5178Y TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-105

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation: $2,650

Total charge for assay with metabolic activation: $2,650

6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intra-peritoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-amino-anthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_2O, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0×10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F_0P will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3×10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3×10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2×10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of

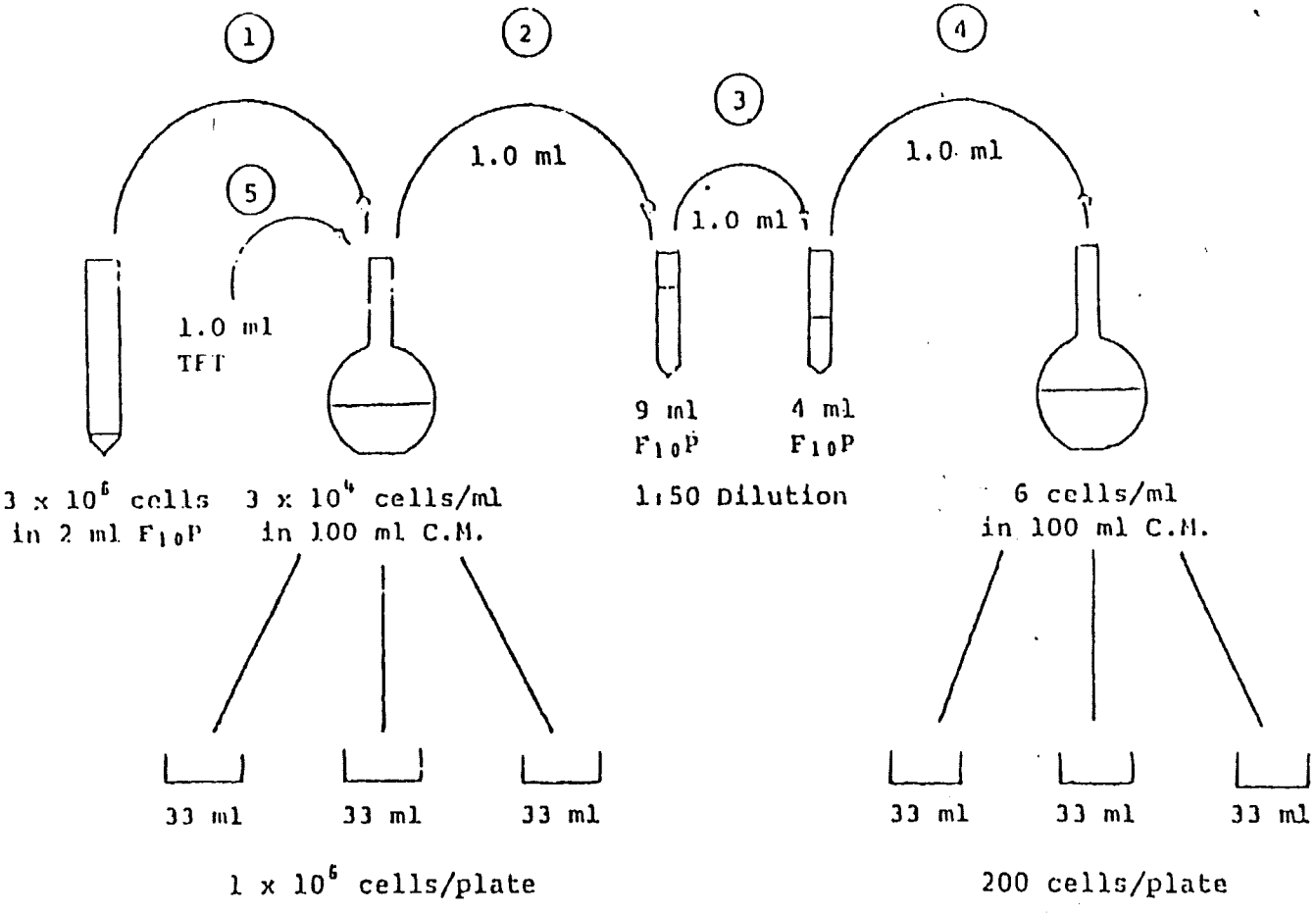


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

MICROBIOLOGICAL ASSOCIATES

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labelled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency?_____
If so, to which agency or agencies? _____

Does the sponsor request that samples of the Test Article dosing solutions be returned?_____

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: 12/10/82 DLC 12/14/82

13.2 Proposed Initiation Date: 1/13/83

13.3 Scheduled Completion Date: 2/18/83

13.4 Report Submission to Sponsor Date: 2/18/83

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

Carol Stark
SPONSOR'S AUTHORIZED REPRESENTATIVE

11/22/82
DATE PROTOCOL APPROVED BY SPONSOR

Paul E. Kinley — 11/24/82
STUDY DIRECTOR — DATE

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136) and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \text{Average Counts (corrected for coincidence)} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{cells/ml})\ (20\ \text{ml})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = 20\ \text{ml} - \text{Volume of Cells Retained}$$

---

2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

---

3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138) and L5178Y TK+/- Assay - Daily Counts (Positive Controls) (Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^{5} = \text{Average Counts (corrected for coincidence)} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{Cells/ml})\ (20\ \text{ml}^{*})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20\ \text{ml}^{*}) - (\text{Volume of Cells Retained})$$

* The final volume is adjusted to 10 ml on the third day of expression.

---

4. Tables 2, 3, and 4 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^{6} \text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left(\begin{array}{c}\text{Mutant Frequency}\\ \text{of Treated Cultures}\end{array}\right) - \left(\begin{array}{c}\text{Average Mutant Frequency}\\ \text{of Solvent Controls}\end{array}\right)$$

---

5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

$$\text{\% Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\text{\% Growth} = \frac{(\text{\% Suspension Growth})\ (\text{\% Cloning Growth})}{100}$$

Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-106 (MA #T1854) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures (Experiment 1854B2) were cloned over a range of test article concentrations which produced from 6% to 101% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 1% to 88% Total Growth.

The highest test article concentration cloned in the non-activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. The result is not considered significant since mutant frequencies observed at such highly toxic levels may be due to epigenetic events. Four S-9 activated cultures exhibited mutant frequencies which were significantly different from the mean mutant frequency of the solvent controls.

The results indicate that under the conditions of this test, the test article CMA-106 produced a negative response in the absence of exogenous metabolic activation and a positive response in the presence of exogenous metabolic activation.

Results

The Initial Toxicity Test (Table 1) performed on test article CMA-106 (MA #T1854) in the absence of S-9 indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.01 µl/ml to 0.00013 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.01, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018, 0.0010, 0.00075 or 0.00056 µl/ml test article. These concentrations produced a range in Suspension Growth of 27% to 109%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 17% to 120%. However, the toxic response at the higher doses was erratic and an acceptable range in toxicity was not achieved. Therefore the assay was repeated over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, nine cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, 0.0012, 0.00089 or 0.00067 µl/ml test article. These concentrations produced a range in Suspension Growth of 13% to 100%. The Cloning Data are presented in Tables 6 and 7 and the Total Compound Toxicity Data are presented in Tables 8 and 9. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2.

One culture that was cloned exhibited a mutant frequency which was 2.3 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 22% to 101%.

An Initial Toxicity Test (Table 10) was conducted in the presence of S-9 on test article CMA-106 (MA #T1854). The results indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05 µl/ml to 0.005 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.031, 0.024, 0.018, 0.011 or 0.005 µl/ml. These concentrations produced a range in Suspension Growth of 6% to 99%. The Cloning Data are presented in Tables 11 and 12 and the Total Compound Toxicity Data are presented in Tables 13 and 14. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 3. Cultures treated with 0.031, 0.024, or 0.018 µl/ml test article exhibited mutant frequencies which ranged from 16.3 to 2.0 times the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 1% to 72%.

None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 78% to 88%.

Study No. 1854 701006

Table 1

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1854.701006 — Study Number
Paul E. Kirby — Study Director
100 μl/ml to 0.001 μl/ml — Dose Range
CMA-106 (T1854) — Test Article Identity
Acetone — Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation	100 μl/ml	0.020	0.101	0.0	0%
	10 μl/ml	0.223	0.381	0.0	0%
	5.0 μl/ml	0.070	0.269	0.0	0%
	1.0 μl/ml	0.016	0.055	0.0	0%
	0.5 μl/ml	0.050	0.100	0.0	0%
	0.1 μl/ml	0.059	0.089	0.0	0%
	0.05 μl/ml	0.036	0.046	0.0	0%
	0.01 μl/ml	0.412	0.490	2.2	16%
	0.005 μl/ml	0.507	0.859	4.8	35%
	0.001 μl/ml	1.154	1.134	14.5	105%
	Solvent 1	1.147	1.061	13.5	13.9
	Solvent 2	1.108	1.152	14.2	
With S-9 Activation					
	Solvent 1				
	Solvent 2				

+ Culture Lost

Table Prepared By James J. Clarke — Signature — 1-13-83 — Date

Form No. WL-233 Workbook Page No. 42 Report Page No IV-4

Form No. WL-41
2/25/82

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1854.701006	Paul E. Kirby	1854B1
Study Number	Study Director	Experiment Number
CMA-706 (T1854)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced Mutant * Frequency
0.01ul/ml	34	33	44	37	191	149	148	163	0.5	0.1
0.0075ul/ml	24	25	33	27	124	112	115	117	0.5	0.1
0.0056ul/ml	33	36	35	35	179	214	174	189	0.4	0.0
0.0042ul/ml	52	42	45	46	197	201	171	190	0.5	0.1
0.0032ul/ml	47	44	38	43	194	171	184	183	0.5	0.1
0.0024ul/ml	42	38	45	42	221	201	212	211	0.4	0.0
0.0018ul/ml	57	34	49	47	199	176	179	185	0.5	0.1*
0.0013ul/ml	+	+	+		+	+	+			
0.0010ul/ml	61	29	37	42	206	184	185	192	0.4	0.0
0.00075ul/ml	36	26	41	34	166	201	182	183	0.4	0.0
0.00056ul/ml	33	30	46	36	214	203	+	209	0.3	-0.1
Solvent 1	36	34	30	33	149	171	176	165	0.4	0.4
Solvent 2	36	36	53	42	187	185	210	194	0.4	

Plates Counted By: Eliana M. Carey 2-7-83 (Signature & Date)

Calculations Performed By: Eliana M. Carey 2-7-83 (Signature & Date)

* Per 10^4 surviving cells

\+ Culture Lost

* Entry Error 2-7-83

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Study No. 1854-706

Form No. WL-128

Workbook Page No. 32 Report Page No. IV-6

Study No. 1854.701006

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1854.701006	Paul E. Kirby	1854B1
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced * Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	190	221	210	207	13	16	13	14	29.6	29.2
0.5µl/ml	428	370	375	391	102	91	102	98	8.0	7.6
SOLVENT 1	38	40	36	38	202	188	214	201	0.4	0.4
SOLVENT 2	41	42	50	44	187	200	223	203	0.4	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1										
SOLVENT 2										

Not Applicable - Eleanor M. Carey 2-7-83

Plates Counted By: Eleanor M. Carey 2-7-83 (Signature & Date)

Calculations Performed By: Eleanor M. Carey 2-2-83 (Signature & Date)

*Per 10^6 surviving cells

+ Culture Lost

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1854.701006	Paul E. Kirby	1854B1
Study Number	Study Director	Experiment Number
CMA-106 (T1854)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.01 ul/ml	0.669	1.253	9.3	51%	163	91%	46%
0.0075 ul/ml	0.474	0.930	4.9	27%	117	65%	17%
0.0056 ul/ml	0.818	1.471	13.4	73%	189	105%	77%
0.0042 ul/ml	0.998	1.639	18.2	100%	190	106%	105%
0.0032 ul/ml	0.987	1.673	18.3	101%	183	102%	102%
0.0024 ul/ml	0.978	1.672	18.2	100%	211	118% **	117%
0.0018 ul/ml	1.020	1.700	19.3	106%	185	103%	109%
* 0.0013 ul/ml	0.992	1.675	18.5	101%	+		
0.0010 ul/ml	1.062	1.653	19.5	107%	192	107%	114%
0.00075 ul/ml	1.031	1.724	19.7	109%	183	102%	110%
0.00056 ul/ml	1.002	1.687	18.8	103%	209	116%	120%
SOLVENT 1	1.022	1.623	18.4 } 18.2		165 } 180		
SOLVENT 2	0.997	1.628	18.0 }		194 }		

** Calculation error [illegible] 4/20/83

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

+ Culture Lost

++ Too Toxic to Clone

* This culture was lost after 2nd day readjustment and was thus not cloned. EMC 2-7-83.

Tables prepared and calculations performed by: Eleanor M. Carey 2-7-83

(Signature & Date)

(4-19-83)

Study No. T1854.701006

Workbook Page No. 339 Report Page No. IV-7

Table re-copied for presentation purposes EMC 4-19-83

Form No. WL-227

Form No. WL-229

Table 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1854.701006 — Study Number

Paul E. Kirby — Study Director

1854B1 — Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml	0.566	0.804	5.1	26%	14	7%	2%
0.5 µl/ml	0.840	1.313	12.3	62%	98	49%	30%
SOLVENT 1	1.092	1.591	19.3 } 19.8		201 } 202		
SOLVENT 2	1.140	1.604	20.3 }		203 }		
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml							
5.0 µg/ml							
SOLVENT 1							
SOLVENT 2							

Not Applicable - Clean M. Carey 2-7-83

Tables prepared and calculations performed by: Elaine M. Carey 2-7-83

(Signature & Date)

\+ Culture Lost

++ Too Toxic to Clone

Workbook Page No. 34 Report Page No. IV-8

Study No. 1854.701006

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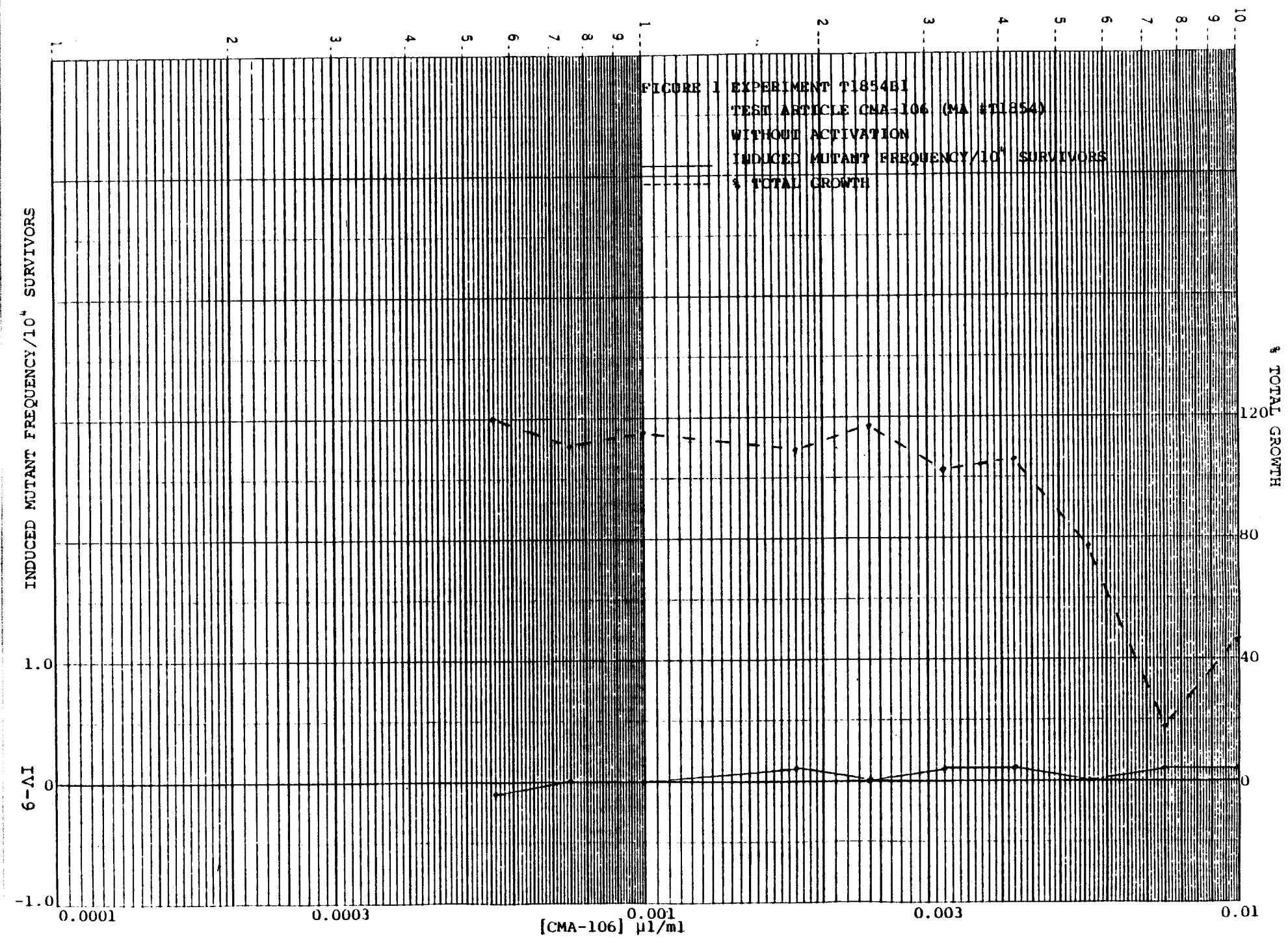

Form No. MA-41
2/16/83

Workbook Page No. 51A

Report Page No. IV-10

Table recopied for presentation purposes.
AMKB 2/23/83

Table 6

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1854.701006	Paul E Kirby	1854B2
Study Number	Study Director	Experiment Number
CMA-106 (T1854)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced Mutant * Frequency
0.0067 ul/l	44	34	+	39	+	88	87	88	0.9	0.5
0.0050 ul/l	23	31	28	27	159	156	129	147	0.4	0.0
0.0038 ul/l	22	24	28	25	172	147	182	167	0.3	-0.1
0.0028 ul/l	31	33	37	34	198	184	173	185	0.4	0.0
0.0021 ul/l	34	38	35	36	+	171	161	166	0.4	0.0
0.0016 ul/l	38	28	27	31	188	184	171	181	0.3	-0.1
0.0012 ul/l	31	22	25	26	199	213	194	202	0.3	-0.1
0.00089 ul/l	25	17	27	23	207	+	187	197	0.2	-0.2
0.00067 ul/l	31	31	42	35	174	186	182	181	0.4	0.0
Solvent 1	38	38	41	39	187	196	194	192	0.4	0.4
Solvent 2	29	40	38	36	188	200	203	197	0.4	

Plates Counted By: Karen Joan Porras 2/22/83 [2/23/83]
(Signature & Date)

Calculations Performed By: Andrea N. Rogers-Bach 2/22/83
(Signature & Date)

* Per 10^4 surviving cells + Culture Lost

Study No. T1854.701006

Form No. WL-128

Workbook Page No. 52 Report Page No. IV-11

Study No. 1854 701006

Table 7

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1854 701006	Paul E. Kirby	1854B2
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced * Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	213	218	220	217	14	22	+	18	24.1	23.6
0.5µl/ml	402	443	414	421	87	77	78	81	10.4	9.9
SOLVENT 1	35	41	61	47	202	172	175	183	0.5	0.5
SOLVENT 2	38	38	26	34	183	164	175	174	0.4	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml		Not	Applicable		Karen J. Pons 2/22/83					
5.0 µg/ml										
SOLVENT 1										
SOLVENT 2										

Plates Counted By: Karen Joan Pons 2/22/83 (Signature & Date)

Calculations Performed By: Anita M Rogers-Brown (Signature & Date) 2/23/83

*Per 10^4 surviving cells

+ Culture Lost

Table 8

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1854.701006	Paul E Kirby	1854B2
Study Number	Study Director	Experiment Number
CMA-106 (T1854)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.012 ml/ml	0.073	0.090	0.0	0%	++		
0.0089 ml/ml	0.132	0.089	0.0	0%	++		
0.0067 ml/ml	0.384	0.625	2.7	13%	88	45%	6%
0.0050 ml/ml	0.506	1.050	5.9	29%	147	75%	22%
0.0038 ml/ml	0.870	1.345	13.0	65%	167	86%	55%
0.0028 ml/ml	1.014	1.595	18.0	89%	185	95%	85%
0.0021 ml/ml	1.001	1.621	18.0	90%	166	85%	76%
0.0016 ml/ml	1.056	1.684	19.8	98%	181	93%	91%
0.0012 ml/ml	1.053	1.662	19.4	97%	202	104%	100%
0.00089 ml/ml	1.059	1.711	20.1	100%	197	101%	101%
0.00067 ml/ml	1.031	1.672	19.2	95%	181	93%	88%
SOLVENT 1	1.065	1.731	20.5	20.1	192	195	
SOLVENT 2	1.052	1.678	19.6		197		

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost

++ Too Toxic to Clone

Tables prepared and calculations performed by: [signature] (Signature & Date) 2/23/83

Study No. T1854.701006

Workbook Page No. 53

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Form No. MA-227

Form No. WL-229
2/25/82

Workbook Page No. 54 Report Page No. IV-13

Study No. 1854 701006

Table 9

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1854.701006
Study Number

Paul E Kirby
Study Director

1854B2
Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml	0.823	0.712	6.5	30%	18	10%	3%
0.5 µl/ml	0.810	1.344	12.1	55%	81	45%	25%
SOLVENT 1	1.152	1.704	21.8	21.9	183	179	
SOLVENT 2	1.188	1.660	21.9		174		
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml							
5.0 µg/ml							
SOLVENT 1							
SOLVENT 2							

not applicable AHH 2/23/83

Tables prepared and calculations performed by: [signature] 2/23/83
(Signature & Date)

\+ Culture Lost
++ Too Toxic to Clone

K•E SEMI-LOGARITHMIC • 2 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO MADE IN U.S.A.

46 4970

FIGURE 2 EXPERIMENT T1854B2
TEST ARTICLE CMA [illegible] (MA #T1854)
WITHOUT ACTIVATION
——— INDUCED MUTANT FREQUENCY/10^4 SURVIVORS
- - - - - % TOTAL GROWTH

INDUCED MUTANT FREQUENCY/10^4 SURVIVORS

% TOTAL GROWTH

1.0
0
-1.0
120
80
40
0

0.0001 0.0003 0.001 0.003 0.01

[CMA-106] µl/ml

Study No. T1854.701006

Table 10

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1854.701006	Paul E. Kirby	10.0 µl/ml to 0.001 µl/ml
Study Number	Study Director	Dose Range
CMA-106 (T1854)		Acetone
Test Article Identity		Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation					
	Solvent 1				
	Solvent 2				
With S-9 Activation	10.0 µl/ml	0.598 *	0.798 *	0.0	0%
	5.0 µl/ml	0.048	0.060	0.0	0%
	1.0 µl/ml	0.065	0.155	0.0	0%
	0.5 µl/ml	0.054	0.133	0.0	0%
	0.1 µl/ml	0.024	0.025	0.0	0%
	0.05 µl/ml	0.019	0.029	0.0	0%
	0.01 µl/ml	0.849	1.074	10.1	79%
	0.005 µl/ml	0.813	1.242	11.2	88%
	0.001 µl/ml	0.986	1.239	13.6	106%
	Solvent 1	0.988	1.157	12.7	12.8
	Solvent 2	0.960	1.202	12.8	

Not Applicable [illegible] 3/3/83

+ Culture Lost * counts represent debris [illegible] 3-3-83

Table Prepared By [signature] 3/3/83
Signature Date

Form No. MA-233 Workbook Page No. 64 Report Page No. IV-15
2/16/83

Table 11

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1854.701006	Paul E. Kirby	1854B3
Study Number	Study Director	Experiment Number
CMA-106 (T1854)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Avg. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Avg. #/ Plate	Mutant* Frequency	Induced Mutant* Frequency
Solvent 1	48	60	50	53	199	235	+	217	0.5	0.6
Solvent 2	69	49	57	58	194	185	+	190	0.6	
0.031 mM A	201	182	+	192	57	60	69	62	6.2	5.6
0.031 mM B	153	182	199	178	42	31	+	37	9.8	9.2
0.024 mM A	+	+	+		158	149	+	154		
0.024 mM B	151	166	+	159	136	166	+	151	2.1	1.5
0.018 mM A	105	99	91	98	196	178	+	187	1.1	0.5
0.018 mM B	101	115	105	107	185	188	172	182	1.2	0.6
0.016 mM A	96	90	81	89	172	187	171	177	1.0	0.4
0.016 mM B	102	111	+	107	+	+	+			
0.005 mM A	66	74	75	72	161	200	+	181	0.8	0.2
0.005 mM B	+	+	+		+	+	+			
A										
B										

Plates Counted By: Leah Griswold 3/28/83
(Signature & Date)

Calculations Performed By: Anita N Rogers-Bank
(Signature & Date) 3/29/83

* Per 10^4 surviving cells

\+ Culture Lost

Study No. T1854.701006

TABLE 12

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1854 701006 — Study Number

Paul E. Kirby — Study Director

1854B3 — Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1		Not Applicable								
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	260	287	+	274	65	+	+	68	8.0	7.4
5.0 µg/ml	+	+	+		+	+	+			
SOLVENT 1	62	+	+	62	+	+	+			
SOLVENT 2	62	57	+	60	188	191	+	190	0.6	

Plates Counted By: Leah Griswold 5/28/83 (Signature & Date)

Calculations Performed By: Anita M Hagen-Bank (Signature & Date) 3/29/83

*Per 10^6 surviving cells

+ Culture Lost

Study No. T1854 701006

Workbook Page No. 82 Report Page No. IV-17

Form No. MA-230

Workbook Page No. 83

Report Page No. IV-18

Study No. T1854.701006

TABLE 13

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1854.701006	Paul E Kirby	1854B3
Study Number	Study Director	Experiment Number
CMA-106 (T1854)	Acetone	Induced Rat Liver S9 ~~None~~ AMKB 3/29/83
Test Article Identity	Solvent	Metabolic Activation Incorrect data sheet used

Test Article Concentration	Cell Concentration X 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Avg. V. C.	% Control	% Total Growth
SOLVENT 1	0.862	1.823	17.5	16.6	217	204	
SOLVENT 2	0.774	1.830	15.7		190		
0.044 ml/ml A	0.054	0.034	0.0	0%	++		
0.044 ml/ml B	0.044	0.036	0.0	0%	++		
0.037 ml/ml A	0.197	0.141	0.0	0%	++		
0.037 ml/ml B	0.186	0.156	0.0	0%	++		
0.031 ml/ml A	0.241	0.390	1.3	8%	62	30%	2%
0.031 ml/ml B	0.244	0.312	1.0	6%	37	18%	1%
0.024 ml/ml A	0.462	1.636	8.4	51%	154	75%	38%
0.024 ml/ml B	0.510	1.534	8.7	52%	151	74%	39%
0.018 ml/ml A	0.710	1.793	14.1	85%	187	92%	78%
0.018 ml/ml B	0.670	1.802	13.4	81%	182	89%	72%
0.011 ml/ml A	0.813	1.821	16.4	99%	177	87%	86%
0.011 ml/ml B	0.769	1.815	15.5	93%	+		
0.005 ml/ml A	0.804	1.823	16.3	98%	+		
0.005 ml/ml B	0.821	1.801	16.4	99%	181	89%	88%
A							
B							

\+ Culture Lost

++ Too Toxic to Clone

Tables prepared and calculations performed by: Anita N Rogers-Bardle

(Signature & Date) 3/29/83

Table 14

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1854.701006	Paul E Kirby	1854B3
Study Number	Study Director	Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml							
0.5 µl/ml							
SOLVENT 1							
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.521	1.087	6.3	35%	68	36%	12%
5.0 µg/ml	0.673	1.694	12.7	70%	+		
SOLVENT 1	0.767	1.971	16.8 } 18.1		+		
SOLVENT 2	0.928	1.870	19.3 }		190		

not applicable AMKB 3/29/83

Tables prepared and calculations performed by: Andrea M. Kopp-Bach

(Signature & Date) 3/29/83

\+ Culture Lost

++ Too Toxic to Clone

Study No. T1854.701006

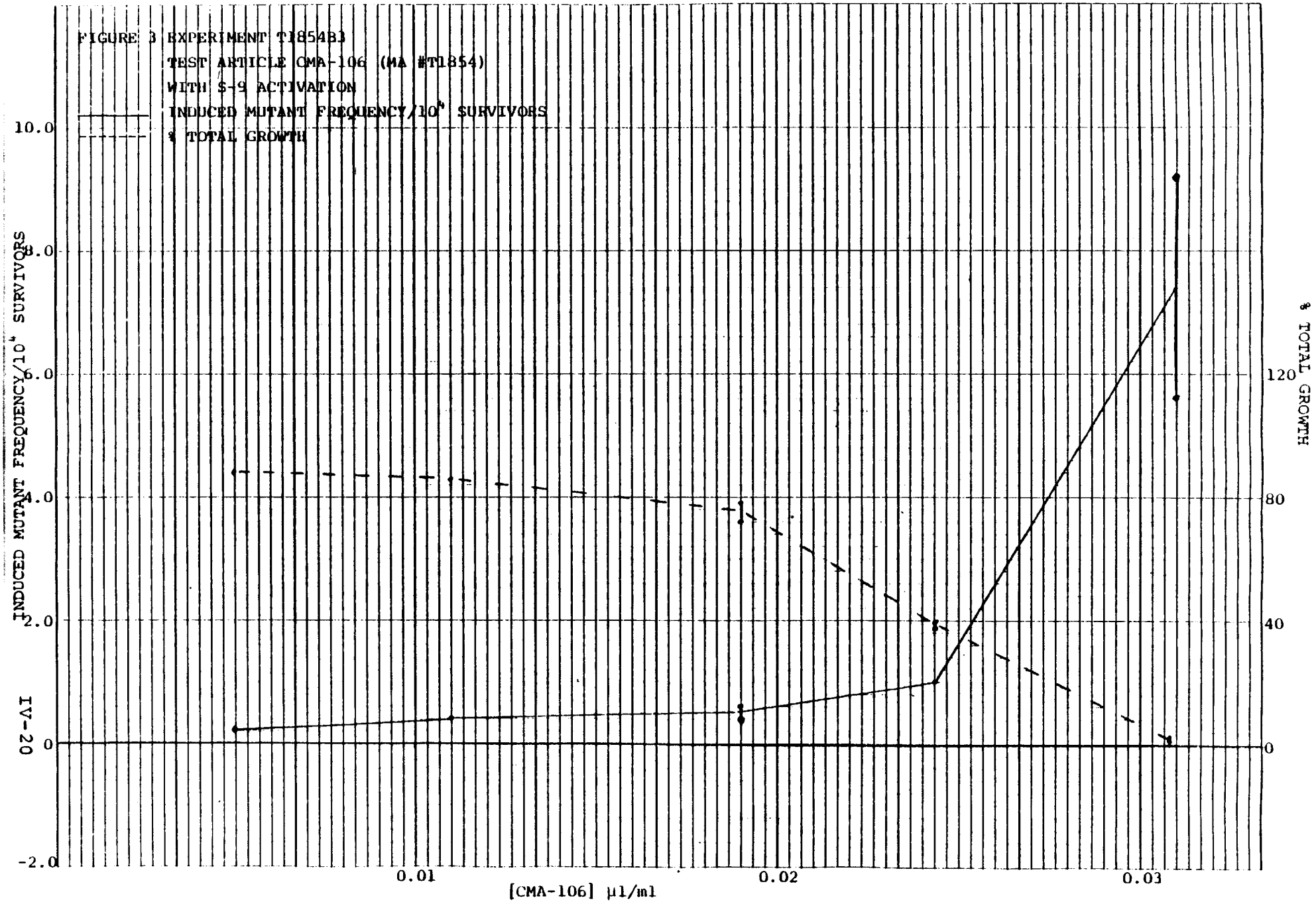
FIGURE 3 EXPERIMENT T1854B3
TEST ARTICLE CMA-106 (MA #T1854)
WITH S-9 ACTIVATION
INDUCED MUTANT FREQUENCY/10⁴ SURVIVORS
% TOTAL GROWTH
INDUCED MUTANT FREQUENCY/10⁴ SURVIVORS
% TOTAL GROWTH
10.0
8.0
6.0
4.0
2.0
0
-2.0
120
80
40
0
0.01
0.02
0.03
[CMA-106] µl/ml

Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-106 (MA #T1854) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest concentration cloned in the nonactivated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. The result is not considered significant since mutant frequencies observed at such highly toxic doses may be due to epigenetic events. Four S-9 activated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-106 produced a negative response in the absence of exogenous metabolic activation and a positive response in the presence of exogenous metabolic activation.

APPENDIX

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Rec'd by RA/QA 10/8/82. N.K./fbc

L5178Y TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-106

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:
$2,650

Total charge for assay with metabolic activation:
$2,650

6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intra-peritoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-amino-anthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_2O, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0×10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F_0P will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3×10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3×10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2×10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of

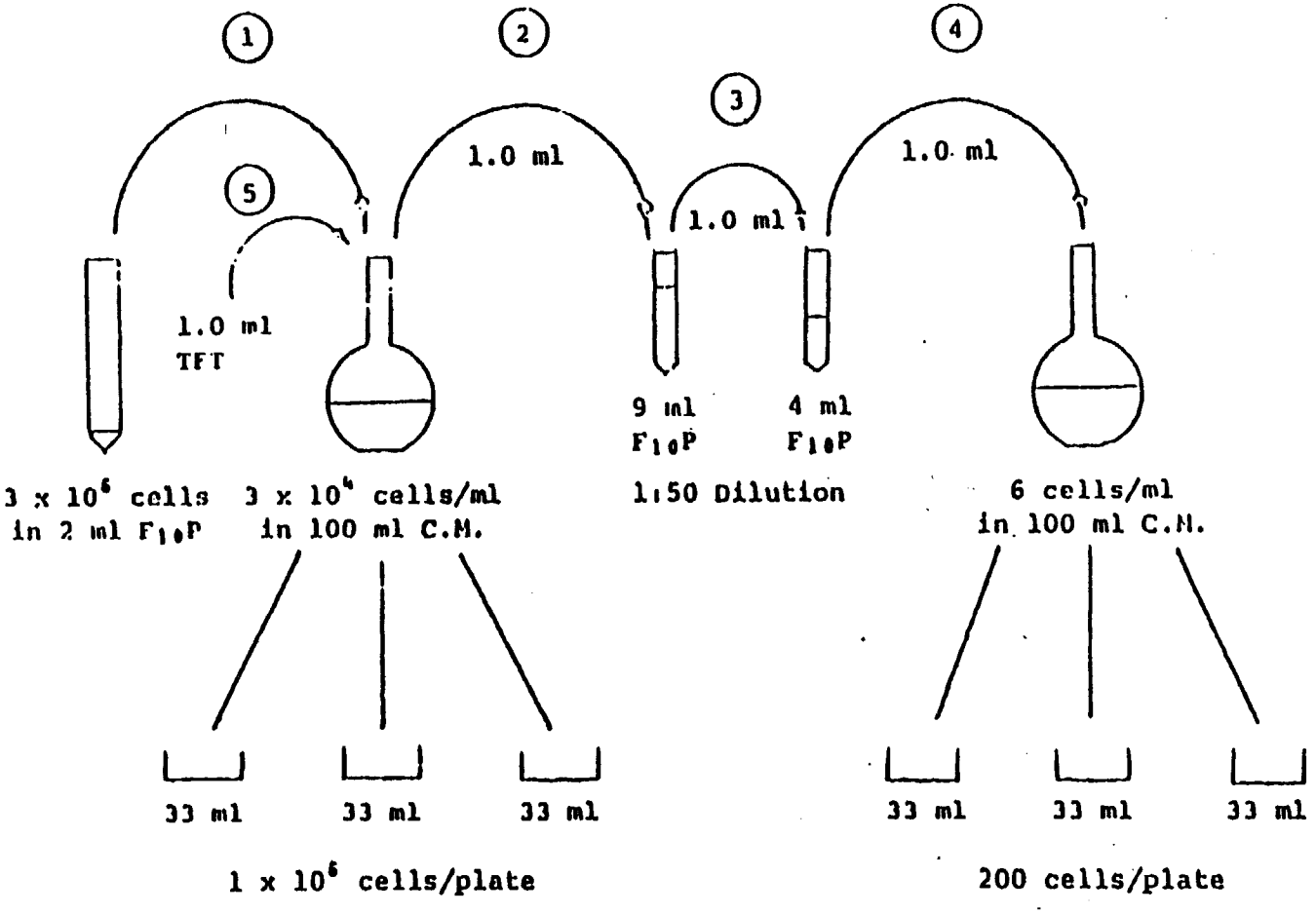


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

MICROBIOLOGICAL ASSOCIATES

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency?_____
If so, to which agency or agencies? _____

Does the sponsor request that samples of the Test Article dosing solutions be returned?_____

13.0 SCHEDULE OF EVENTS

GK 4/20/83

13.1 Test Material Received Date: 1/11/83 ~~11/24/82~~

13.2 Proposed Initiation Date: December 20, 1982

13.3 Scheduled Completion Date: February 11, 1983

13.4 Report Submission to Sponsor Date: February 11, 1983

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

SPONSOR'S AUTHORIZED REPRESENTATIVE

11/22/82
DATE PROTOCOL APPROVED BY SPONSOR

STUDY DIRECTOR — DATE 11/24/82

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a) anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136) and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \text{Average Counts (corrected for coincidence)} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{cells/ml})\ (20\ \text{ml})}{\text{(No. of Cells/ml)}}$$

$$\text{Volume of Media to Add} = 20\ \text{ml} - \text{Volume of Cells Retained}$$

---

2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

---

3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138) and L5178Y TK+/- Assay - Daily Counts (Positive Controls) (Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^{5} = \text{Average Counts (corrected for coincidence)} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{Cells/ml})\ (20\ \text{ml}^{*})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20\ \text{ml}^{*}) - (\text{Volume of Cells Retained})$$

* The final volume is adjusted to 10 ml on the third day of expression.

---

4. Tables 2, 3, and 4 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^{6}\text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left(\text{Mutant Frequency of Treated Cultures}\right) - \left(\text{Average Mutant Frequency of Solvent Controls}\right)$$

---

5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

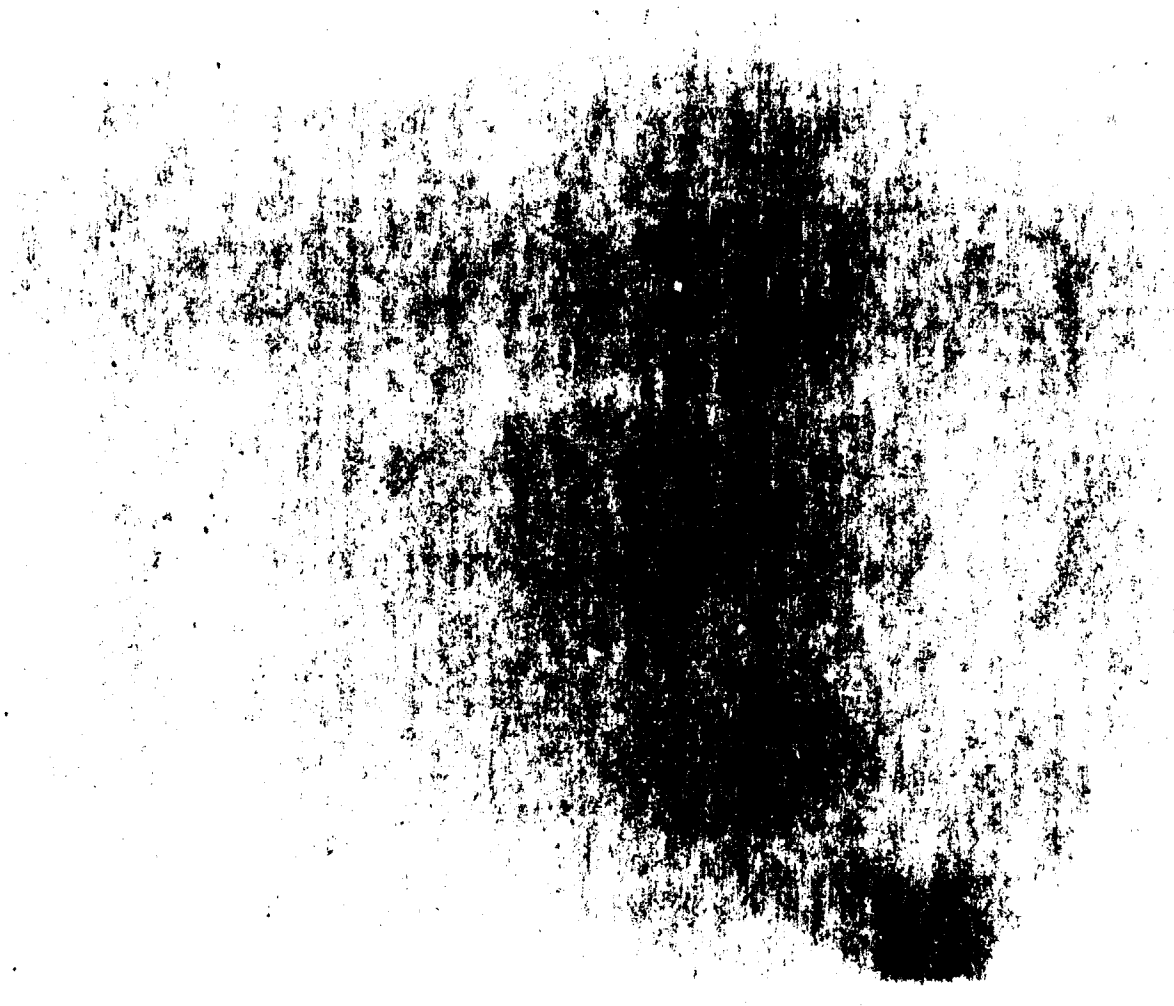
$$\text{\% Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (~t'd.)

$$\text{\% Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\text{\% Growth} = \frac{(\text{\% Suspension Growth})\ (\text{\% Cloning Growth})}{100}$$



Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-107 (MA #T1857) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 47% to 123% Total Growth in one assay and from 2% to 97% Total Growth in a second assay. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 8% to 95% Total Growth.

One nonactivated culture having less than 10% Total Growth exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant due to the high toxicity observed. Five S-9 activated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that under the conditions of this test, test article CMA-107 produced a negative response in the absence of exogenous metabolic activation and a positive response in the presence of metabolic activation.

Results

The Initial Toxicity Test (Table 1) performed on test article CMA-107 (MA #T1857) in the absence of S-9 indicated a threshold level of complete toxicity at 0.1 ml/ml. Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.01 µl/ml to 0.00013 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018, 0.0013, 0.0010 or 0.00075 µl/ml test article. These concentrations produced a range in Suspension Growth of 47% to 105%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 47% to 123%. However, the toxicity observed in the treated cultures did not cover the optimum range (10% to 90% Total Growth). The assay was repeated over a concentration range of 0.1 µl/ml to 0.0013 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.024, 0.018, 0.013, 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024 or 0.0018 µl/ml test article. These concentrations produced a range in Suspension Growth of 8% to 96%. The Cloning Data are presented in Tables 6 and 7 and the Total Compound Toxicity Data are presented in Tables 8 and 9. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2.

One of the cultures that were cloned (0.024 µl/ml) exhibited a mutant frequency which was 2.8 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 2%.

None of the remaining cultures that were cloned exhibited mutant frequencies that were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 19% to 97%.

An Initial Toxicity Test (Table 10) was conducted in the presence of S-9 on test article CMA-107 (MA #T1857). The results indicated a threshold level of complete toxicity at 0.1 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.1 µl/ml to 0.01 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.061, 0.049, 0.036, 0.023 or 0.01 µl/ml. These concentrations produced a range in Suspension Growth of 17% to 94%. The Cloning Data are presented in Tables 11 and 12 and the Total Compound Toxicity Data are presented in Tables 13 and 14. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 3.

Five of the cultures that were cloned (0.061, 0.049, 0.036, 0.023 µl/ml) exhibited mutant frequencies which were 13.2 to 2.0 times the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 8% to 84%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 86% to 95%.

Study No. 1857 701006

Table 1

701 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL COMPOUND TOXICITY TEST

1857.701006	Paul E. Kirby	100 ul/ml to 0.001 ul/ml
Study Number	Study Director	Dose Range
CMA-107 (T1857)		Acetone
Test Article Identity		Solvent

	Test Article Concentration	Cell Concentration (X 10^5) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation	* 100 ul/ml	1.944	1.878	0.0	0%
	* 10 ul/ml	0.790	0.997	0.0	0%
	1.0 ul/ml	0.014	0.043	0.0	0%
	0.1 ul/ml	0.106	0.195	0.0	0%
	0.01 ul/ml	0.918	0.538	2.5	21%
	0.001 ul/ml	0.967	1.198	12.2	103%
	Solvent 1	0.982	1.094	11.9 / 11.9	
	Solvent 2	1.003	1.063	11.8	
With S-9 Activation					
			1-7-83		
		Not Applicable ff			
	Solvent 1				
	Solvent 2				

+ Culture Lost

Table Prepared By: Janice L. Johnson 1-7-83
Signature Date

* These cultures are toxic, the high counts are due to debris. ff 1-7-83

Workbook Page No. 13 Report Page No. V-4

Form No. WL-232
2/22/82

Form No. WL-41
2/25/82

Workbook Page No. 30

Report Page No. V-5

Study No. T1857.701006

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1857.701006 Study Number	Paul E. Tinsley Study Director	1857B1 Experiment Number
CMA-107 (T1857) Test Article Identity	Acetone Solvent	None Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
0.01ul/ml	38	30	+	34	140	147	163	150	0.5	0.0
0.0075ul/ml	22	22	26	23	166	135	169	157	0.3	-0.2
0.005ul/ml	34	38	18	30	175	159	183	172	0.3	-0.1
0.0042ul/ml	50	31	30	37	162	170	130	154	0.5	0.0
0.0032ul/ml	27	16	26	23	136	129	122	129	0.4	-0.1
0.0024ul/ml	20	25	+	23	129	176	165	157	0.3	-0.2
0.0018ul/ml	33	29	24	29	211	171	196	193	0.3	-0.2
0.0013ul/ml	28	27	23	26	160	117	152	143	0.4	-0.1
0.0010ul/ml	35	36	41	37	177	189	183	183	0.4	-0.1
0.00075ul/ml	35	37	39	37	161	154	182	166	0.4	-0.1
Solvent 1	35	33	29	32	136	141	154	144	0.5 ≥0.5	
Solvent 2	33	24	39	32	151	153	165	156	0.4	

Plates Counted By: Eleanor M. Carey 2-7-83 (Signature & Date)

Calculations Performed By: Eleanor M. Carey 2-7-83 (Signature & Date)

* Per 10^4 surviving cells

\+ Culture Lost

Form No. WL-128

Workbook Page No. 31 Report Page No. V-6

Study No: T1857 701006

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1857.701006 — Study Number

Paul E. Zisley — Study Director

1857B1 — Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Pl 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	160	163	190	171	7	11	13	10	33.1	32.6* ~~33.7~~
0.5µl/ml	281	262	308	284	67	62	84	71	8.0	7.5* ~~7.6~~
SOLVENT 1	27	24	24	25	322	334	308	321	0.2** 0.4	
SOLVENT 2	35	26	37	33	134	139	149	141	0.5	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1										
SOLVENT 2										

Not Applicable - Eliana M. Carey 2-7-83

Plates Counted By: Eliana M. Carey 2-7-83 (Signature & Date)

Calculations Performed By: Eliana M. Carey 2-7-83 (Signature & Date)

*Per 10^6 surviving cells

+ Culture Lost

* Values corrected to reflect amended average mutant frequency of the Solv. [illegible]

** This value not used in calculating average mutant frequency since the VC count was too high. AHB 4/18/83

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1857.701006	Paul E. Kirby	1857B1
Study Number	Study Director	Experiment Number
CMA-107 (T1857)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth *
	Day 1	Day 2	Total	% Control	Ave. V. C.	% Control	
0.01 ul/ml	0.605	1.326	8.9	47%	150	100%	47%
0.0075 ul/ml	0.774	1.563	13.4	70%	157	105%	74%
0.0056 ul/ml	1.094	0.825	10.0	53%	172	115%	60%
0.0042 ul/ml	0.988	1.685	18.5	97%	154	103%	99%
0.0032 ul/ml	1.037	1.736	20.0	105%	129	86%	90%
0.0024 ul/ml	0.938	1.634	17.0	89%	157	105%	93%
0.0018 ul/ml	0.962	1.691	18.1	95%	193	129%	122%
0.0013 ul/ml	0.941	1.640	17.1	90%	143	95%	86%
0.0010 ul/ml	0.992	1.746	19.2	101%	183	122%	123%
0.00075 ul/ml	0.954	1.593	16.9	88%	166	111%	98%
SOLVENT 1	0.996	1.741	19.3	719.1	144	150	
SOLVENT 2	0.992	1.715	18.9		156		

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost

++ Too Toxic to Clone

Tables prepared and calculations performed by: Eleanor M. Casey 2-7-83
(Signature & Date)

Form No. WL-227 2/82

Workbook Page No. 37 Report Page No. V-7

Study No. T1857.701006

Form No. MS-229

Table 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1857.701006 — Study Number
Paul E. Kirby — Study Director
1857B1 — Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml	0.708	0.807	-6.3	-27%	10	7%	2%
0.5 µl/ml	0.834	1.300	12.0	51%	71	50%	26%
SOLVENT 1	1.430	1.680	26.7	23.5	321*		
SOLVENT 2	1.097	1.654	20.2		141		
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml							
5.0 µg/ml							
SOLVENT 1							
SOLVENT 2							

Not Applicable - Eleana M. Carey 2-7-83

Tables prepared and calculations performed by: Eleana M. Carey 2-7-83
(Signature & Date)

\+ Culture Lost
++ Too Toxic to Clone

* This value was not used in calculating % control & % Total growth, because it [illegible]

Workbook Page No. 27 Report Page No. V-8

Study No. T1857.701006

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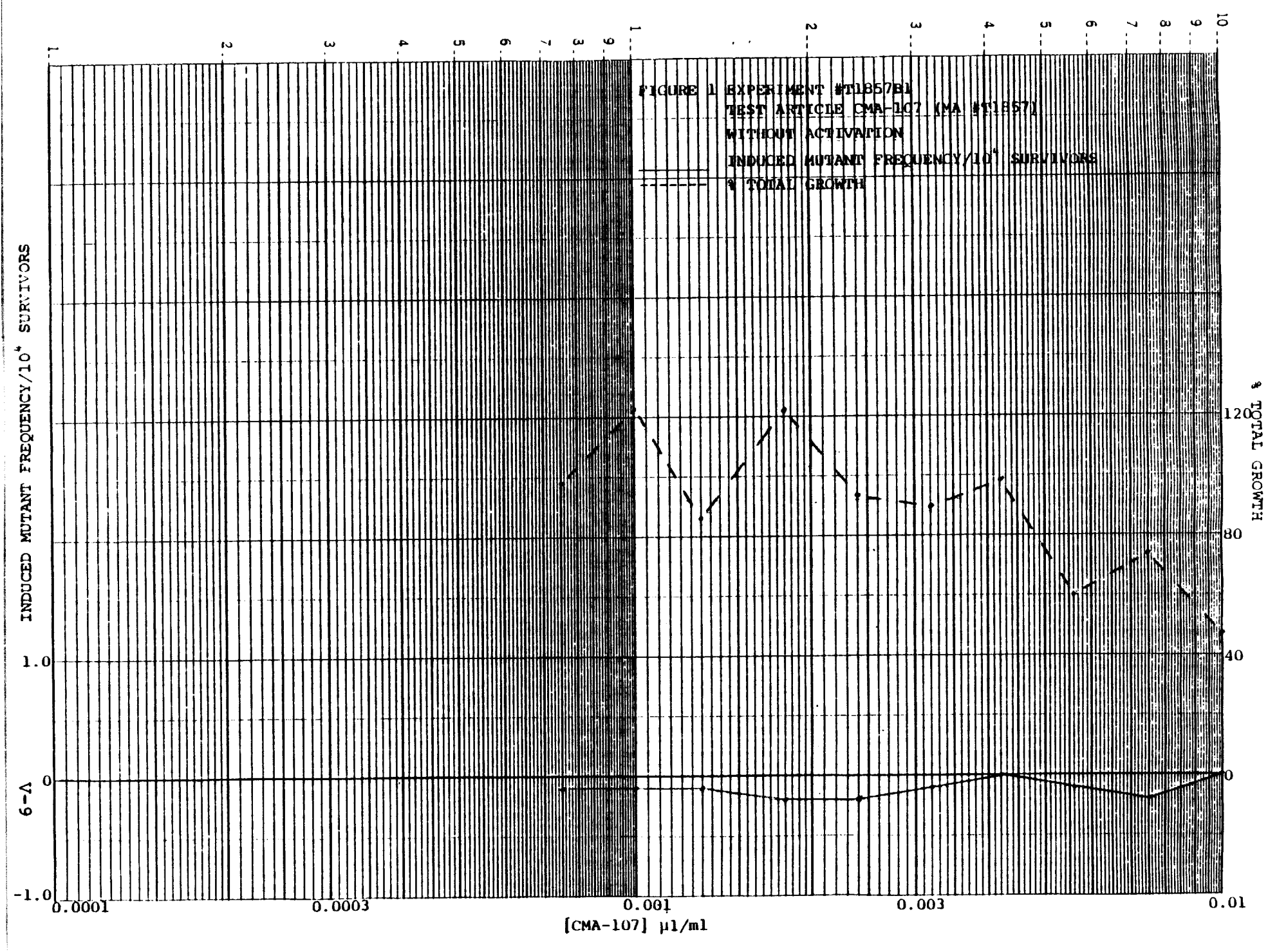

Form No. WL-41
2/25/82

Workbook Page No. 51

Report Page No. V-10

Study No. T 1857.701006

Table 6

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1857.701006	Paul E Kirby	1857B2
Study Number	Study Director	Experiment Number
CMA-107(T1857)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
0.024 µl/ml	33	38	25	32	45	46	46	46	1.4	0.9
0.018 µl/ml	30	38	31	33	130	90	124	116	0.6	0.1
0.013 µl/ml	27	26	37	33	136	148	139	141	0.5	0.0
0.010 µl/ml	30	45	33	36	178	163	163	168	0.4	-0.1
0.0075 µl/ml	47	39	48	46	178	183	198	186	0.5	0.0
0.0056 µl/ml	37	34	35	35	163	174	191	176	0.4	-0.1
0.0042 µl/ml	30	41	42	38	142	175	162	160	0.5	0.0
0.0032 µl/ml	25	28	31	30	153	164	171	163	0.4	-0.1
0.0024 µl/ml	61	40	37	43	†	175	158	167	0.5	0.0
0.0018 µl/ml	45	52	44	47	183	207	177	189	0.5	0.0
Solvent 1	44	31	45	40	189	211	189	196	0.4	0.5
Solvent 2	42	40	42	41	175	175	185	178	0.5	

Plates Counted By: Karen J. Ponso 2/23/83 (Signature & Date) ; Calculations Performed By: Jane Clarke 2/23/83 (Signature & Date)

* Per 10^4 surviving cells † Culture Lost

Secondary review KJP 2/23/83

Table 7

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1857.701006	Paul E. Kirby	1857B2
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	164	184	188	179	11	22	9	14	26.1	25.6
0.5µl/ml	430	415	456	435	113	97	84	98	8.9	8.4
SOLVENT 1	34	35	31	33	146	135	154	145	0.5	0.5
SOLVENT 2	47	39	37	40	160	+	175	168	0.5	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1										
SOLVENT 2										

Not Applicable

Plates Counted By: Karen J. Pomo 2/23/83 (Signature & Date)

Calculations Performed By: Karen J. Pomo 2/23/83 (Signature & Date)

*Per 10^4 surviving cells

+ Culture Lost

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Study No. T 1857.701006

Form No. WL-227

Table 8

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1857.701006	Paul E. Kirby	1857B2
Study Number	Study Director	Experiment Number
CMA-107 (T1857)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

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Study No. T1857 701006

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.042 µl/ml	0.097	0.185	0.0	0%	++		
0.032 µl/ml	0.090	0.128	0.0	0%	++		
0.024 µl/ml	0.281	0.412	1.4	8%	46	25%	2%
0.018 µl/ml	0.486	0.922	5.0	30%	116	62%	19%
0.013 µl/ml	0.589	1.089	7.1	43%	141	75%	32%
0.010 µl/ml	0.935	1.465	15.2	92%	168	90%	82%
0.0075 µl/ml	0.927	1.527	15.7	95%	186	99%	94%
0.0056 µl/ml	0.973	1.390	15.0	91%	176	94%	85%
0.0042 µl/ml	0.903	1.406	14.1	85%	160	86%	73%
0.0032 µl/ml	0.967	1.470	15.8	95%	163	87%	83%
0.0024 µl/ml	0.897	1.545	15.4	93%	167	89%	83%
0.0018 µl/ml	0.935	1.528	15.9	96%	189	101%	97%
SOLVENT 1	0.962	1.536	16.4 } 16.6		196 } 187		
SOLVENT 2	0.950	1.578	16.7 }		178 }		

* % Total Growth = $\frac{(\%\text{ Suspension Growth})\ (\%\text{ Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: [signature] 2/23/83
(Signature & Date)

Form No. WL-229 2/25/82

Table 9

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1857.701006 — Study Number

Paul E. Kirby — Study Director

185782 — Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml	0.489	0.820	4.5	26%	14	9%	2%
0.5 µl/ml	0.721	1.280	10.3	60%	98	62%	38%
SOLVENT 1	1.016	1.577	17.8 } 17.0		145 } 157		
SOLVENT 2	0.994	1.520	16.8 }		168 }		
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml							
5.0 µg/ml							
SOLVENT 1							
SOLVENT 2							

Not Applicable JE 2/23/83

Tables prepared and calculations performed by: Jane Clarke 2/23/83
(Signature & Date)

\+ Culture Lost
++ Too Toxic to Clone

Workbook Page No. 54 Report Page No. V-13

Study No. T1857.701006

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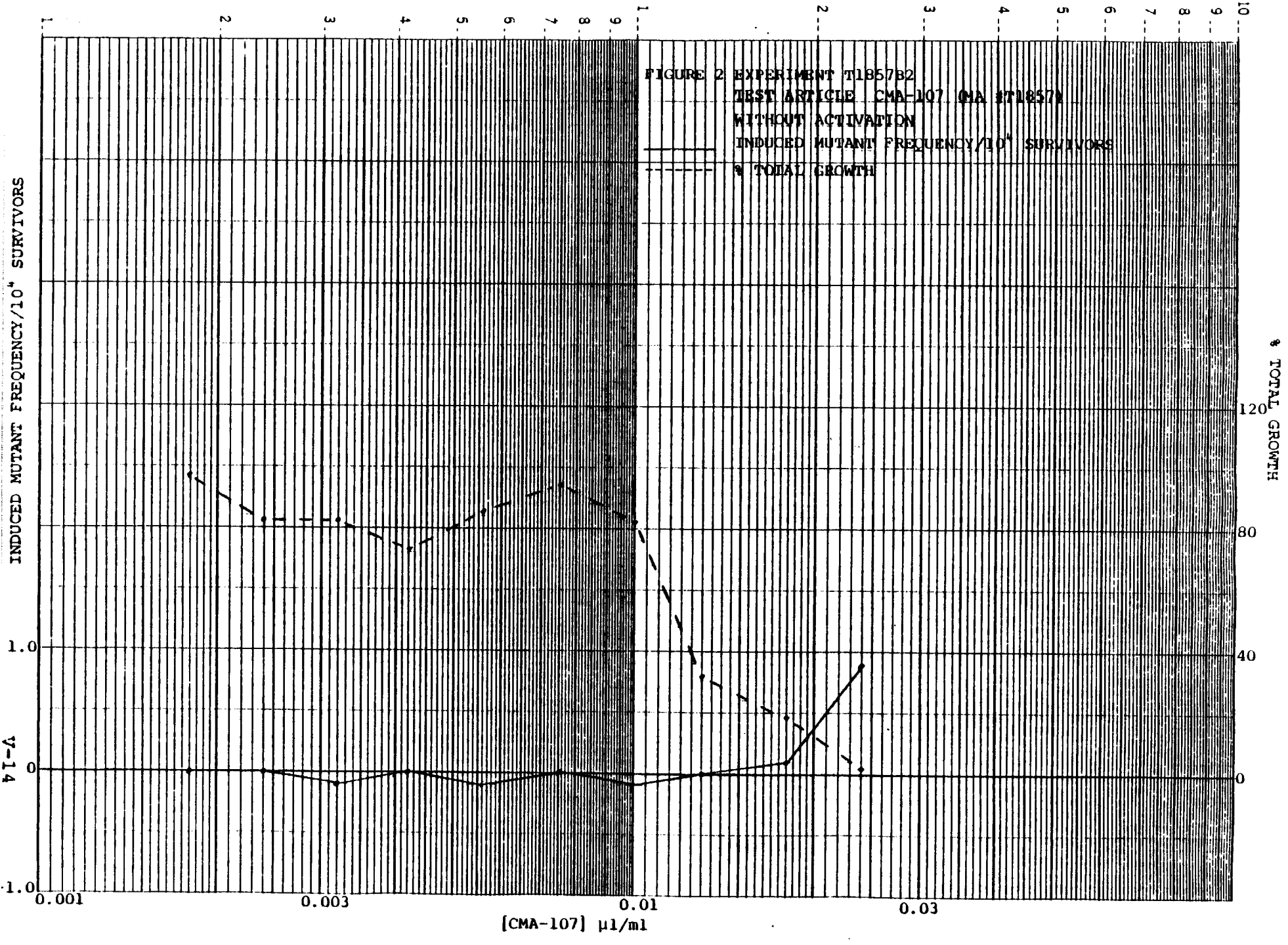

Study No. T857.701006

Table 10

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1857.701006 — Study Number
Paul E Kirby — Study Director
10.0 µl/ml to 0.001 µl/ml — Dose Range
CMA-107 (T857) — Test Article Identity
Acetone — Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Cell Concentration (x10⁶) Day 2	Suspension Growth Total	Suspension Growth % of Control
Without Activation					
	Solvent 1				
	Solvent 2				
With S-9 Activation	10.0 µl/ml	0.347*	0.293*	0.0	0%
	5.0 µl/ml	0.092	0.038	0.0	0%
	1.0 µl/ml	0.124	0.150	0.0	0%
	0.5 µl/ml	0.078	0.087	0.0	0%
	0.1 µl/ml	0.018	0.018	0.0	0%
	0.05 µl/ml	0.320	0.947	3.4	27%
	0.01 µl/ml	1.012	1.037	11.7	94%
	0.005 µl/ml	1.071	1.009	12.0	97%
	0.001 µl/ml	1.064	1.051	12.4	100%
	Solvent 1	1.080	1.112	13.3 } 12.4	
	Solvent 2	1.011	1.016	11.4 }	

Not Applicable 3-4-83

+ Culture Lost *Counts indicate debris not cells 3-3-83

Table Prepared By _____ (Signature) 3/3/83 (Date)

Form No. MA-233 Workbook Page No. 64 Report Page No. V-15

Table 11

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1857.701006	Paul E Kirby	1857B3
Study Number	Study Director	Experiment Number
CMA-107 (T1857)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Avg. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Avg. #/ Plate	Mutant* Frequency	Induced Mutant* Frequency
Solvent 1	62	+	+	62	189	162	194	182	0.7	0.5
Solvent 2	30	36	+	33	189	+	+	189	0.3	
0.066 µl/l A	269	233	+	251	+	+	+			
0.066 µl/l B	284	293	+	2889	96	84	82	87	6.6	6.1
0.049 µl/l A	134	149	155	146	159	165	+	162	1.8	1.3
0.049 µl/l B	+	+	+		+	+	+			
0.036 µl/l A	113	101	+	107	153	163	+	158	1.4	0.9
0.036 µl/l B	123	+	+	123	137	182	+	160	1.5	1.0
0.023 µl/l A	65	80	108	84	173	167	+	170	1.0	0.5
0.023 µl/l B	84	88	97	90	219	191	+	205	0.9	0.4
0.01 µl/l A	75	68	86	76	190	151	173	171	0.9	0.4
0.01 µl/l B	81	76	77	78	186	194	+	190	0.8	0.3
A										
B										

Plates Counted By: Leah Griswold 3/28/83
(Signature & Date)

Calculations Performed By: [illegible] 3/29/83
(Signature & Date)

* entry error [illegible] 3/29/83

+ Culture Lost

* Per 10^4 surviving cells

Workbook Page No. 81 Report Page No. V-16

1857.701006

TABLE 12

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1857.701006 — Study Number

Paul E Kirby — Study Director

1857B3 — Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced * Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1										
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	237	~~255~~ 1 +	+	237	88	84	108	93	5.1	4.6
5.0 µg/ml	271	253	253	258	158	153	167	159	3.2	2.7
SOLVENT 1	40	45	38	41	197	158	191	182	0.5	0.5
SOLVENT 2	48	+	+	48	189	187	191	189	0.5	

not attributable to MLD 3/29/83

Plates Counted By: Leah Griswold 3/28/83 (Signature & Date)

Calculations Performed By: Anita M Logan-Bank (Signature & Date) 3/29/83

*Per 10^6 surviving cells

+ Culture Lost

Notebook Page No. 82 Report Page No. V-17

Table 13

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1857.701006	Paul E Kirby	1857B3
Study Number	Study Director	Experiment Number
CMA-107 (T1857)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration X 10^6 cells Day 1	Day 2	Suspension Growth	% Control	Cloning Growth Avg. V. C.	% Control	% Total Growth
SOLVENT 1	0.986	1.824	20.0	18.8	182	186	
SOLVENT 2	0.888	1.769	17.5		189		
0.087 ul/ml A	0.096	0.069	0.0	0%	++		
0.087 ul/ml B	0.090	0.072	0.0	0%	++		
0.074 ul/ml A	0.228	0.178	0.0	0%	++		
0.074 ul/ml B	0.193	0.137	0.0	0%	++		
0.061 ul/ml A	0.369	1.112	4.6	24%	+		
0.061 ul/ml B	0.309	0.935	3.2	17%	87	47%	8%
0.049 ul/ml A	0.565	1.724	10.8	58%	162	87%	50%
0.049 ul/ml B	0.607	1.743	11.8	63%	+		
0.036 ul/ml A	0.758	1.822	15.3	82%	158	85%	69%
0.036 ul/ml B	0.797	1.764	15.6	83%	160	86%	71%
0.023 ul/ml A	0.826	1.874	17.2	92%	170	91%	84%
0.023 ul/ml B	0.764	1.797	15.3	81%	205	110%	89%
0.010 ul/ml A	0.832	1.911	17.7	94%	171	92%	86%
0.010 ul/ml B	0.869	1.805	17.4	93%	190	102%	95%
A							
B							

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: [signature] (Signature & Date) 3/29/83

Study No. T1857.701006

Workbook Page No. 83

Report Page No. V-18

Form No. MA-231

Table 14

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1857.701006	Paul E. Kirby	1857B3
Study Number	Study Director	Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml							
0.5 µl/ml							
SOLVENT 1							
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.555	1.052	6.5	42%	93	50%	21%
5.0 µg/ml	0.740	1.690	13.9	90%	159	85%	77%
SOLVENT 1	0.665	1.804	13.3	15.5	182	186	
SOLVENT 2	0.883	1.804	17.7		189		

not applicable MRB 3/29/83

Tables prepared and calculations performed by: [signature] 3/29/83

(Signature & Date)

\+ Culture Lost

++ Too Toxic to Clone

Study No. T1857 701006

K·E 10 X 10 TO THE INCH • 7 X 10 INCHES
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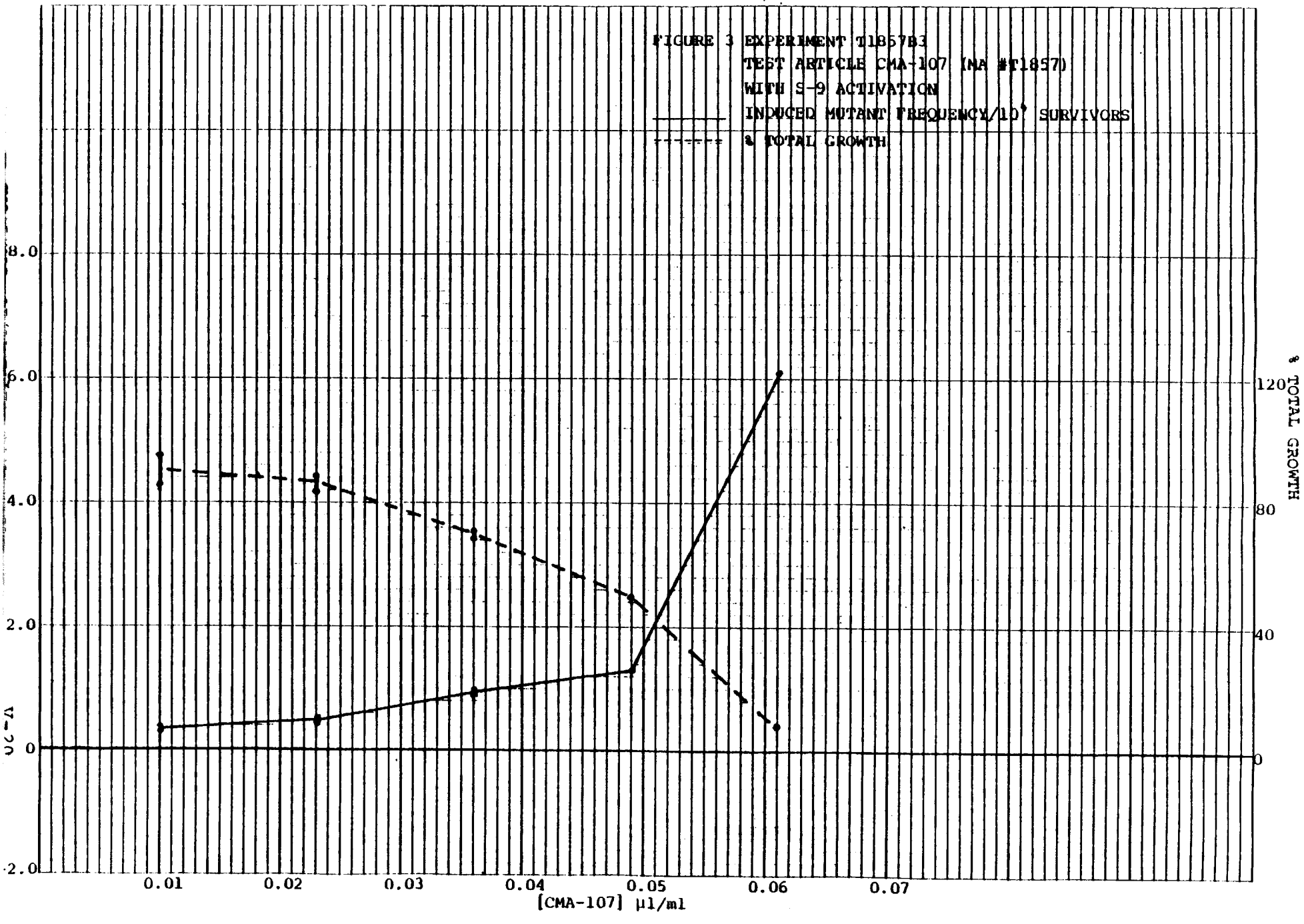


Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-107 (MA #T1857) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. One nonactivated culture having less than 10% Total Growth exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant due to the high toxicity observed. Five S-9 activated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-107 produced a negative response in the absence of exogenous metabolic activation and a positive response in the presence of metabolic activation.

APPENDIX

RECEIVED OCT 0 5 1982

Rec'd by
RA/QA 12/8/82.
N.K./ltr

L5178Y TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-107

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:
$2,650

Total charge for assay with metabolic activation:
$2,650

6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intra-peritoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-amino-anthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_2O, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0×10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F_0P will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3 x 10^6 cells/ml.

7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3 x 10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3 x 10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2 x 10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of

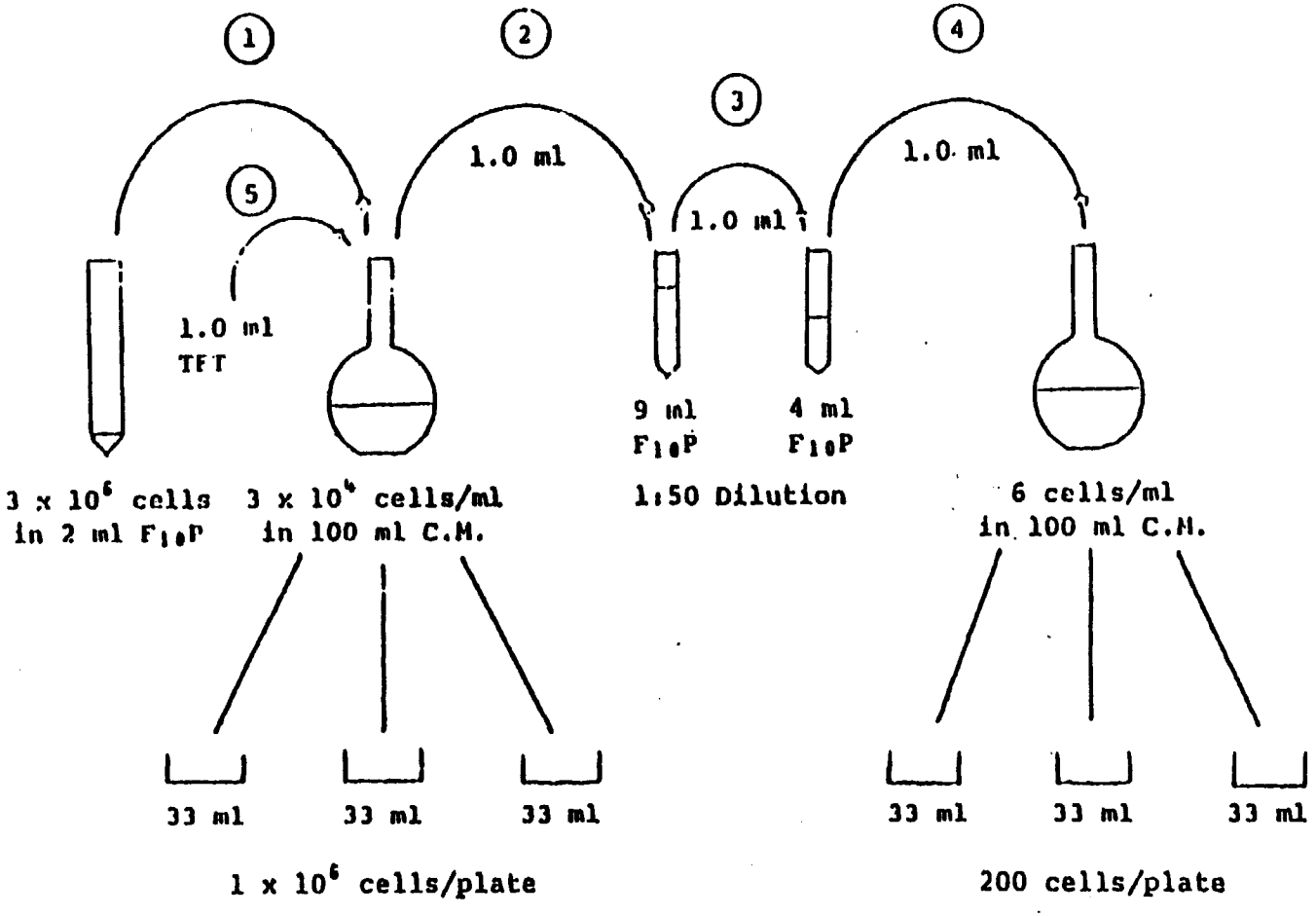


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

MICROBIOLOGICAL ASSOCIATES

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data
After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency?_____
If so, to which agency or agencies? _____

Does the sponsor request that samples of the Test Article dosing solutions be returned?_____

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: 11/24/82

13.2 Proposed Initiation Date: January 4, 1983

13.3 Scheduled Completion Date: February 25, 1983

13.4 Report Submission to Sponsor Date: February 25, 1983

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

Carol Stark
SPONSOR'S AUTHORIZED REPRESENTATIVE

11/24/82
DATE PROTOCOL APPROVED BY SPONSOR

Paul E. Kinley 11/24/82
STUDY DIRECTOR DATE

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136) and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \text{Average Counts (corrected for coincidence)} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{cells/ml})\ (20\ \text{ml})}{\text{(No. of Cells/ml)}}$$

$$\text{Volume of Media to Add} = 20\ \text{ml} - \text{Volume of Cells Retained}$$

---

2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

---

3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138) and L5178Y TK+/- Assay - Daily Counts (Positive Controls) (Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^{6} = \text{Average Counts (corrected for coincidence)} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{Cells/ml})\ (20\ \text{ml}^{*})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20\ \text{ml}^{*}) - (\text{Volume of Cells Retained})$$

* The final volume is adjusted to 10 ml on the third day of expression.

---

4. Tables 2, 3, and 4 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^{8}\text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left(\text{Mutant Frequency of Treated Cultures}\right) - \left(\text{Average Mutant Frequency of Solvent Controls}\right)$$

---

5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

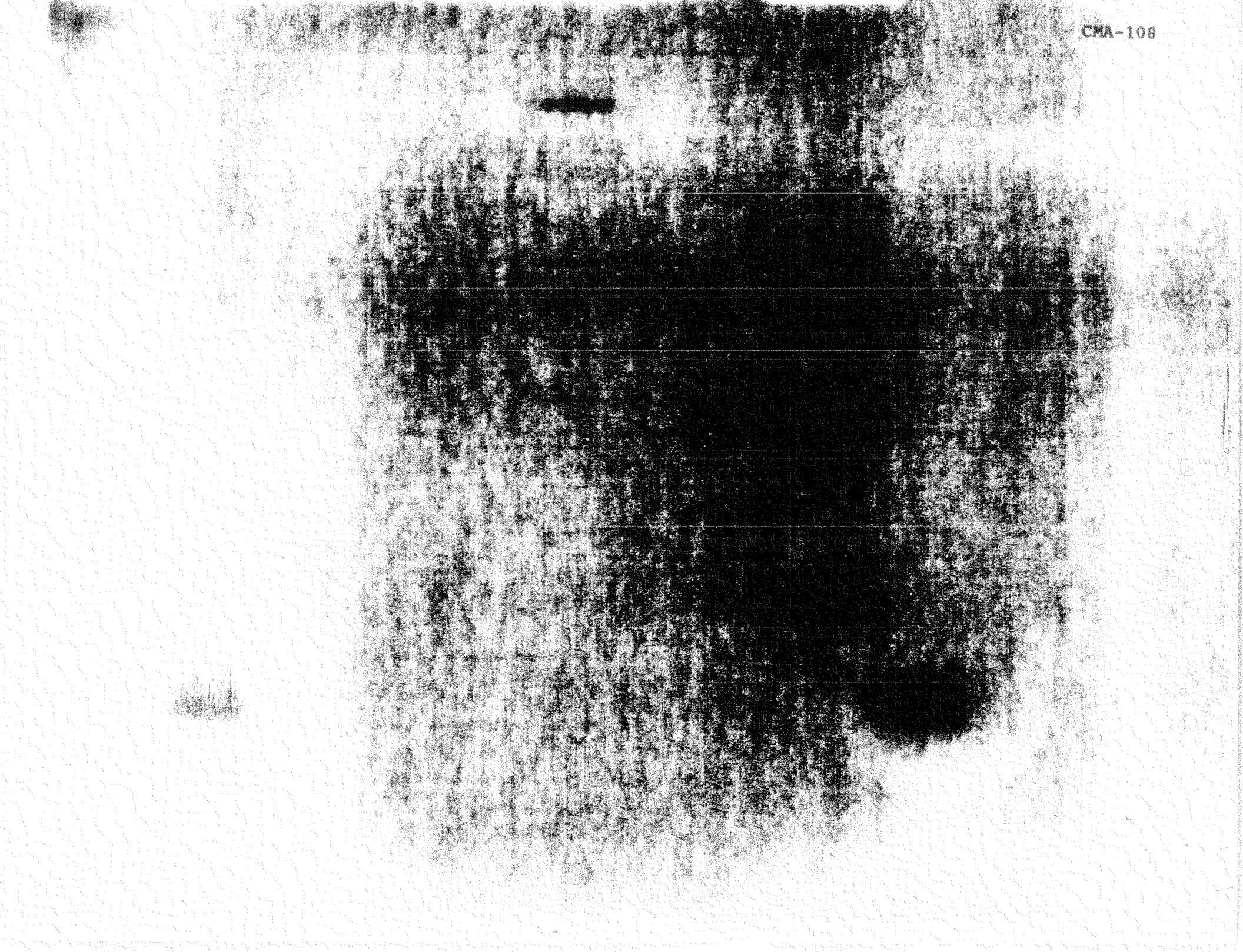
$$\text{\% Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

$$\text{\% Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\text{\% Growth} = \frac{(\text{\% Suspension Growth})\ (\text{\% Cloning Growth})}{100}$$



Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-108 (MA #T1852) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 6% to 105% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 12% to 103% Total Growth (Experiment 1852B4).

The highest test article concentration cloned in the non-activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. The result is not considered significant since mutant frequencies observed at such highly toxic levels may be due to epigenetic events. One S-9 activated culture having greater than 10% Total Growth exhibited a mutant frequency which was significantly different from the mean mutant frequency of the solvent controls.

The results indicate that under the conditions of this test, the test article CMA-108 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

Results

The Initial Toxicity Test (Table 1) performed on test article CMA-108 (MA #T1852) in the absence of S-9 indicated a threshold level of complete toxicity at 0.1 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.1 µl/ml to 0.0013 µl/ml.

After a two day expression period, seven cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018 or 0.0013 µl/ml test article. These concentrations produced a range in Suspension Growth of 12% to 100%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

One of the cultures that was cloned (0.0075 µl/ml) exhibited a mutant frequency which was 3.8 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 30% to 105%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-108 (MA #T1852). The results indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016 or 0.0012 µl/ml. These concentrations

produced a range in Suspension Growth of 67% to 100%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 70% to 109%.

The positive control cultures of the assay described above were lost to contamination. Therefore, the assay was repeated in the presence of S-9 over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. These cultures were treated with 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, 0.0012 or 0.00089 µl/ml. These concentrations produced a range in Suspension Growth of 17% to 106%. The Cloning Data are presented in Tables 11 and 12 and the Total Compound Toxicity Data are presented in Tables 13 and 14. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 3.

One culture (0.012 µl/ml) that was cloned exhibited a mutant frequency that was 3.4 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 12%. None of the remaining cultures that were cloned exhibited mutant frequencies that were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 69% to 103%.

T1852 701006

Table 1

701 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL COMPOUND TOXICITY TEST

T1852.701006	Paul E Kirby	100 ul/ml – 0.001 ul/ml
Study Number	Study Director	Dose Range
CMA-108 (T1852)		Acetone
Test Article Identity		Solvent

	Test Article Concentration	Cell Concentration (X 10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation	100 ul/ml	1.181**	1.238**	0.0	0%
	10 ul/ml	0.688**	0.556**	0.0	0%
	1.0 ul/ml	0.014	0.014	0.0	0%
	0.1 ul/ml	0.021	0.009	0.0	0%
	0.01 ul/ml	0.420	0.582	2.7	17%
	0.001 ul/ml	1.102	1.305	16.0	101%
	Solvent 1	1.093	1.281	15.6	15.9* ~~15.8~~
	Solvent 2	1.112	1.303	16.1	
With S-9 Activation					
	Solvent 1				
	Solvent 2				

not applicable AMRB 12/21/82

+ Culture Lost

Table Prepared By: Andrea N Rogers-Backe 12/21/82
Signature Date

** no viable cells present. Counts are compound precipitate. AMRB 12/21/82

* calculation error. AMRB 4/28/83

Workbook Page No. 13 Report Page No. VI-4

Form No. WL-232
2/22/82

Form No. WL-41
2/25/82

Workbook Page No. 30

Report Page No. VI-5

Study No. T1852.701006

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1852.701006 — Study Number
Paul E. Kirby — Study Director
1852B1 — Experiment Number
CMA-108 (T1852) — Test Article Identity
Acetone — Solvent
None — Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
0.0075 #/ml	49	51	69	56	76	57	91	75	1.5	1.1
0.0056 #/ml	48	52	55	52	164	141	135	147	0.7	0.3
0.0042 #/ml	47	38	47	44	162	153	162	159	0.6	0.2
0.0032 #/ml	42	27	36	35	164	172	137	158	0.4	0.0
0.0024 #/ml	36	42	42	40	163	165	158	162	0.5	0.1
0.0018 #/ml	48	27	+	38	162	153	171	162	0.5	0.1
0.0013 #/ml	48	29	35	37	165	159	167	164	0.5	0.1
Solvent 1	25	41	23	30	173	170	159	167	0.4	0.4
Solvent 2	+	+	+	+	155	147	134	145	**	

Plates Counted By: Jane Clarke 1/31/83 (Signature & Date)
Calculations Performed By: Jane Clarke 2/1/83 (Signature & Date)

* Per 10^4 surviving cells + Culture Lost ** cannot be calculated JC 2/1/83

Form No. WL-128

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1852.701006	Paul B. Kirby	1852B1
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced * Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	187	202	195	195	13	22	12	16	24.9	24.4
0.5µl/ml	346	309	321	325	78	72	79	76	8.5	8.0
SOLVENT 1	38	36	34	36	143	150	174	156	0.5	0.5
SOLVENT 2	35	38	38	37	151	146	163	153	0.5	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1	Not Applicable JH 2-1-83									
SOLVENT 2										

Plates Counted By: Jane D. Clarke 1/31/83 (Signature & Date)

Calculations Performed By: Jane D. Clarke 2/1/83 (Signature & Date)

*Per 10^8 surviving cells

+ Culture Lost

Workbook Page No. 31 Report Page No. VI-6

Study No. T1852.701005

Form No. WL-227
7/.../82

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1852.701006	Paul E. Kirby	1852B1
Study Number	Study Director	Experiment Number
LMA-108 (T1852	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^5 cells/ml		Suspension Growth		Cloning Growth		% Total Growth *
	Day 1	Day 2	Total	% Control	Ave. V. C.	% Control	
0.013 µl/ml	0.008	0.022	0.0	0%	++		
0.010 µl/ml	0.008	0.018	0.0	0%	++		
0.0025 µl/ml	0.368	0.486	2.0	12%	75	48%	6%
0.0056 µl/ml	0.574	0.799	5.1	32%	147	94%	30%
0.0042 µl/ml	0.889	1.136	11.2	71%	159	102%	72%
0.0032 µl/ml	0.934	1.228	12.7	80%	158	101%	81%
0.0024 µl/ml	0.984	1.326	14.5	91%	162	104%	95%
0.0018 µl/ml	1.052	1.360	15.9	100%	162	104%	104%
0.0013 µl/ml	1.082	1.323	15.9	100%	164	105%	105%
SOLVENT 1	1.098	1.294	15.8 / 15.9		167 / 156		
SOLVENT 2	1.083	1.325	15.9		145		

Workbook Page No. 32 Report Page No. VI-7

Study No. 1 1852 701006

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: [signature] 4/1/83
(Signature & Date)

Form No. TL-229
2/25/82

Table 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1852.701006 — Study Number
Paul E. Kirby — Study Director
1852B' — Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml	0.501	0.762	4.2	25%	16	10%	3%
0.5 µl/ml	0.826	1.158	10.6	63%	76	49%	31%
SOLVENT 1	1.089	1.373	16.6 16.8		156 155		
SOLVENT 2	1.094	1.395	17.0		153		
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml							
5.0 µg/ml							
SOLVENT 1							
SOLVENT 2							

Not Applicable JL 2/1/83

Tables prepared and calculations performed by: Jane Clarke 2/1/83
(Signature & Date)

\+ Culture Lost
++ Too Toxic to Clone

Workbook Page No. 33 Report Page No. VI-8

Study No. T1852.701006

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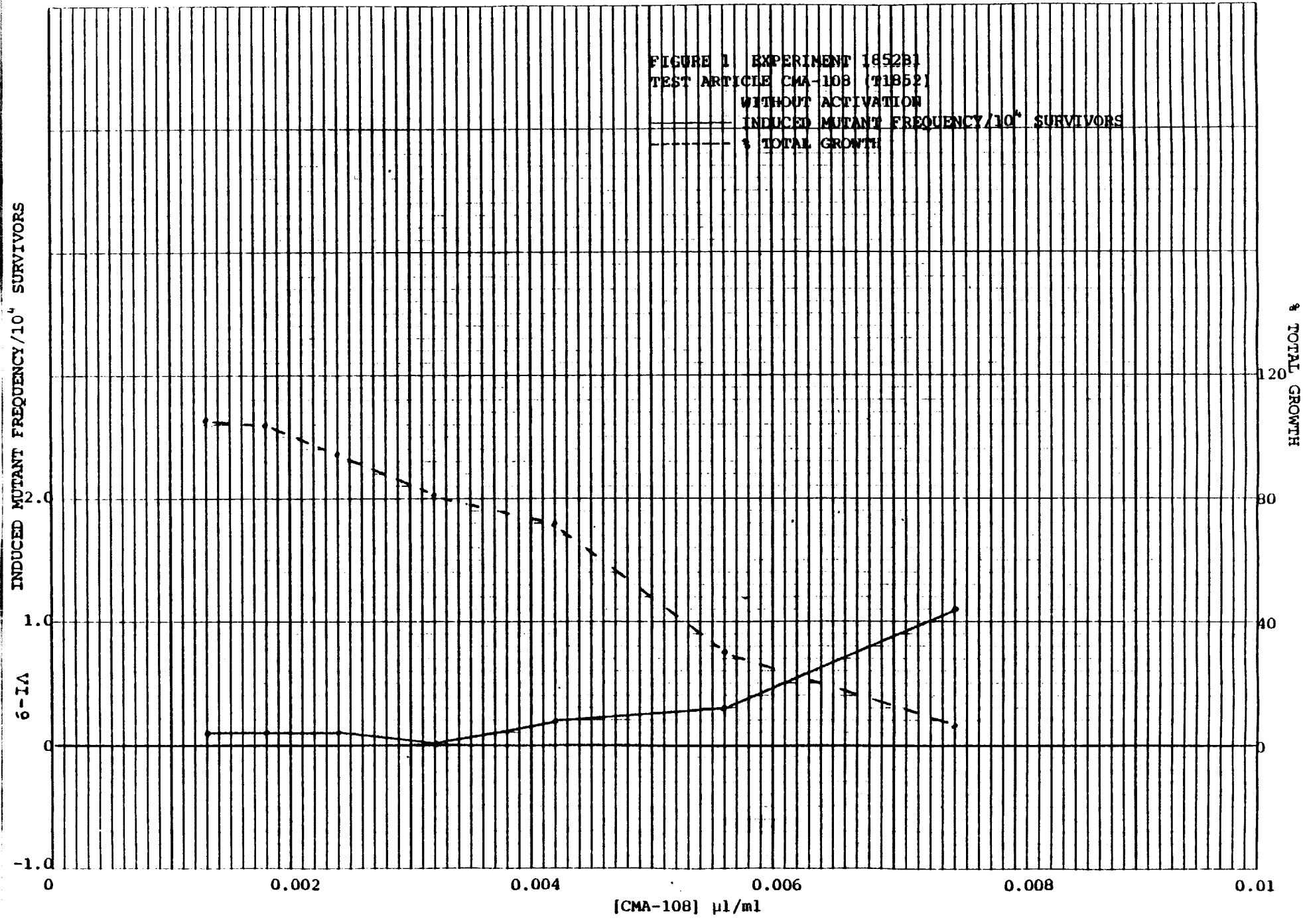

Study No. T1852 701006

Table 6

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1852 701006	Paul E. Kirby	10 ul/ml → 0.001 ul/ml
Study Number	Study Director	Dose Range
CMA-108 (T1852)		Acetone
Test Article Identity		Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation					
	Solvent 1				
	Solvent 2				
With S-9 Activation	10 ul/ml	0.315*	0.209	0.0	0%
	5.0 ul/ml	0.161	0.125	0.0	0%
	1.0 ul/ml	0.022	0.011	0.0	0%
	0.5 ul/ml	0.030	0.010	0.0	0%
	0.1 ul/ml	0.011	0.011	0.0	0%
	0.05 ul/ml	0.017	0.008	0.0	0%
	0.01 ul/ml	0.694	1.394	10.7	85%
	0.005 ul/ml	0.783	1.405	12.2	97%
	0.001 ul/ml	0.798	1.489	13.2	105%
	Solvent 1	0.822	1.390	12.7 } 12.6	
	Solvent 2	0.779	1.436	12.4 }	

Not Applicable - Eleanor M. Carey

+ Culture Lost
* no viable cells present. SMRD 2/18/83

Table Prepared By Eleanor M. Carey 2-18-83
Signature Date

Form No. MA-233 Workbook Page No. 43 Report Page No. VI-10

Table 7

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1852.701006	Paul E. Kirby	1852B2
Study Number	Study Director	Experiment Number
CMA-108 (T1852)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
0.016 ul/ml	46	51	+	49	213	224	197	211	0.5	0.2
0.012 ul/ml	45	51	44	47	200	170	177	182	0.5	0.2
0.0089 ul/ml	48	48	+	35	194	202	182	193	0.4	0.1
0.0067 ul/ml	40	19	+	30	198	214	189	200	0.3	0.0
0.005 ul/ml	35	34	44	38	199	183	221	201	0.4	0.1
0.0038 ul/ml	33	28	33	31	184	237	226	216	0.3	0.0
0.0028 ul/ml	38	47	30	38	184	232	+	209	0.4	0.1
0.0021 ul/ml	29	33	35	32	218	219	223	220	0.3	0.0
0.0016 ul/ml	34	30	+	32	197	212	184	198	0.3	0.0
0.0012 ul/ml	+	+	+		+	+	+			
Solvent 1	24	35	+	30	178	195	+	187	0.3	0.3
Solvent 2	29	27	26	27	222	196	236	216	0.3	

Plates Counted By: [signature] 3-28 (Signature & Date)

Calculations Performed By: [signature] 3-28 (Signature & Date)

* Per 10^4 surviving cells

\+ Culture Lost

Form No. MA-127
2/16/83

Workbook Page No. 60

Report Page No. VI-11

Study No. T1852 701006

Table 8

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1852.701006 — Study Number

Paul E Kirby — Study Director

1852 B2 — Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1										
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	+	+	+		+	+	+			
5.0 µg/ml	+	+	+		+	+	+			
SOLVENT 1	+	+	+		+	+	+			
SOLVENT 2	+	+	+		+	+	+			

Not applicable eff 3-8-83

Plates Counted By: Jennie L. Johnson 3-8-83 (Signature & Date)

Calculations Performed By: (Signature & Date)

*Per 10^4 surviving cells

+ Culture Lost

Workbook Page No. 61 Report Page No. VI-12

Study No. T1852.701006

Form No. MA-228
2/16/83

Table 9

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1852.701006	Paul E. Kirby	1852 B2
Study Number	Study Director	Experiment Number
CMA-108(CT1852)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.028 ul/ml	0.052	0.055			++		
0.021 ul/ml	0.153	0126			++		
0.016 ul/ml	0.586	1.461	9.5	67%	211	105%	70%
0.012 ul/ml	0.712	1.495	11.8	84%	182	90%	75%
0.0089 ul/ml	0.744	1.475	12.2	86%	193	96%	83%
0.0067 ul/ml	0.763	1.582	13.4	95%	200	99%	94%
0.0050 ul/ml	0.777	1.559	13.5	95%	201	100%	95%
0.0038 ul/ml	0.788	1.586	13.9	94%	216	107%	101%
0.0028 ul/ml	0.847	1.477	13.9	98%	209	104%	102%
0.0021 ul/ml	0.827	1.544	14.2	100%	220	107%	107%
0.0016 ul/ml	0.847	1.501	14.1	100%	198	98%	98%
0.0012 ul/ml	0.915	1.477			+		
SOLVENT 1	0.808	1.589	14.3 } 14.2		187 } 202		
SOLVENT 2	0.837	1.505	14.0 }		216 }		

* % Total Growth = $\frac{(\%\text{ Suspension Growth})\ (\%\text{ Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: Janice L. Johnson 3-8-83
(Signature & Date)

Study No. T1852 701006

Workbook Page No. 62 Report Page No. VI-13

Table 10

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1852.701006	Paul E. Kirby	1852B2
Study Number	Study Director	Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml							
0.5 µl/ml							
SOLVENT 1							
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.582	1.071	6.9	45%	+		
5.0 µg/ml	0.731	1.335	10.8	64%	+		
SOLVENT 1	0.898	1.602	16.0 } 15.3		+		
SOLVENT 2	0.908	1.443	14.6		+		

Not Applicable JJ 3-8-83

Tables prepared and calculations performed by: [signature] 3-8-83 (Signature & Date)

\+ Culture Lost

++ Too Toxic to Clone

Study No. T1852 701006

Form No. MA-229

Workbook Page No. 63

Report Page No. VI-14

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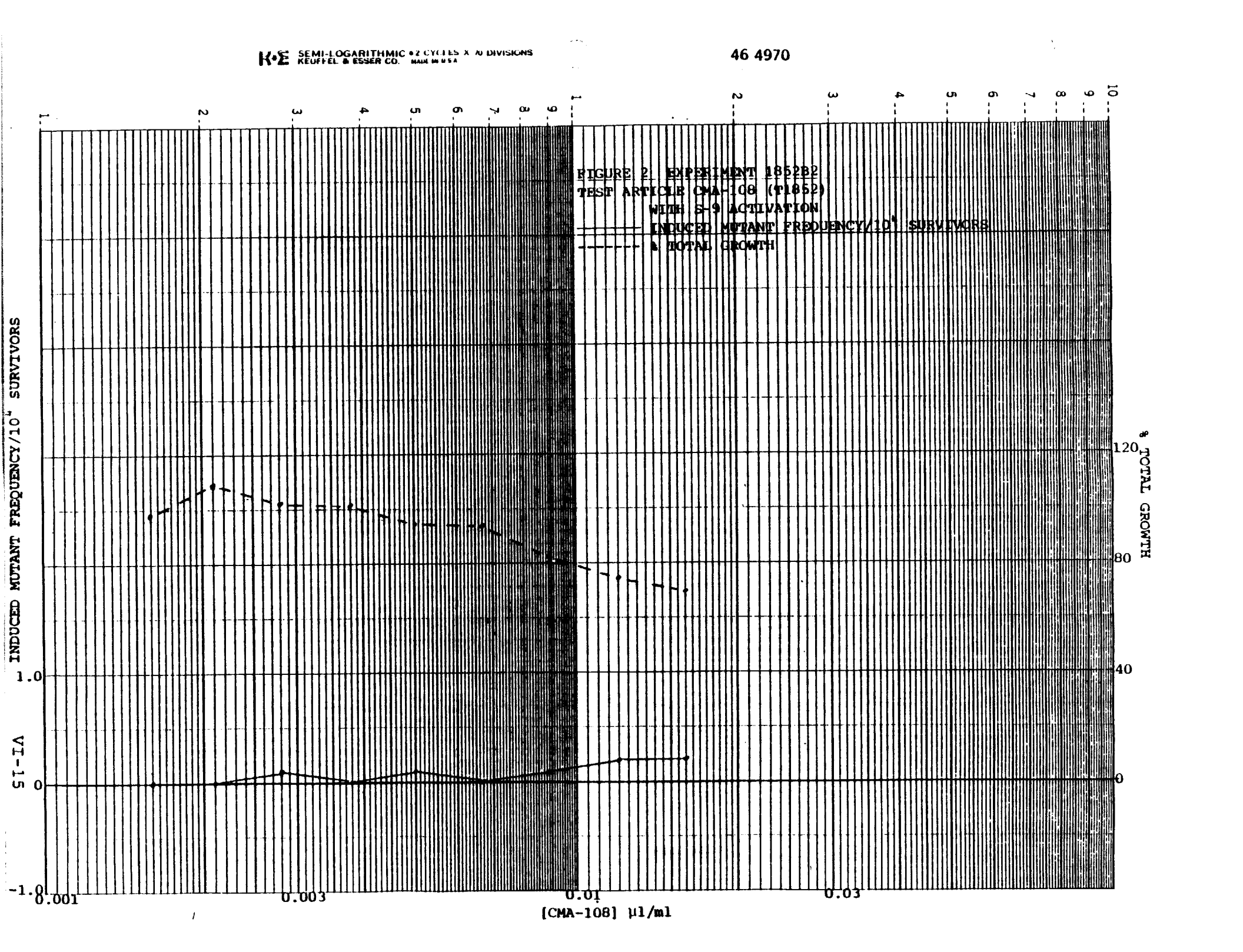

Table 11

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1852.701006	Paul E Kirby	1852B4
Study Number	Study Director	Experiment Number
CMA-108 (T1852)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced Mutant Frequency*
0.012 µl/ml	111	112	116	113	127	136	139	134	1.7	1.2
0.0089 µl/ml	63	63	57	61	161	150	173	161	0.8	0.3
0.0067 µl/ml	50	+	56	53	179	163	163	168	0.6	0.1
0.005 µl/ml	51	59	63	58	153	184	175	171	0.7	0.2
0.0038 µl/ml	~~28~~ + **	48	56	52	198	199	210	202	0.5	0.0
0.0028 µl/ml	28	55	49	44	194	153	201	183	0.5	0.0
0.0021 µl/ml	42	54	32	43	160	191	165	172	0.5	0.0
0.0016 µl/ml	52	33	41	42	165	182	160	169	0.5	0.0
0.0012 µl/ml	28	27	33	29	161	142	154	152	0.4	-0.1
0.00089 µl/ml	42	38	55	45	159	176	198	178	0.5	0.0
Solvent 1	39	45	44	43	161	190	177	176	0.5 } 0.5	
Solvent 2	30	40	44	38	185	198	190	191	0.4 }	

Plates Counted By: Karen J. Poms 4/4/83 (Signature & Date)

Calculations Performed By: Jane J. Clarke 4/4/83 (Signature & Date)

** entry error KJP

Form No. MA-127

Workbook Page No. 100

Report Page No. VI-16

Study No. T1852 701006

Table 12

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1852.701006	Paul E. Kirby	1852B4
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1										
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	135	111	130	125	29	26	24	26	9.5	9.1
5.0 µg/ml	207	200	185	197	105	103	131	113	3.5	3.1
SOLVENT 1	45	37	41	41	187	208	191	195	0.4 } 0.4	
SOLVENT 2	30	36	34	33	196	186	194	192	0.3	

Not Applicable KJP 4/4/83

Plates Counted By: Karen J. Poms 4/4/83 (Signature & Date)

Calculations Performed By: [signature] 4/4/83 (Signature & Date)

*Per 10^6 surviving cells

+ Culture Lost

Workbook Page No. 101 Report Page No. VI-17

Form No. MA-228 7/16/82

Table 13

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1852 701006	Paul E. Kirby	1852B4
Study Number	Study Director	Experiment Number
CMA-108 (T1852)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^5 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.021 µl/ml	0.014	0.080	0.0	0%	++		
0.016 µl/ml	0.031	0.044	0.0	0%	++		
0.012 µl/ml	0.262	0.764	2.5	17%	134	73%	12%
0.0089 µl/ml	0.805	1.353	12.1	79%	161	88%	69%
0.0067 µl/ml	0.846	1.381	13.0	85%	168	91%	77%
0.0050 µl/ml	0.893	1.354	13.4	88%	171	93%	82%
0.0038 µl/ml	0.910	1.405	14.2	93%	202	110%	102%
0.0028 µl/ml	0.894	1.341	13.3	87%	183	99%	87%
0.0021 µl/ml	0.920	1.393	14.2	93%	172	93%	87%
0.0016 µl/ml	0.873	1.321	12.9	85%	169	92%	78%
0.0012 µl/ml	0.943	1.448	15.2	99%	152	83%	82%
0.00089 µl/ml	1.008	1.452	16.3	106%	178	97%	103%
SOLVENT 1	1.031	1.376	15.8 / 15.3		176 / 184		
SOLVENT 2	0.987	1.347	14.8		191		

Study No. T1852 701006

Workbook Page No. 102 Report Page No. VI-18

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: [signature] 7/4/83
(Signature & Date)

Table 14

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1852.701006 — Study Number | Paul E. Kirby — Study Director | 1852B4 — Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V.C.	% Control	% Total Growth
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml							
0.5 µl/ml							
SOLVENT 1	Not Applicable		4-4-83				
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.493	0.582	3.2	22%	26	13%	3%
5.0 µg/ml	0.732	1.107	9.0	62%	113	58%	36%
SOLVENT 1	0.981	1.299	14.2 | 14.5		195 | 194		
SOLVENT 2	1.000	1.323	14.7		192		

Tables prepared and calculations performed by: Jan Clarke 4/4/83 (Signature & Date)

\+ Culture Lost

++ Too Toxic to Clone

Study No. T1852.701006

Page No. M1-229 Workbook Page No. 103 Report Page No. VI-19

K•E SEMI-LOGARITHMIC • 3 CYCLES X 70 DIVISIONS
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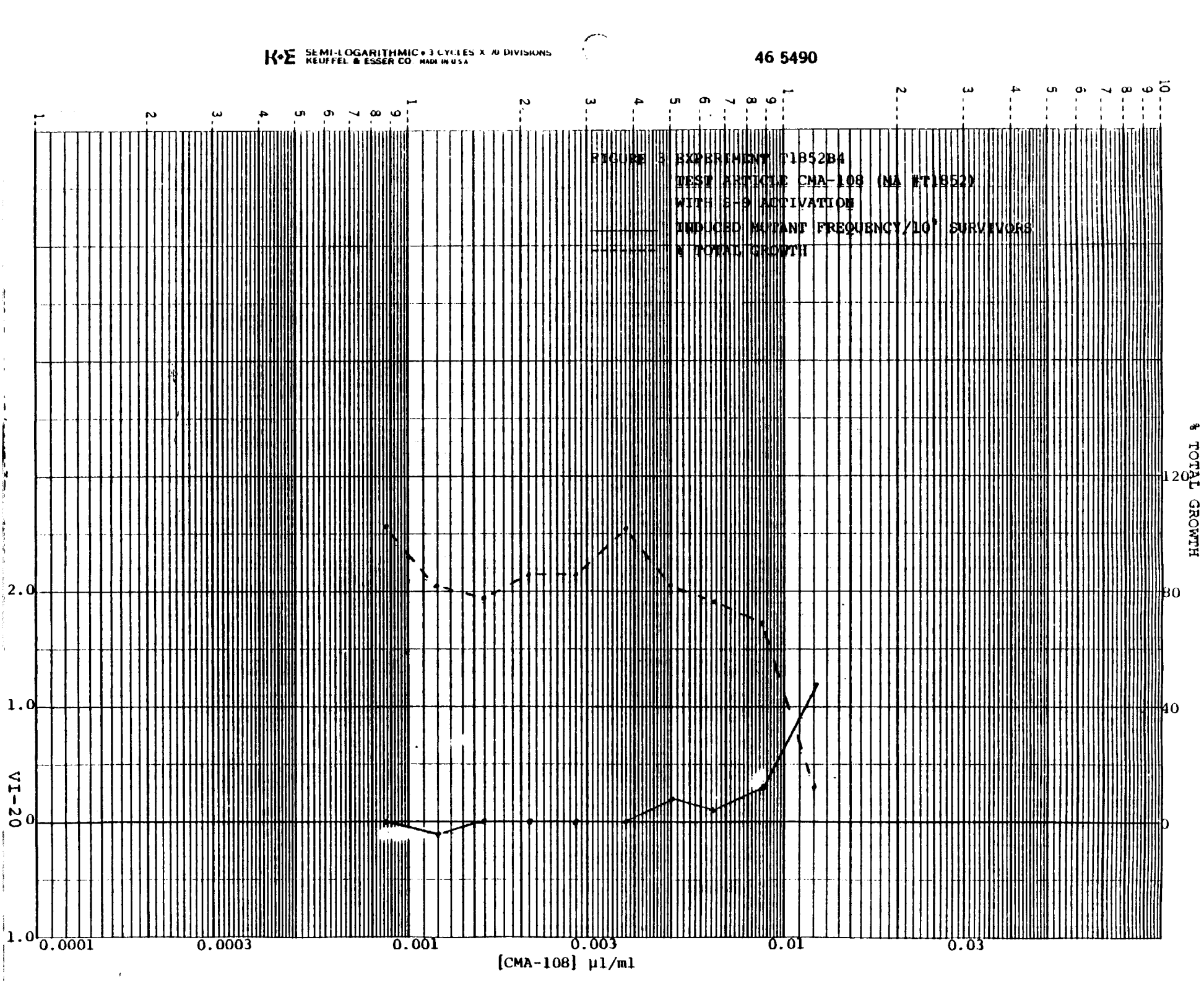


Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-108 (MA #T1852) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest concentration cloned in the nonactivated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%. The result is not considered significant since mutant frequencies observed at such highly toxic doses may be due to epigenetic events. One S-9 activated culture that was cloned exhibited a mutant frequency which was significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-108 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of exogenous metabolic activation.

RECEIVED OCT 0 5 1982

Rec'd by
RA/QA 12/8/82.
N.K./PEC

L5178Y $TK^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-108

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:
$2,650

Total charge for assay with metabolic activation:
$2,650

6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-amino-anthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_2O, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0×10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F_0P will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3×10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3×10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2×10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of

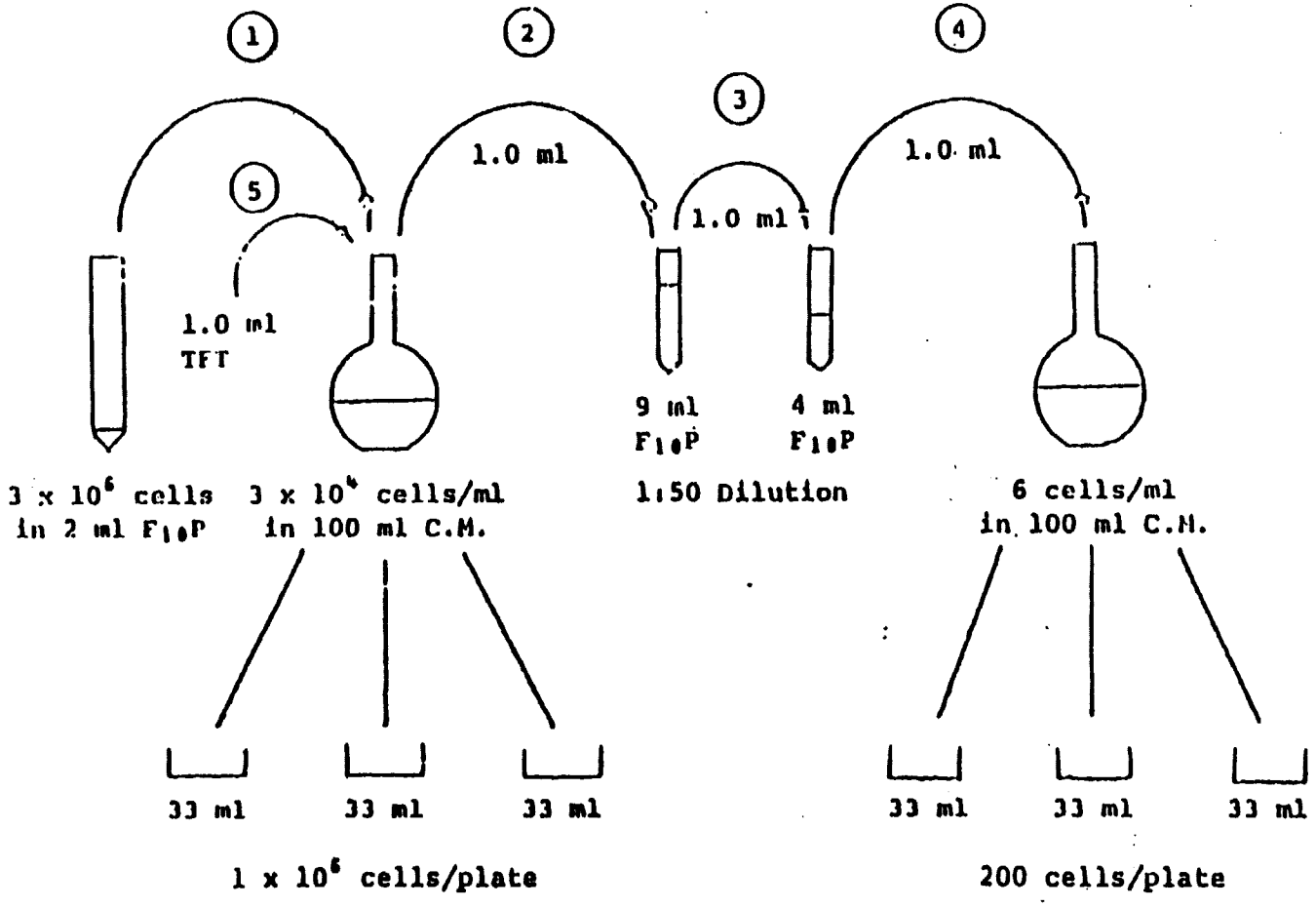


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

MICROBIOLOGICAL ASSOCIATES

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency?_____
If so, to which agency or agencies? _____
_____
Does the sponsor request that samples of the Test Article dosing solutions be returned?_____

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: 11/24/82

13.2 Proposed Initiation Date: December 16, 1982

13.3 Scheduled Completion Date: January 29, 1983

13.4 Report Submission to Sponsor Date: January 29, 1983

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

SPONSOR'S AUTHORIZED REPRESENTATIVE

11/20/82
DATE PROTOCOL APPROVED BY SPONSOR

STUDY DIRECTOR — DATE 11/24/82

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136) and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \text{Average Counts (corrected for coincidence)} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{cells/ml})\ (20\ \text{ml})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = 20\ \text{ml} - \text{Volume of Cells Retained}$$

---

2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

---

3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138) and L5178Y TK+/- Assay - Daily Counts (Positive Controls) (Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^{6} = \text{Average Counts (corrected for coincidence)} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{Cells/ml})\ (20\ \text{ml}^{*})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20\ \text{ml}^{*}) - (\text{Volume of Cells Retained})$$

* The final volume is adjusted to 10 ml on the third day of expression.

---

4. Tables 2, 3, and 4 - L5178Y TK+ - Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^{6} \text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left(\text{Mutant Frequency of Treated Cultures}\right) - \left(\text{Average Mutant Frequency of Solvent Controls}\right)$$

---

5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

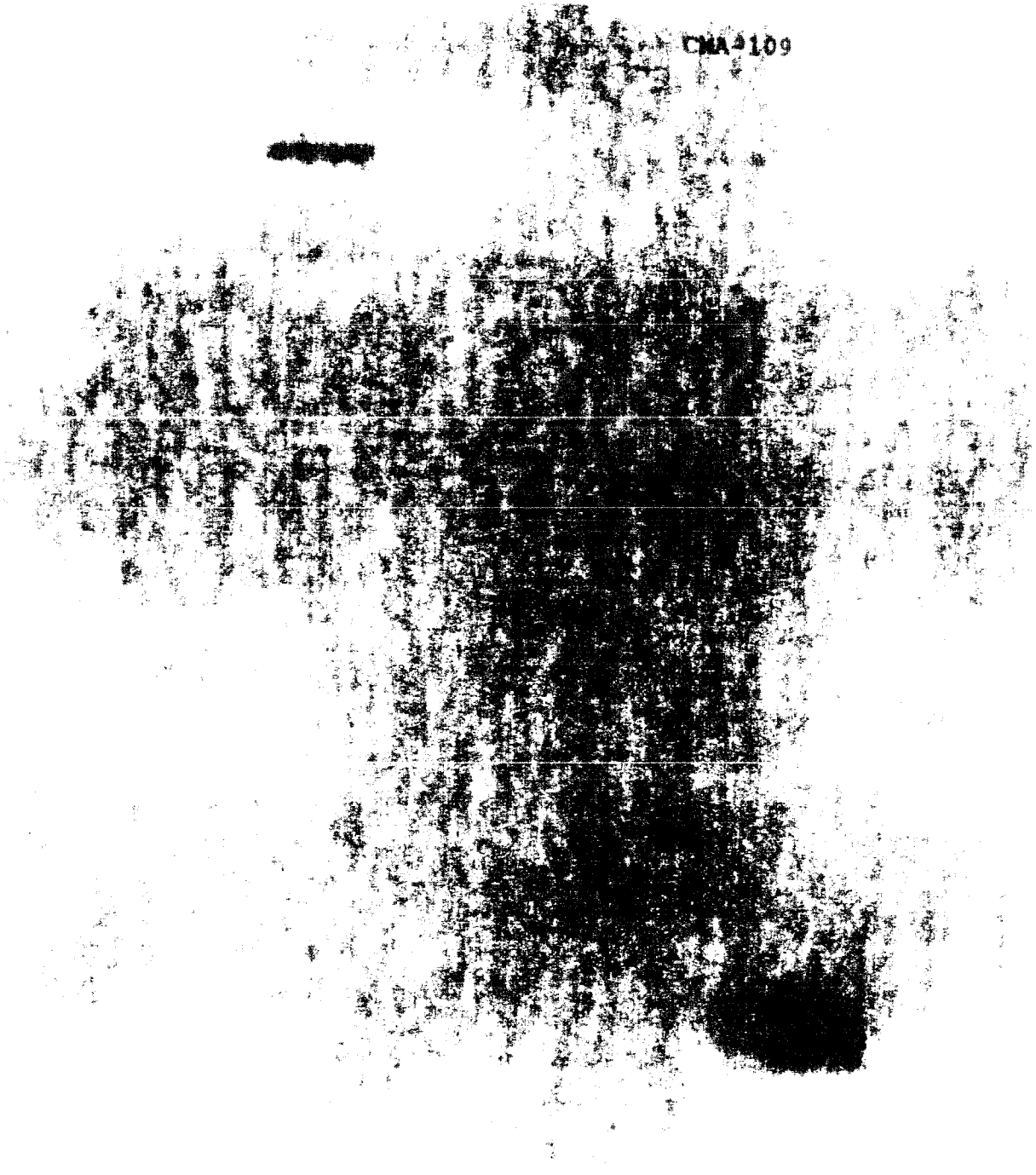
$$\text{\% Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than 0.3×10^{5} cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

$$\text{\% Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\text{\% Growth} = \frac{(\text{\% Suspension Growth})\ (\text{\% Cloning Growth})}{100}$$



Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-109 (MA #T1855) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 2% to 92% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 6% to 125% Total Growth.

The highest test article concentration cloned in the S-9 activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. Two of the nonactivated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. These results are not considered significant as the Total Growth of these cultures was less than 10%. TFR resistance observed at these highly toxic levels may be due to epigenetic events.

The results indicate that under the conditions of this test, test article CMA-109 produced a negative response in the presence and absence of exogenous metabolic activation. In the presence of metabolic activation, the Total Growth of the treated cultures that were cloned did not cover the critical range of survival (10-40%). A precipitous toxic response was induced by the test article. The cultures treated with the two highest concentrations of test article had 6% and 77% Total Growth. It was felt that a repeat assay would not provide any additional information since the difference in concentration between the cultures having 6% and 77% Total Growth was only 0.006 µl/ml.

Results

The Initial Toxicity Test (Table 1) performed on test article CMA-109 (MA #T1855) in the absence of S-9 indicated a threshold level of complete toxicity at 0.1 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.1 µl/ml to 0.0013 µl/ml.

After a two day expression period, nine cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.013, 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018 or 0.0013 µl/ml test article. These concentrations produced a range in Suspension Growth of 11% to 96%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

Two of the cultures (0.013 and 0.010 µl/ml) that were cloned exhibited mutant frequencies which were 10.8 and 2.5 times, respectively, the mean mutant frequency of the solvent controls. The Total Growth of these cultures were 2% and 4%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly different from the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 20% to 92%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-109 (MA #T1855). The results indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.1 µl/ml to 0.0013 µl/ml.*

---

*Three S-9 activated assays were performed. Two of the assays were lost to contamination. The results of the third assay are reported here.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.024, 0.018, 0.013, 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024 or 0.0018 µl/ml. These concentrations produced a range in Suspension Growth of 11% to 82%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2. One culture (0.024 µl/ml) that was cloned exhibited a mutant frequency which was 7.2 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 6%.

None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 77% to 125%.

Study No. T1855 701006

Table 1

701 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL COMPOUND TOXICITY TEST

T1855 701006 — Study Number
Paul E. Kirby — Study Director
100 ul/ml & 0.001 ul/ml — Dose Range
CMA 109 (T1855) — Test Article Identity
Acetone — Solvent

	Test Article Concentration	Cell Concentration (X 10^5) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation	100 ul/ml	1.995	*		
	10 ul/ml	0.465	*		
	1.0 ul/ml	0.068	*		
	0.1 ul/ml	0.047	0.140	0.0	0%
	0.01 ul/ml	0.508	0.823	4.6	80%
	0.001 ul/ml	1.029	1.177	7.5**	129%
	Solvent 1	1.009	1.148	7.6**	5.8
	Solvent 2	0.927	0.621	4.1**	
With S-9 Activation					
	Solvent 1				
	Solvent 2				

not available MFB 1/13/83

+ Culture Lost

Table Prepared By: Andrea N Rogers-Back — Signature — 1/13/83 — Date

* Cultures not counted on the 2nd day MFB 1/13/83

** Values take into account incorrect adjustment on day 1. MFB 1/13/83

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Form No. WL-232
2/22/82

Form No. WL-41
2/25/82

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Study No. T1855 701006

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1855, 701006	Paul E. Kirby	1855B1
Study Number	Study Director	Experiment Number
CMA-109 (T1855)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
0.013 μl/ml	72	56	65	64	31	33	26	30	4.3	3.9
0.010 μl/ml	37	41	39	39	77	77	81	78	1.0	0.6
0.0075 μl/ml	26	41	50	39	114	123	104	114	0.7	0.3
0.0056 μl/ml	51	63	61	58	189	199	183	190	0.6	0.2
0.0042 μl/ml	50	76	79	68	182	188	183	184	0.7	0.3
0.0032 μl/ml	33	41	42	39	+	187	194	191	0.4	0.0
0.0024 μl/ml	62	64	80	69	201	206	211	206	0.7	0.3
0.0018 μl/ml	52	52	59	54	214	189	197	200	0.5	0.1
0.0013 μl/ml	63	65	68	65	213	208	182	201	0.7	0.3
Solvent 1	48	42	48	46	194	208	212	205	0.4	
Solvent 2	+	+	+		208	219	211	213		

Plates Counted By: Eleanor M. Carey 1-31-83 (Signature & Date)

Calculations Performed By: [signature] 2-1-83 (Signature & Date)

* Per 10^6 surviving cells

\+ Culture Lost

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1855, 701006	Paul E Kirby	1855B1
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	177	173	184	178	18	15	23	19	19.1	18.5
0.5µl/ml	347	349	348	348	102	90	99	97	7.2	6.6
SOLVENT 1	51	55	47	51	212	162	182	185	0.6	0.6
SOLVENT 2	61	48	57	55	232	223	230	228	0.5	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1										
SOLVENT 2										

Not APPLICABLE - Jim McMonsette 1-31-83

Plates Counted By: Eleanor M. Carey 1-31-83 (Signature & Date)

Calculations Performed By: Jim McMonsette 2-1-83 (Signature & Date)

*Per 10^4 surviving cells

+ Culture Lost

Study No. T1855 70100

Form No. WI-227
2/22/82

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1855.701006	Paul E Kuby	1855B1
Study Number	Study Director	Experiment Number
CMA-109 (T1855)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^4 cells/ml		Suspension Growth		Cloning Growth		% Total Growth *
	Day 1	Day 2	Total	% Control	Avg. V. C.	% Control	
0.029 µl/ml	0.009	0.024	~~60.0~~ **	0%	++		
0.018 µl/ml	0.075	0.078	0.0	0%	++		
0.013 µl/ml	0.243	0.482	1.6	11%	30	14%	2%
0.010 µl/ml	0.192	0.532	1.8	12%	78	37%	4%
0.0075 µl/ml	0.512	0.958	5.9	37%	114	55%	20%
0.0056 µl/ml	0.730	1.276	10.3	69%	190	91%	63%
0.0090 µl/ml	0.862	1.360	13.0	87%	184	88%	77%
0.0032 µl/ml	0.969	1.305	14.1	94%	191	91%	86%
0.0024 µl/ml	0.901	1.195	12.0	80%	206	99%	79%
0.0018 µl/ml	0.967	1.292	13.9	93%	200	96%	89%
0.0013 µl/ml	0.961	1.337	14.3	96%	201	96%	92%
SOLVENT 1	0.983	1.376	15.0	99	205		
SOLVENT 2	0.963	1.374	14.7		213		

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Study No. T1855 701006

* % Total Growth = $\frac{(\%\text{ Suspension Growth})\ (\%\text{ Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: [signature] 1-31-83
(Signature & Date)

Table 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1855 701006	Paul E Kirby	1855 B1
Study Number	Study Director	Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml	0.520	0.773	4.5	27%	19	9%	2%
0.5 µl/ml	0.719	1.143	9.1	55%	97	47%	26%
SOLVENT 1	1.053	1.358	15.9	16.6	185	~~206.5~~*	
SOLVENT 2	1.145	1.355	17.2		228	207	
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml							
5.0 µg/ml							
SOLVENT 1							
SOLVENT 2							

NOT APPLICABLE [signature] 2-1-83

Tables prepared and calculations performed by: [signature] 2-1-83

(Signature & Date)

Study No. T1855 701006

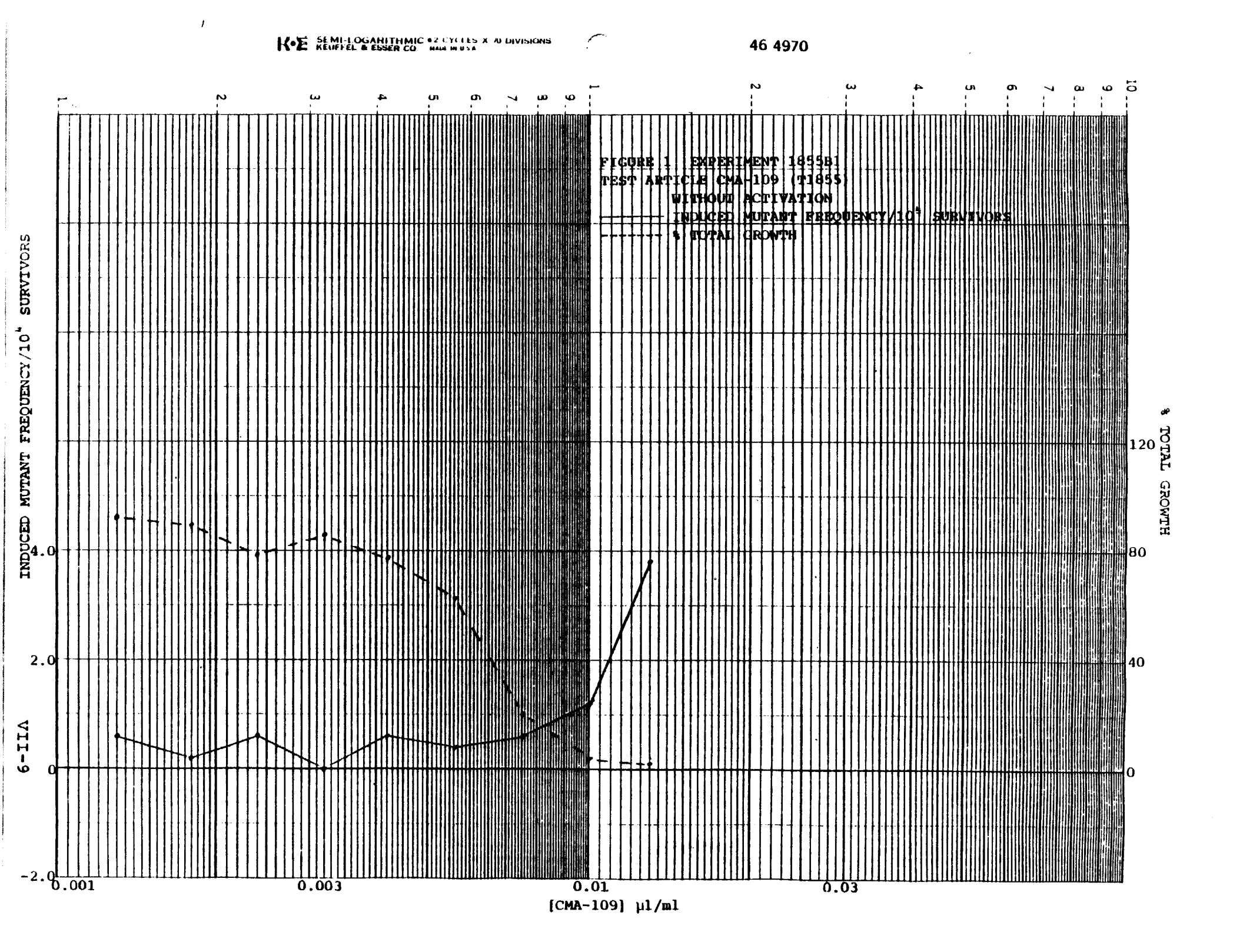
+ Culture Lost

++ Too Toxic to Clone

* ENTRY ERROR [initials] 1-31-83

VTT-8

K•E SEMI-LOGARITHMIC 2 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO MADE IN U.S.A.



Study No. T T1855.701006

Table 6

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1855.701006	Paul E. Kirby	10ul/ml to 0.001ul/ml
Study Number	Study Director	Dose Range
CMA-109 (T1855)		Acetone
Test Article Identity		Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation					
	Solvent 1				
	Solvent 2				
With S-9 Activation *	10ul/ml	0.652	0.253	0.0	0%
	5.0ul/ml	0.008	0.005	0.0	0%
	1.0ul/ml	0.072	0.121	0.0	0%
	0.5ul/ml	0.030	0.060	0.0	0%
	0.1ul/ml	0.012	0.013	0.0	0%
	0.05ul/ml	0.148	0.138	0.0	0%
	0.01ul/ml	0.876	1.049	10.2	89%
	0.005ul/ml	0.946	1.029	10.8	94%
	0.001ul/ml	0.966	1.058	11.4	99%
	Solvent 1	0.953	1.104	11.7 } 11.5	
	Solvent 2	1.015	0.995	11.2 }	

not applicable JK 2-23-83

+ Culture Lost

* this concentration is toxic high counts are due to [illegible]

Table Prepared By Janine L. Johnson 2-23-83

Signature Date

Form No. MA-127
2/16/

Table 7

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1855.701006 — Study Number
Paul E. Kirby — Study Director
1855B4 — Experiment Number
CMA-109(T1855) — Test Article Identity
Acetone — Solvent
Induced Rat Liver S-9 — Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
0.024 ul/ml	151	148	151	150	75	67	68	70	4.3	3.7
0.018 ul/ml	98	69	87	85	177	175	175	176	1.0	0.4
0.013 ul/ml	80	69	78	76	171	158	165	165	0.9	0.3
0.010 ul/ml	63	69	85	72	178	155	147	160	0.9	0.3
0.0075 ul/ml	78	64	62	68	143	143	160	149	0.9	0.3
0.0056 ul/ml	73	67	85	75	196	202	190	196	0.8	0.2
0.0042 ul/ml	55	62	68	62	150	160	172	161	0.8	0.2
0.0032 ul/ml	75	66	72	71	141	165	171	159	0.9	0.3
0.0024 ul/ml	51	58	53	54	143	115	148	135	0.8	0.2
0.0018 ul/ml	51	39	55	48	170	160	179	170	0.6	0.0
Solvent 1	46	47	46	46	115	136	138	130	0.7	0.6
Solvent 2	25	33	33	30	115	111	108	111	0.5	

Plates Counted By: Eliana M. Cary 4-4-83 (Signature & Date)
Calculations Performed By: Eliana M. Cary 4-4-83 (Signature & Date)

* Per 10^6 surviving cells + Culture Test

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Study No. 1855 7

Table 8

L5178Y TK+/- MC LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1855.701006	Paul E. Kirby	185584
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced * Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1										
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	137	101	+	119	36	35	44	38	6.2	5.6
5.0 µg/ml	170	173	166	170	85	99	101	95	3.6	3.0
SOLVENT 1	36	36	37	36	100	105	112	106	0.7 } 0.6	
SOLVENT 2	33	36	47	39	154	148	154	152	0.5 }	

Not Applicable - Eleanor M. Carey 4-4-83

Plates Counted By: Eleanor M. Carey 4-4-83 (Signature & Date)

Calculations Performed By: M. Carey 4-4-83 (Signature & Date)

*Per 10^6 surviving cells

+ Culture lost

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Study No. T1855.701006

Form No. HA-228
2/15/83

Table 9

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1855.701006	Paul E. Kirby	185584
Study Number	Study Director	Experiment Number
CMA-109(T1855)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^4 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.042 ul/ml	0.035	0.026	0.0	0%	++		
0.032 ul/ml	0.037	0.032	0.0	0%	++		
0.024 ul/ml	0.281	0.582	1.9	11%	70	58%	6%
0.018 ul/ml	0.656	1.314	9.6	52%	176	146%	77%
0.013 ul/ml	0.821	1.330	12.1	66%	165	137%	91%
0.010 ul/ml	0.915	1.387	14.1	77%	160	133%	103%
0.0075 ul/ml	0.972	1.281	13.8	76%	149	124%	94%
0.0056 ul/ml	0.966	1.305	14.0	77%	196	163%	125%
0.0042 ul/ml	0.951	1.352	14.3	78%	161	134%	105%
0.0032 ul/ml	1.015	1.322	14.9	82%	159	132%	108%
0.0024 ul/ml	~~1.019~~ *0.993	1.279	14.1	77%	135	112%	87%
0.0018 ul/ml	1.019	1.199	13.6	74%	170	141%	105%
SOLVENT 1	1.075	1.564	18.7 } 18.3		130 } 121		
SOLVENT 2	1.022	1.564	17.8 }		111 }		

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

*Entry Error
Emd 4-4-83

Tables prepared and calculations performed by: Eleanor M. Casey 4-4-83
(Signature & Date)

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Study No. 1855 701006

Table 10

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1855.701006 — Study Number
Paul E. Kirby — Study Director
1855B4 — Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml							
0.5 µl/ml							
SOLVENT 1	Not Applicable - Eleanor M. Cary 4-4-83						
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.534	0.670	4.0	25%	38	29%	7%
5.0 µg/ml	0.765	1.115	9.5	60%	95	74%	44%
SOLVENT 1	1.088	1.311	15.8 7159		106 7129		
SOLVENT 2	1.096	1.311	16.0		152		

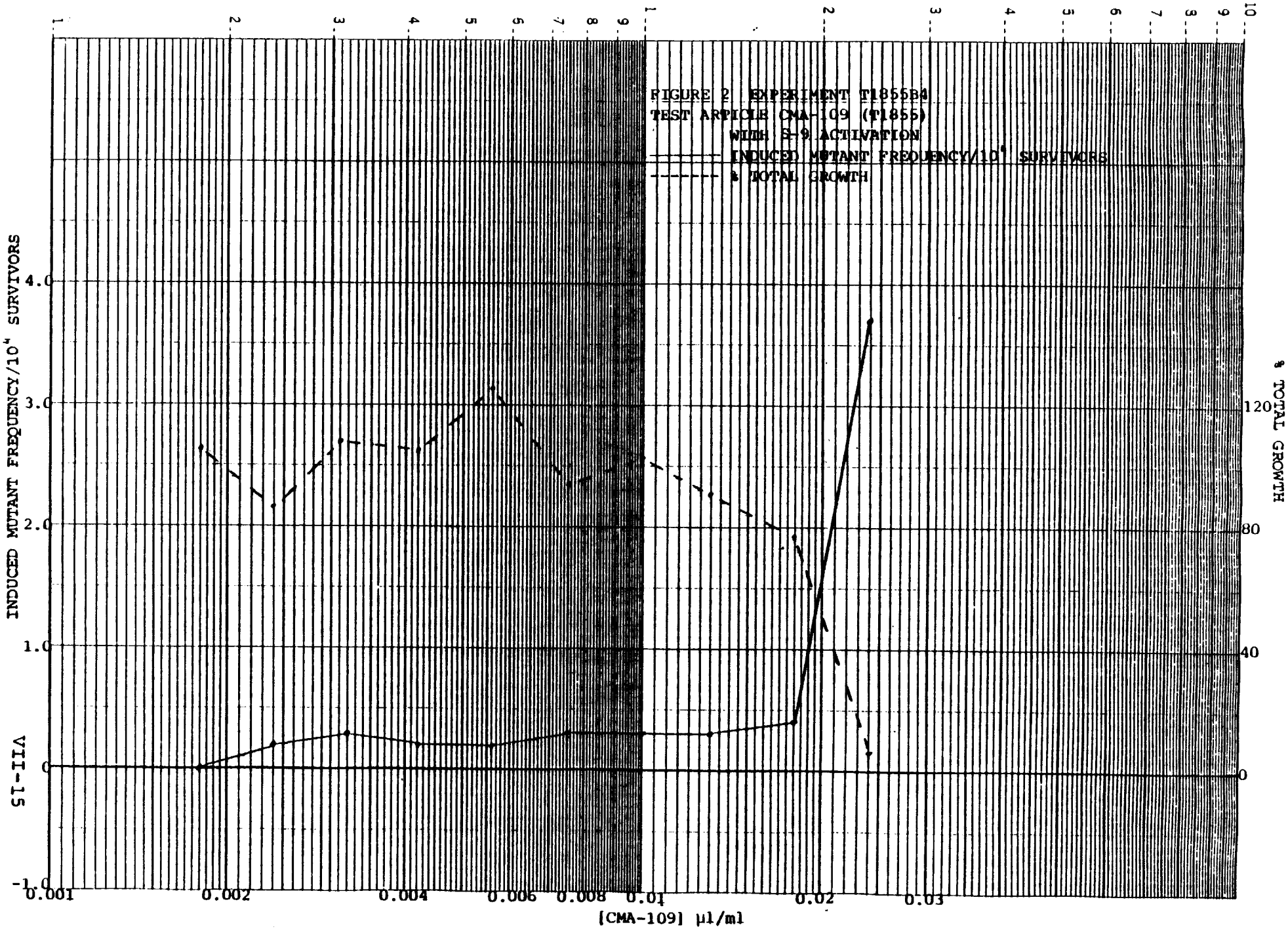
Tables prepared and calculations performed by: Eleanor M. Cary 4-4-83
(Signature & Date)

\+ Culture Lost
++ Too Toxic to Clone

Study No. 1 1855 701006

K•E SEMI-LOGARITHMIC • 2 CYCLES X 10 DIVISIONS
KEUFFEL & ESSER CO MADE IN U.S.A.

FIGURE 2 EXPERIMENT T1855B4
TEST ARTICLE CMA-109 (T1855)
WITH S-9 ACTIVATION
——— INDUCED MUTANT FREQUENCY/10^4 SURVIVORS
- - - - - - % TOTAL GROWTH



VII-15

Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-109 (MA #T1855) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest test article concentration cloned in the S-9 activated cultures and the two highest test article concentrations cloned in the non-activated cultures exhibited mutant frequencies which were more than twice the mean mutant frequency of the solvent controls. These results are not considered significant as the Total Growth of these cultures was less than 10%. Mutations observed at these highly toxic levels may be due to epigenetic events. The results indicate that under the conditions of this test, test article CMA-109 produced a negative response in the presence and absence of exogenous metabolic activation. In the presence of metabolic activation, the Total Growth of the treated cultures that were cloned did not cover the critical range of survival (10-40%). A precipitous toxic response was induced by the test article. The cultures treated with the two highest concentrations of test article had 6% and 77% Total Growth. It was felt that a repeat assay would not provide any additional information since the difference in concentration between the cultures having 6% and 77% Total Growth was only 0.006 µl/ml. Thus the results obtained for treatment in the presence of metabolic activation do not permit an accurate assessment of the compound's mutagenic potential.

Rec'd. by
RA/QA 12/8/82.
N.K. /jkc

RECEIVED OCT 0 5 1982

L5178Y TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-109

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:
$2,650

Total charge for assay with metabolic activation:
$2,650

6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intra-peritoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-amino-anthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_20, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0×10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F_0P will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3×10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3×10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2×10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of

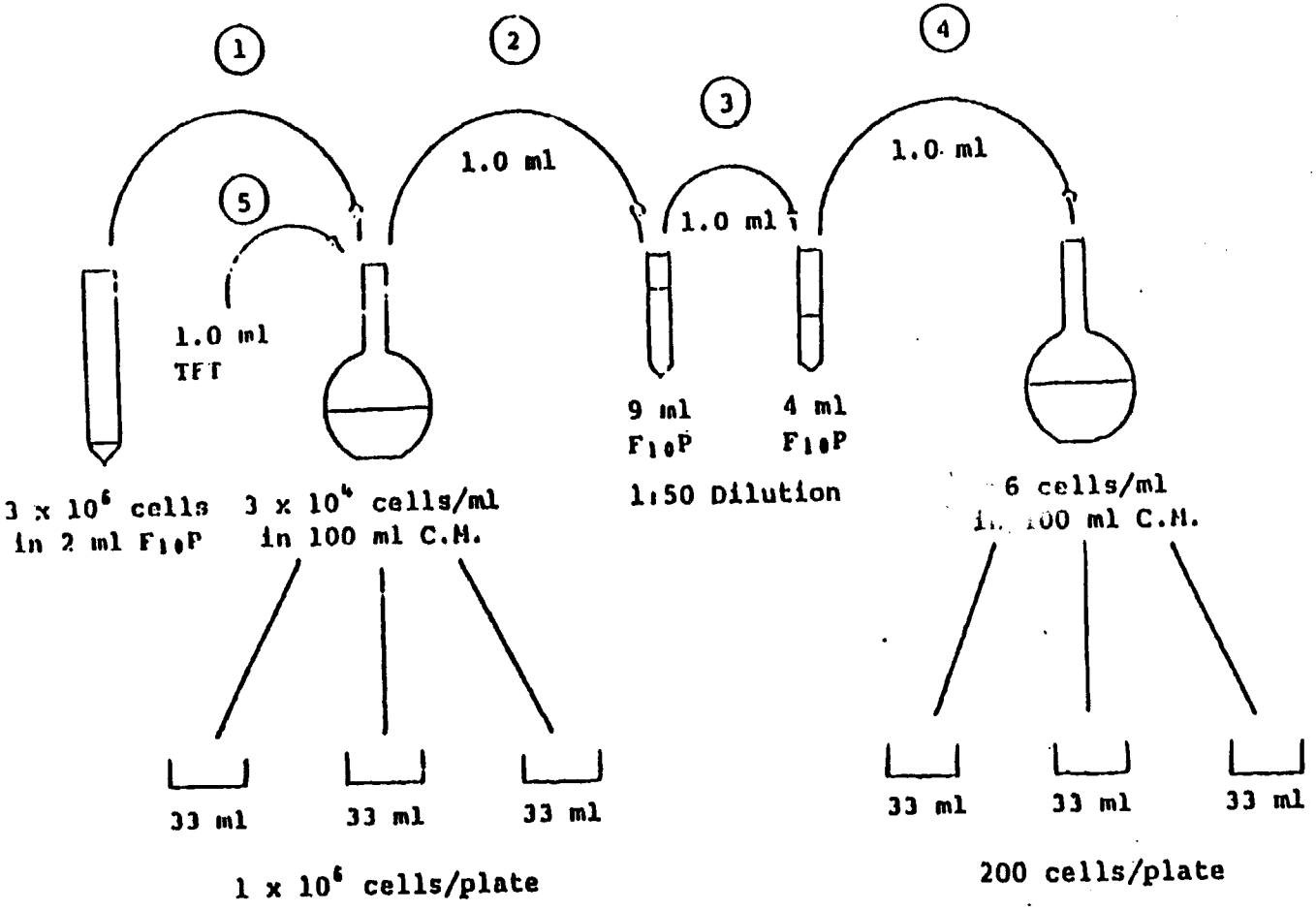


Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

MICROBIOLOGICAL ASSOCIATES

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency?_____
If so, to which agency or agencies? _____

Does the sponsor request that samples of the Test Article dosing solutions be returned?_____

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: 11/24/82

13.2 Proposed Initiation Date: December 28, 1982

13.3 Scheduled Completion Date: February 18, 1983

13.4 Report Submission to Sponsor Date: February 18, 1983

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

SPONSOR'S AUTHORIZED REPRESENTATIVE

11/24/82
DATE PROTOCOL APPROVED BY SPONSOR

STUDY DIRECTOR — DATE 11/24/82

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136) and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \text{Average Counts (corrected for coincidence)} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{cells/ml})\ (20\ \text{ml})}{\text{(No. of Cells/ml)}}$$

$$\text{Volume of Media to Add} = 20\ \text{ml} - \text{Volume of Cells Retained}$$

---

2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

---

3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138) and L5178Y TK+/- Assay - Daily Counts (Positive Controls) (Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^{5} = \text{Average Counts (corrected for coincidence)} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{Cells/ml})\ (20\ \text{ml}^{*})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20\ \text{ml}^{*}) - (\text{Volume of Cells Retained})$$

* The final volume is adjusted to 10 ml on the third day of expression.

---

4. Tables 2, 3, and 4 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^{6} \text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left(\text{Mutant Frequency of Treated Cultures}\right) - \left(\text{Average Mutant Frequency of Solvent Controls}\right)$$

---

5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentracion}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

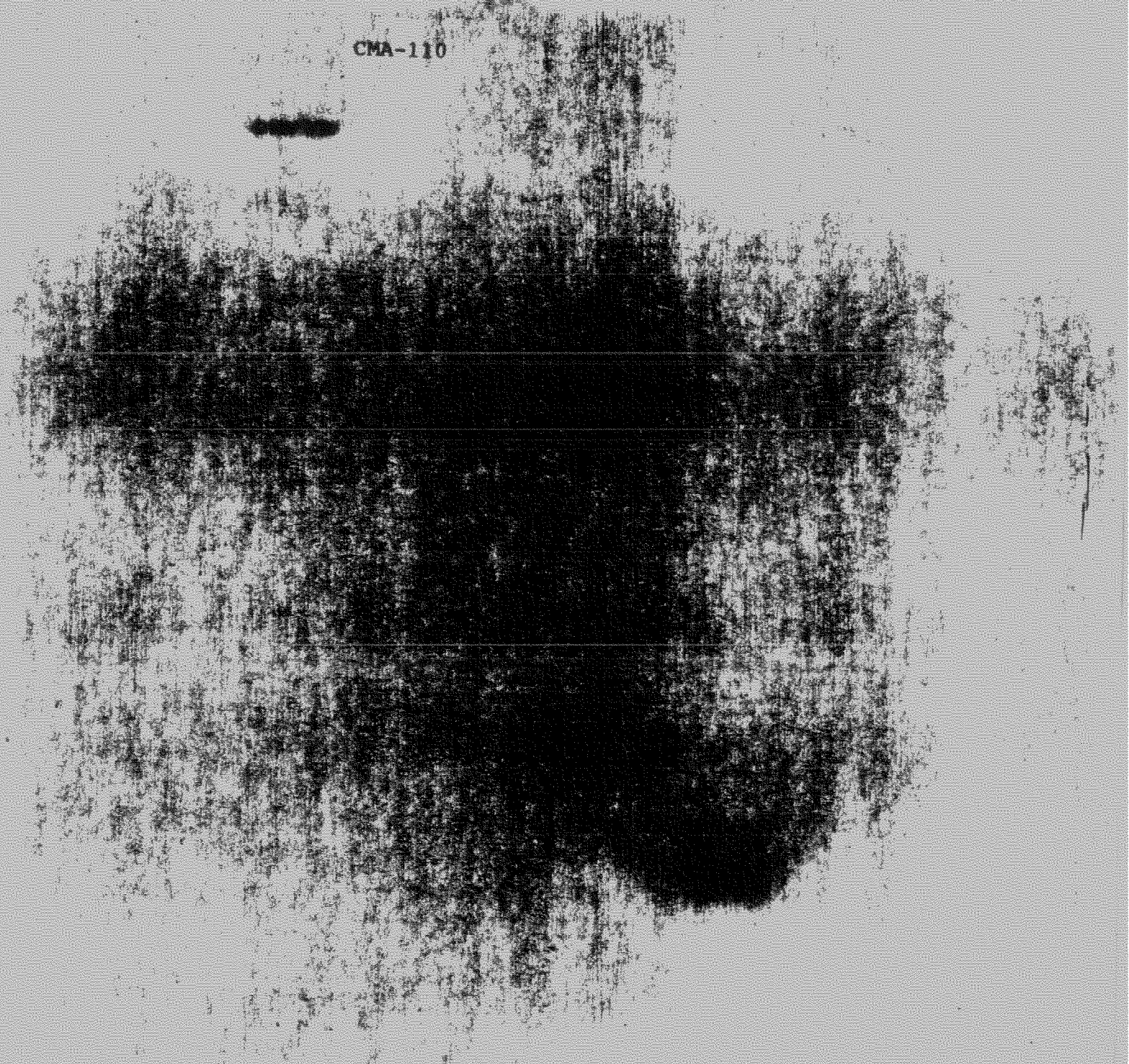
$$\text{\% Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

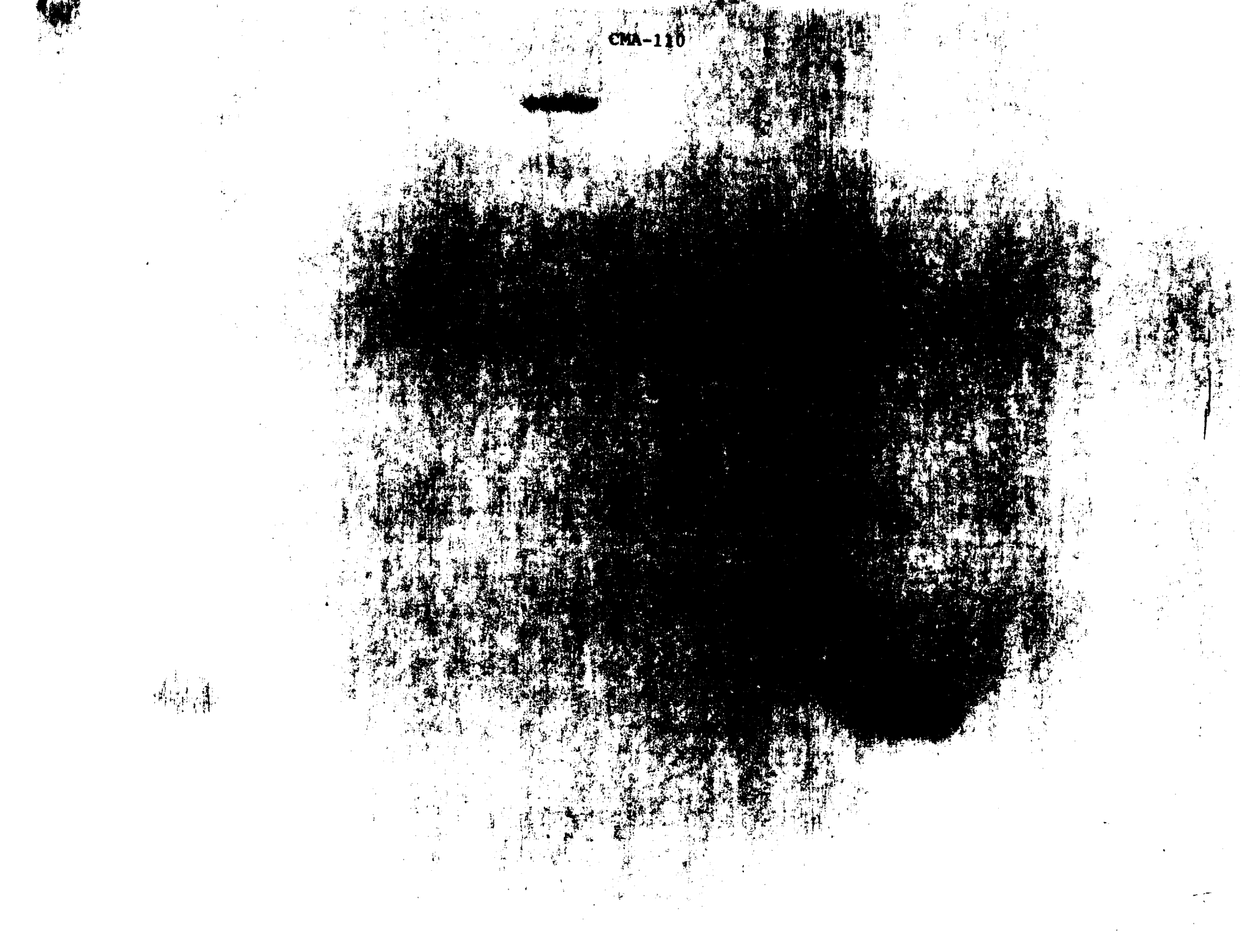
Cultures containing less than 0.3×10^{6} cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

$$\text{\% Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\text{\% Growth} = \frac{(\text{\% Suspension Growth})\ (\text{\% Cloning Growth})}{100}$$





Form No. MA-127
2/16/83

Workbook Page No. 96 Report Page No. IX-11

Study No. T 1867 701006

TABLE 7

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T 1867 701006
Study Number

Paul E. Kirby
Study Director

186784
Experiment Number

(T 1867) CMA 111
Test Article Identity

Acetone
Solvent

Induced Rat Liver S-9
Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced Mutant * Frequency
0.024 ml/ml	66	66	52	61	135	177	159	157	0.8	0.4
0.018 ml/ml	37	33	42	37	104	117	122	114	0.7	0.3
0.013 ml/ml	25	30	27	27	79	78	78	78	0.7	0.3
0.010 ml/ml	33	26	35	31	116	103	123	114	0.5	0.1
0.0075 ml/ml	29	30	30	30	170	172	117	153	0.4	0.0
0.0056 ml/ml	25	28	35	29	148	154	146	149	0.4	0.0
0.0042 ml/ml	36	30	+	33	161	152	+	157	0.4	0.0
0.0032 ml/ml	28	22	16	22	115	110	108	111	0.4	0.0
0.0024 ml/ml	30	28	25	28	159	146	158	154	0.4	0.0
0.0018 ml/ml	38	33	33	35	134	198	147	160	0.4	0.0
⊗ ~~0.0013 ml/ml~~										
Solvent 1	18	34	20	24	124	129	115	123	0.4 } 0.4	
Solvent 2	24	17	24	22	141	129	134	135	0.3	

Plates Counted By: Ava P Budag 4-18-83
(Signature & Date)

Calculations Performed By: Ava P Budag 4-18-83
(Signature & Date)

⊗ culture not cloned APB 4-18-83

Culture Lost

Table 8

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1867.701006	Paul E. Kirby	186784
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1										
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	116	135	124	125	96	88	86	90	2.8	2.4
5.0 µg/ml	105	96	91	97	88	81	88	86	2.3	1.9
SOLVENT 1	17	24	31	24	113	136	110	120	0.4	
SOLVENT 2	25	20	+	23	122	160	+	141	0.3	

Plates Counted By: Ann P Burday 4-18-83 (Signature & Date)

Calculations Performed By: Ann P Burday 4-19-83 (Signature & Date)

*Per 10^6 surviving cells

+ Culture Lost

Study No. T1867.701006

TX-12

Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-111 (MA #T1867) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures that were cloned were treated with a range of test article concentrations which produced from 2% to 116% Total Growth. The S-9 activated cultures that were cloned were treated with a range of test article concentrations which produced from 27% to 111% Total Growth.

One nonactivated culture that was cloned exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant since the Total Growth of this culture was 2%. TFT resistance observed at these highly toxic levels may be due to epigenetic events. One S-9 activated culture that was cloned exhibited a mutant frequency which was significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that, under the conditions of this test, test article CMA-111 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

Results

The Initial Toxicity Test (Table 1) conducted on test article CMA-111 (MA #T1867) in the absence of S-9 indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in the mutagenesis assay in the absence of S-9 over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, or 0.0012 µl/ml. These concentrations produced a range in Suspension Growth of 11% to 100%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. These data are also presented graphically in Figure 1.

One nonactivated culture that was cloned (0.016 µl/ml) exhibited a mutant frequency which was 3.4 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 2%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 8% to 116%.

The Initial Toxicity Test (Table 6) conducted an test article CMA-111 (MA #T1867) in the presence of S-9 indicated a threshold level of complete toxicity at 0.1 µl/ml. Based on these data, the test article was tested in the mutagenesis assay over a range of concentrations from 0.1 µl/ml to 0.0013 µl/ml.*

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.024, 0.018, 0.013, 0.010, 0.0075, 0.0056, 0.0042,

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*Three S-9 activated assays were performed. Considerable contamination was experienced in two assays and the results of these assays will not be reported. The results of the third assay are discussed in this report.

0.0032, 0.0024 or 0.0018 µl/ml. These concentrations produced a range in Suspension Growth of 22% to 93%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. These data are also presented graphically in Figure 2.

One culture that was cloned (0.024 µl/ml) exhibited a mutant frequency which was 2.0 times the mean mutant frequency of the solvent control. The Total Growth of this culture was 27%. None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly different from the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 43% to 111%.

Study No. T1867 701006

Table 1

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1867.701006 (Study Number) — Paul E. Kirby (Study Director) — 1004g/ml to 0.001 4g/ml (Dose Range)

CMA-111 (T1867) (Test Article Identity) — Acetone (Solvent)

	Test Article Concentration	Cell Concentration ($\times 10^6$) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation	100 4g/0	0.207	0.126	0.0	0%
	10 4g/ml	0.171	0.147	0.0	0%
	5.0 4g/ml	0.071	0.063	0.0	0%
	1.0 4g/ml	0.002	0.001	0.0	0%
	0.5 4g/ml	0.005	0.017	0.0	0%
	0.1 4g/ml	0.022	0.036	0.0	0%
	0.05 4g/ml	0.012	0.009	0.0	0%
	0.01 4g/ml	0.982	1.192	13.0	92%
	0.005 4g/ml	1.061	1.337	15.8	112%
	0.001 4g/ml	1.023	1.192	13.5	96%
	Solvent 1	1.008	1.247	14.0 | 14.1	
	Solvent 2	0.984	1.245	14.1	
With S-9 Activation					
	Solvent 1				
	Solvent 2				

+ Culture Lost

Table Prepared By _____ 1-21-83
Signature Date

Form No. WL-41
2/25/82

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1867.701006	Paul E. Kirby	1867B1
Study Number	Study Director	Experiment Number
CMA-111 (T1867)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced Mutant * Frequency
0.016 ul/ml	326 **	48	51	44	39	35	37	37	2.4	1.7
0.012 ul/ml	55	36	49	47	89	98	84	90	1.0	0.3
0.0089 ul/ml	40	23	37	33	166	175	147	163	0.4	-0.3
0.0067 ul/ml	44	48	42	45	201	201	187	196	0.5	-0.2
0.0050 ul/ml	36	42	50	43	227	197	208	211	0.4	-0.3
0.0038 ul/ml	35	44	45	41	231	231	207	223	0.4	-0.3
0.0028 ul/ml	38	73	55	55	225	224	218	222	0.5	-0.2
0.0021 ul/ml	34	49	45	43	264	203	194	220	0.4	-0.3
0.0016 ul/ml	26	42	36	35	177	160	172	170	0.4	-0.3
0.0012 ul/ml	44	55	38	46	179	198	182	186	0.5	-0.2
Solvent 1	71	67	56	65	170	171	203	181	0.7	0.7
Solvent 2	51	55	68	58	207	178	220	202	0.6	

Plates Counted By: [signature] 2-8-83 (Signature & Date)

Calculations Performed By: [signature] 2-8-83 (Signature & Date)

* Per 10^6 surviving cells + Culture Lost

** Contaminated 2-7-83

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Study No. T1867.701006

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

71867 701006	Paul E. Kirby	1867B1
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	109	131	112	116	6	13	12	10	22.9	21.8
0.5µl/ml	343	329	332	335	62	72	61	65	10.3	9.7
SOLVENT 1	69	63	63	65	199	231	214	215	0.6	0.6
SOLVENT 2	57	57	64	59	207	220	201	209	0.6	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1	not applicable			ff 2-7-83						
SOLVENT 2										

Plates Counted By: Janine L. Johnson 2-7-83 (Signature & Date)

Calculations Performed By: Janine L. Johnson 2-7-83 (Signature & Date)

*Per 10^6 surviving cells

+ Culture Lost

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Study No. T1562 701003

Form No. WL-227
2/22/82

Workbook Page No. 32

Report Page No. IX-7

Study No. T1867 701006

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1867.701006	Paul E. Kilby	1867B1
Study Number	Study Director	Experiment Number
CMA-111 (T1867)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.028 ul/ml	0.019	0.026			++		
0.021 ul/ml	0.246	0.238			++		
0.016 ul/ml	0.362	0.477	1.9	11%	37	19%	2%
0.012 ul/ml	0.417	0.629	2.9	17%	90	47%	8%
0.0089 ul/ml	0.523	1.026	6.0	36%	163	85%	30%
0.0067 ul/ml	0.879	1.498	14.6	87%	196	102%	89%
0.0050 ul/ml	0.955	1.453	15.4	92%	211	110%	101%
0.0038 ul/ml	0.916	1.525	15.5	93%	223	116%	108%
0.0028 ul/ml	0.963	1.571	16.8	100%	222	116%	116%
0.0021 ul/ml	0.969	1.503	16.1	96%	220	115%	110%
0.0016 ul/ml	0.972	1.544	16.7	100%	170	89%	88%
0.0012 ul/ml	0.888	1.523	15.0	90%	186	97%	87%
SOLVENT 1	0.970	1.542	16.6 } 16.8		181 } 192		
SOLVENT 2	1.036	1.469	16.9		202		

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost

++ Too Toxic to Clone

Tables prepared and calculations performed by: [signature] 2-7-83

(Signature & Date)

Form No. WL-229

Table 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1867.701006 — Study Number

Paul E. Kirby — Study Director

186781 — Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. P.C.	% Control	
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml	0.972	0.757	4.0	23%	10	5%	1%
0.5 µl/ml	0.70	1.177	9.2	54%	65	31%	17%
SOLVENT 1	1.023	1.474	16.8	169	215	212	
SOLVENT 2	1.007	1.507	16.9		209		
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	Not Applicable as SB 4-16-87						
5.0 µg/ml							
SOLVENT 1							
SOLVENT 2							

Tables prepared and calculations performed by Janice Johnson 7-8-87

(Signature & Date)

\+ Culture Lost

++ Too Toxic to Clone

Workbook Page No. 33 Report Page No. IX-8

Study No. T1867.701006

Study No. T1867

Table 6

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

INITIAL TOXICITY TEST

T1867.701006	Paul E. Kirby	100 ul/ml to 0.0
Study Number	Study Director	Dose Range
CMA-111 (T1867)		Acetone
Test Article Identity		Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation					
	Solvent 1				
	Solvent 2				
With S-9 Activation *	100 ul/ml	0.948	1.640	0.0	0%
*	10 ul/ml	0.374	0.391	0.0	0%
	5.0 ul/ml	0.047	0.016	0.0	0%
	1.0 ul/ml	0.051	0.030	0.0	0%
	0.5 ul/ml	0.026	0.043	0.0	0%
	0.1 ul/ml	0.016	0.018	0.0	0%
	0.05 ul/ml	0.309	0.796	2.7	26%
	0.01 ul/ml	0.908	0.976	9.8	95%
	0.005 ul/ml	0.898	1.041	10.4	100%
	0.001 ul/ml	0.910	1.120	11.3	109%
	Solvent 1	0.955	1.007	10.7 } 10.4	
	Solvent 2	0.899	1.008	10.1 }	

not applicable JJ 2-23-83

+ Culture Lost

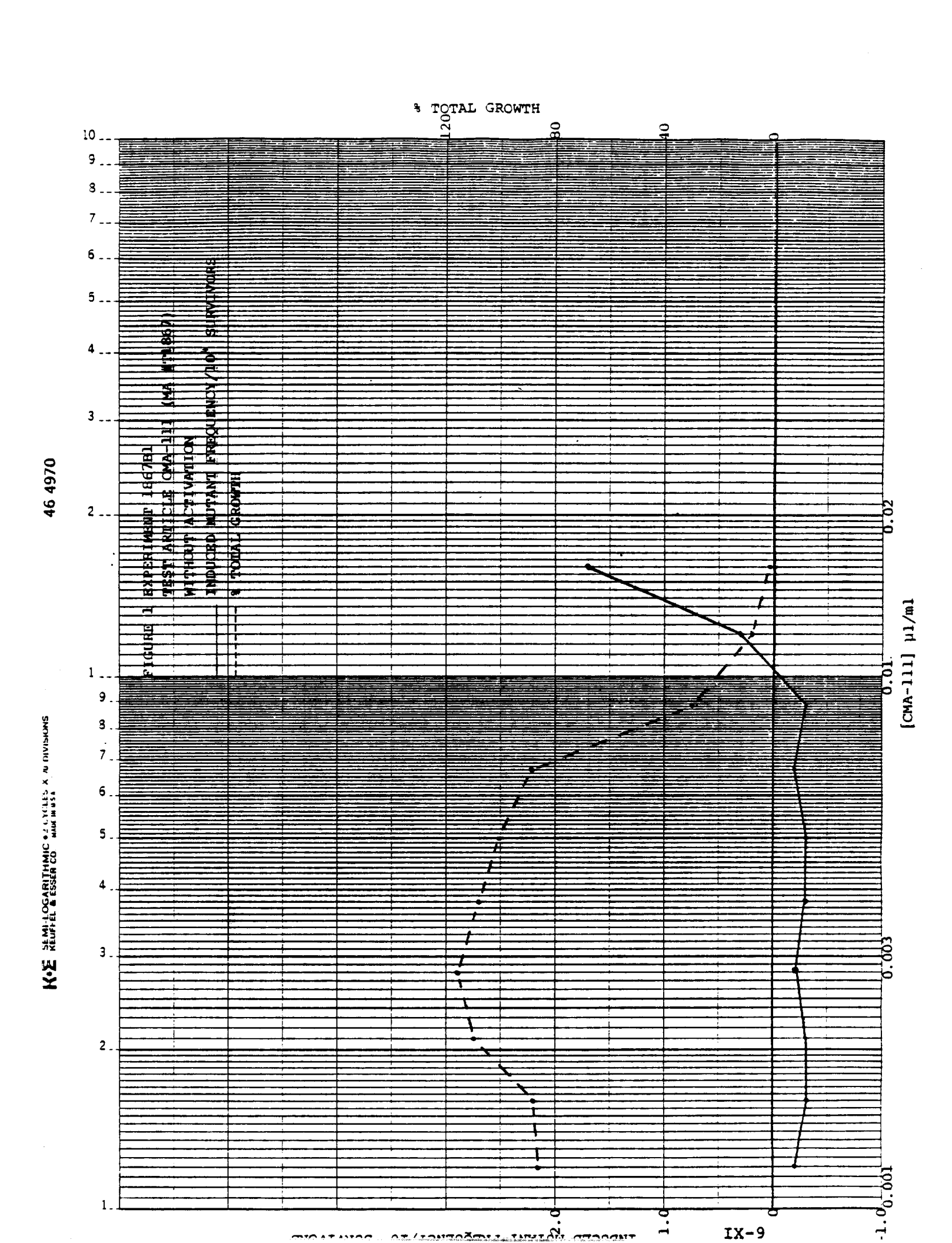
* these concentrations are toxic - high counts are due to debris JJ 2-23-83

Table Prepared By Janine L. Johnson 2-23-83

Signature Date

Form No. MA-233 Workbook Page No. 43 Report Page No. IX-10

2/16/83


% TOTAL GROWTH
120
80
40
0
10
9
8
7
6
5
4
3
2
1
9
8
7
6
5
4
3
2
1
0.02
0.01
0.003
0.001
[CMA-111] µl/ml
2.0
1.0
0
-1.0
FIGURE 1 EXPERIMENT 1667BL
TEST ARTICLE CMA-111 (MA #71867)
WITHOUT ACTIVATION
INDUCED MUTANT FREQUENCY/10⁶ SURVIVORS
% TOTAL GROWTH
46 4970
K·E SEMI-LOGARITHMIC
KEUFFEL & ESSER CO

Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-110 (MA #T1851) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 1% to 114% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 34% to 110% Total Growth in one experiment and from 11% to 86% Total Growth in a second experiment.

The highest test article concentration cloned in the non-activated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant since the Total Growth of this culture was less than 10%. TFT resistance observed at these highly toxic levels may be due to epigenetic events. In the first experiment with S-9 activation, one culture exhibited a mutant frequency which was twice the mean mutant frequency of the solvent controls. There were some technical difficulties associated with the second experiment with S-9 activation. However, the results obtained correlated well with the results of the first experiment.

The results indicate that under the conditions of this test, the test article CMA-110 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

Results

The Initial Toxicity Test (Table 1) performed on test article CMA-110 (MA #T1851) in the absence of S-9 indicated a threshold level of complete toxicity at 0.1 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.1 µl/ml to 0.0013 µl/ml.*

After a two day expression period, nine cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.013, 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018 or 0.0013 µl/ml test article. These concentrations produced a range in Suspension Growth of 10% to 99%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

One of the cultures that was cloned (0.013 µl/ml) exhibited a mutant frequency which was 5.0 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 1%. None of the remaining nonactivated cultures exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.** The Total Growth of these cultures ranged from 6% to 114%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-110 (MA #T1851). The results indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned

---

* Two nonactivated assays were performed. In the first assay the cloning efficiency was not optimum and the assay was repeated. Only the results of the second nonactivated assay will be presented here.

** Culture 0.0056 µl/ml exhibited a mutant frequency which was 4.0 times the mean mutant frequency of the solvent controls. This elevated mutant frequency is probably due to the low VC counts since there is no significant increase in the TFT counts for this culture. The suspension growth of the culture was 56%. Thus the increase in mutant frequency is not significant.

were treated with 0.038, 0.028, 0.021, 0.016, 0.012, 0.0089, 0.0067, 0.0050, 0.0038 or 0.0028 µl/ml. These concentrations produced a range in Suspension Growth of 37% to 99%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data are presented in Tables 9 and 10. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2. One culture (0.038 µl/ml) that was cloned exhibited a mutant frequency which was 2.0 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 34%.

None of the remaining cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 78% to 110%.

In an attempt to obtain cultures having Total Growth in the range 11% to 30%, a repeat experiment was performed over a narrower dose range from 0.055 µl/ml to 0.019 µl/ml.***

After a two day expression period, eleven cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.055, 0.050, 0.045, 0.039, 0.035 or 0.029 µl/ml. These concentrations produced a range in Suspension Growth of 15% to 83%. The Cloning Data are presented in Tables 11 and 12 and the Total Compound Toxicity Data are presented in Tables 13 and 14. The data are also presented graphically in Figure 3.

Due to contamination, no solvent control mutant frequencies were obtained from the assay. However, the solvent control mutant frequencies for the positive control compound were obtained. The solvent used for the test article and the positive control compound were the same (Acetone). Applying these solvent control mutants, the induced mutant frequencies for the test article can be calculated (shown on Table 11 in parenthesis). Six cultures

---

*** Two repeat experiments were performed. In the first assay there was considerable contamination. However, the results are presented in this report as a comparison with the results of the initial experiment in the presence of S-9. In the second repeat assay, some difficulty was experienced in the toxic response of the test article and only two doses were cloned. The results will not be presented here.

exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of there cultures ranged from 11% to 83%. None of the remaining cultures exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 57% to 86%.

Study No. ~~T1851~~ 701006

Table 1

701 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL COMPOUND TOXICITY TEST

T1851.701006
Study Number

Paul E Kirby
Study Director

100 ul/ml - 0.001 ul/ml
Dose Range

CMA-110 (T1851)
Test Article Identity

Acetone
Solvent

	Test Article Concentration	Cell Concentration (X 10^5) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation	100 ul/ml	1.278**	2.426**	0.0	0%
	10 ul/ml	0.457**	0.693**	0.0	0%
	1.0 ul/ml	0.020	0.015	0.0	0%
	0.1 ul/ml	0.040	0.088	0.0	0%
	0.01 ul/ml	0.547	0.986	6.0	39%
	0.001 ul/ml	1.006	1.351	15.1	98%
	Solvent 1	1.003	1.395	15.5	15.4
	Solvent 2	1.008	1.370	15.3	
With S-9 Activation					
	Solvent 1				
	Solvent 2				

not attainable MLB 12/21/82

+ Culture Lost

* entry error MLB 12/21/82

Table Prepared By: Audrey M Hayes-Bands 12/21/82
Signature Date

** no viable cells present. Counts are compound precipitate MLB 12/21/82

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Form No. WL-232
2/22/82

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1851.701006	Paul E. Kirby	1851B2
Study Number	Study Director	Experiment Number
CMA-110 (T1851)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced Mutant * Frequency
0.013 µl/ml	19	20	26	22	19	14	18	17	2.5 ~~2.0~~ 6*	2.0
0.010 µl/ml	18	27	22	22	69	74	61	68	0.6 7*	0.2 * ~~0.2~~
0.0075 µl/ml	35	40	37	37	139	128	165	144	0.5	0.0
0.0056 µl/ml	47	44	49	47	49	56	37	46	2.0	1.5
0.0042 µl/ml	34	40	37	37	230	201	198	210	0.4	-0.1
0.0032 µl/ml	37	49	55	47	203	213	217	211	0.4	-0.1
0.0024 µl/ml	40	49	55	48	185	258	258	234	0.4	-0.1
0.0018 µl/ml	41	33	48	41	249	258	262	256	0.3	-0.2
0.0013 µl/ml	40	37	47	41	246	215	202	221	0.4	-0.1
Solvent 1	38	48	48	45	186	200	233	206	0.4	0.5
Solvent 2	51	56	49	52	197	186	243	209	0.5	

Plates Counted By: [signature] 1/24/83 (Signature & Date)

Calculations Performed By: [signature] 1/24/83 (Signature & Date)

* Per 10^4 surviving cells + Culture Lost

* entry error [initials] 1/24/83

Form No. WL-41
2/25/82

Workbook Page No. 50

Report Page No. VIII-6

Study No. T1851.701006

Form No. WL-128
2/25/82

Workbook Page No. 51 Report Page No. VIII-7

Study No. T85L701006

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T85L701006 — Study Number

Paul B. Kirby — Study Director

185B2* — Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	249	227	246	241	24	26	22	24	20.1	19.7
0.5µl/ml	475	524	504	501	120	121	116	119	8.4	8.0
SOLVENT 1	28	34	29	30	185	199	234	206	0.3	0.4
SOLVENT 2	39	44	49	44	231	199	227	219	0.4	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1										
SOLVENT 2										

not applicable MLB 1/24/83

Plates Counted By: [signature] 1/24/83 (Signature & Date)

Calculations Performed By: Andrea M Rogers-Bond 1/24/83 (Signature & Date)

*Per 10^6 surviving cells

+ Culture Lost

* entry error MLB 5/5/83

Form No. WL-227
2/22/83

Workbook Page No. 52

Report Page No. VIII-8

Study No. T1851.701006

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1851.701006	Paul E Kirby	1851B2
Study Number	Study Director	Experiment Number
CMA-110 (T1851)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.024 ul/ml	0.016	0.052	0.0	0%	++		
0.018 ul/ml	0.061	0.049	0.0	0%	++		
0.013 ul/ml	0.322	0.536	1.9	10%	17	8%	1%
0.010 ul/ml	0.426	0.749	3.5	19%	68	33%	6%
0.0075 ul/ml	0.416	0.607	2.8	15%	144	69%	10%
0.0056 ul/ml	0.737	1.289	10.6	56%	46	22%	12%
0.0042 ul/ml	0.934	1.424	14.8	78%	210	101%	79%
0.0032 ul/ml	1.148	1.465	18.7	99%	211	101%	100%
0.0024 ul/ml	1.107	1.374	16.9	89%	234	113%	101%
0.0018 ul/ml	1.079	1.456	17.5	92%	256	123%	114%
0.0013 ul/ml	1.199	1.347	17.9	95%	221	106%	101%
SOLVENT 1	1.210	1.409	18.9	18.9	206	208	
SOLVENT 2	1.150	1.477	18.9		209		

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost

++ Too Toxic to Clone

Tables prepared and calculations performed by: [signature] (Signature & Date) 1/24/83

Form No. WL-229
2/25/82

Workbook Page No. 52 Report Page No. VIII-9

Study No. T1851.701006

Table 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1851.701006 — Study Number
Paul E Kirby — Study Director
185182 — Experiment No.

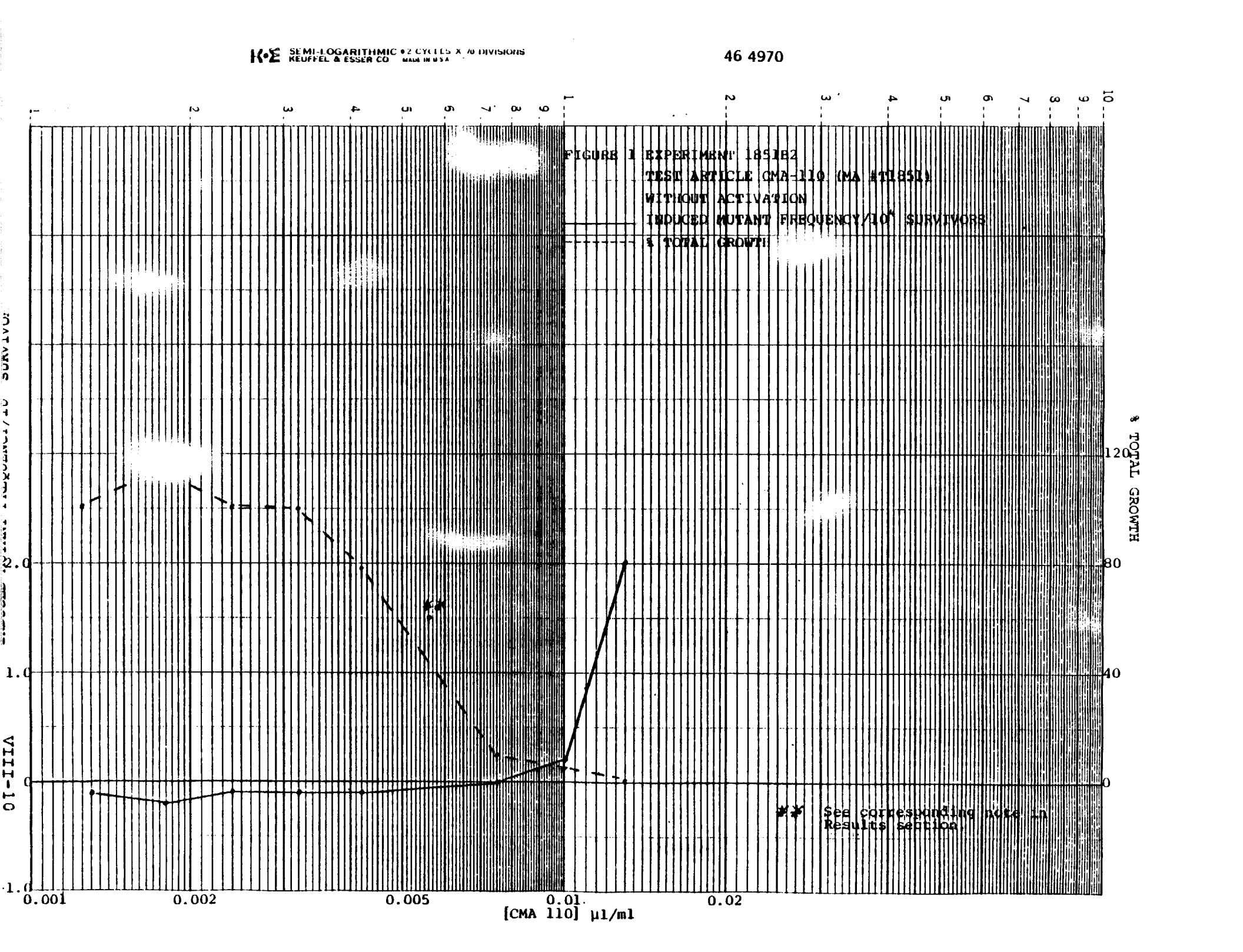
Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml	0.644	0.783	5.6	29%	24	11%	3%
0.5 µl/ml	0.913	1.217	12.3	63%	119	56%	35%
SOLVENT 1	1.176	1.450	18.9	19.6	206	213	
SOLVENT 2	1.222	1.490	20.2		219		
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml							
5.0 µg/ml							
SOLVENT 1							
SOLVENT 2							

not affinable AHB 1/24/83

Tables prepared and calculations performed by: Andrea M Rogers-Bank

(Signature & Date) 1/24/83

\+ Culture Lost
++ Too Toxic to Clone


K&E SEMI-LOGARITHMIC 2 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO MADE IN U.S.A.
FIGURE I EXPERIMENT 1851B2
TEST ARTICLE CMA-110 (MA #T185I)
WITHOUT ACTIVATION
INDUCED MUTANT FREQUENCY/10^5 SURVIVORS
% TOTAL GROWTH
VIII-10
2.0
1.0
0
-1.0
120
80
40
0
0.001
0.002
0.005
0.01
0.02
[CMA 110] µl/ml
** See corresponding note in Results section.

Study No. ~~T1851 70~~ 00

Table 6

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1851.701006 — Study Number
Paul E. Kirby — Study Director
10 ul/ml to 0.001 ul/ml — Dose Range
CMA-110 (T1851 — Test Article Identity
Acetone — Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation					
	Solvent 1				
	Solvent 2				
With S-9 Activation	10 ul/ml	0.108	0.280	0.0	0%
	5.0 ul/ml	0.069	0.071	0.0	0%
	1.0 ul/ml	0.009	0.016	0.0	0%
	0.5 ul/ml	0.014	0.020	0.0	0%
	0.1 ul/ml	0.008	0.011	0.0	0%
	0.05 ul/ml	0.063	0.037	0.0	0%
	0.01 ul/ml	0.934	1.349	14.0	95%
	0.005 ul/ml	0.953	1.406	14.9	101%
	0.001 ul/ml	0.992	1.388	15.3	104%
	Solvent 1	0.981	1.330	14.5 } 14.8	
	Solvent 2	0.968	1.390	15.0 }	

Not applicable JJ 2-7-83

+ Culture Lost

Table Prepared By Janine L. Johnson — Signature — 2-7-83 Date

Form No. WL-233 Workbook Page No. 63 Report Page No. VIII-11

Form No. WL-127

Table 7

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1551.701006	Paul E Kirby	1851B73
Study Number	Study Director	Experiment Number
CMA-110 (T1851)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
0.038 mM	104	74	75	84	172	165	162	166	1.0	0.5
0.028 mM	52	64	56	57	165	155	140	153	0.7	0.2
0.021 mM	45	57	60	54	189	182	155	175	0.6	0.1
0.016 mM	39	55	61	52	198	184	188	191	0.5	0.0
0.012 mM	50	36	41	42	161	184	214	186	0.5	0.0
0.008 mM	50	45	51	49	197	207	221	208	0.5	0.0
0.0062 mM	45	56	39	47	173	179	164	172	0.6	0.1
0.005 mM	29	46	41	39	189	208	187	195	0.4	-0.1
0.0038 mM	44	29	36	36	195	195	164	185	0.4	-0.1
0.0028 mM	37	34	34	35	161	166	+	164	0.4	-0.1
Solvent 1	60	52	38	50	166	184	190	180	0.6	0.5
Solvent 2	46	39	35	40	198	171	183	184	0.4	

Plates Counted By: Jane Clarke 2/22/83 (Signature & Date)

Calculations Performed By: Anne N Rogers-Bank (Signature & Date) 2/22/83

+ Culture Lost

Workbook Page No. 80 Report Page No. VIII-12

Study No. T1551.701006

Form No. WL-128
2/25/82

Workbook Page No. 81 Report Page No. VIII-13

Study No. T1851.701006

Table 8

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1851.701006	Paul E Kirby	1851B23
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1										
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	39	34	41	38	6	7	7	7	11.4	10.9
5.0 µg/ml	183	224	186	198	68	68	78	71	5.5	5.0
SOLVENT 1	49	41	52	47	175	165	176	172	0.6	0.5
SOLVENT 2	35	38	36	36	166	173	188	176	0.4	

not applicable AMLB 2/22/83

Plates Counted By: [signature] 2/22/83 (Signature & Date)

Calculations Performed By: [signature] 2/22/83 (Signature & Date)

*Per 10^4 surviving cells

+ Culture Lost

Form No. WL-228
2/22/82

Workbook Page No. 82 Report Page No. VIII-14

Study No. T1851.701006

Table 9

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

3 entry error [illegible] 2/22/82

T1851 701006	Paul E Kirby	1851B2
Study Number	Study Director	Experiment Number
CMA-110 (T1851)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.050 ul/ml	0.172	0.144	0.0	0%	++		
0.038 ul/ml	0.414	1.106	5.1	37%	166	91%	34%
0.028 ul/ml	0.727	1.567	12.7	92%	153	84%	78%
0.021 ul/ml	0.713	1.488	11.8	86%	175	96%	83%
0.016 ul/ml	0.762	1.516	12.8	94%	191	105%	98%
0.012 ul/ml	0.771	1.565	13.4	98%	186	102%	100%
0.0089 ul/ml	0.778	1.526	13.2	96%	208	114%	110%
0.0067 ul/ml	0.782	1.475	12.8	94%	172	95%	88%
0.0050 ul/ml	0.835	1.468	13.6	99%	195	107%	107%
0.0038 ul/ml	0.773	1.522	13.1	95%	186	102%	98%
0.0028 ul/ml	0.811	1.465	13.2	96%	164	90%	87%
SOLVENT 1	0.827	1.483	13.6 } 13.7		180 } 182		
SOLVENT 2	0.822	1.515	13.8 }		184 }		

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: [signature] 2/22/82
(Signature & Date)

Form No. WL-229
2/25/82

Table 10

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1851.701006
Study Number

Paul E Kirby
Study Director

185162
Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml							
0.5 µl/ml							
SOLVENT 1							
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.361	0.366	1.5	11%	7	4%	0%
5.0 µg/ml	0.510	1.053	6.0	46%	71	41%	19%
SOLVENT 1	0.733	1.534	12.5	13.1	172	174	
SOLVENT 2	0.825	1.493	13.7		176		

not applicable AMKB 2/22/82

Tables prepared and calculations performed by: [signature]

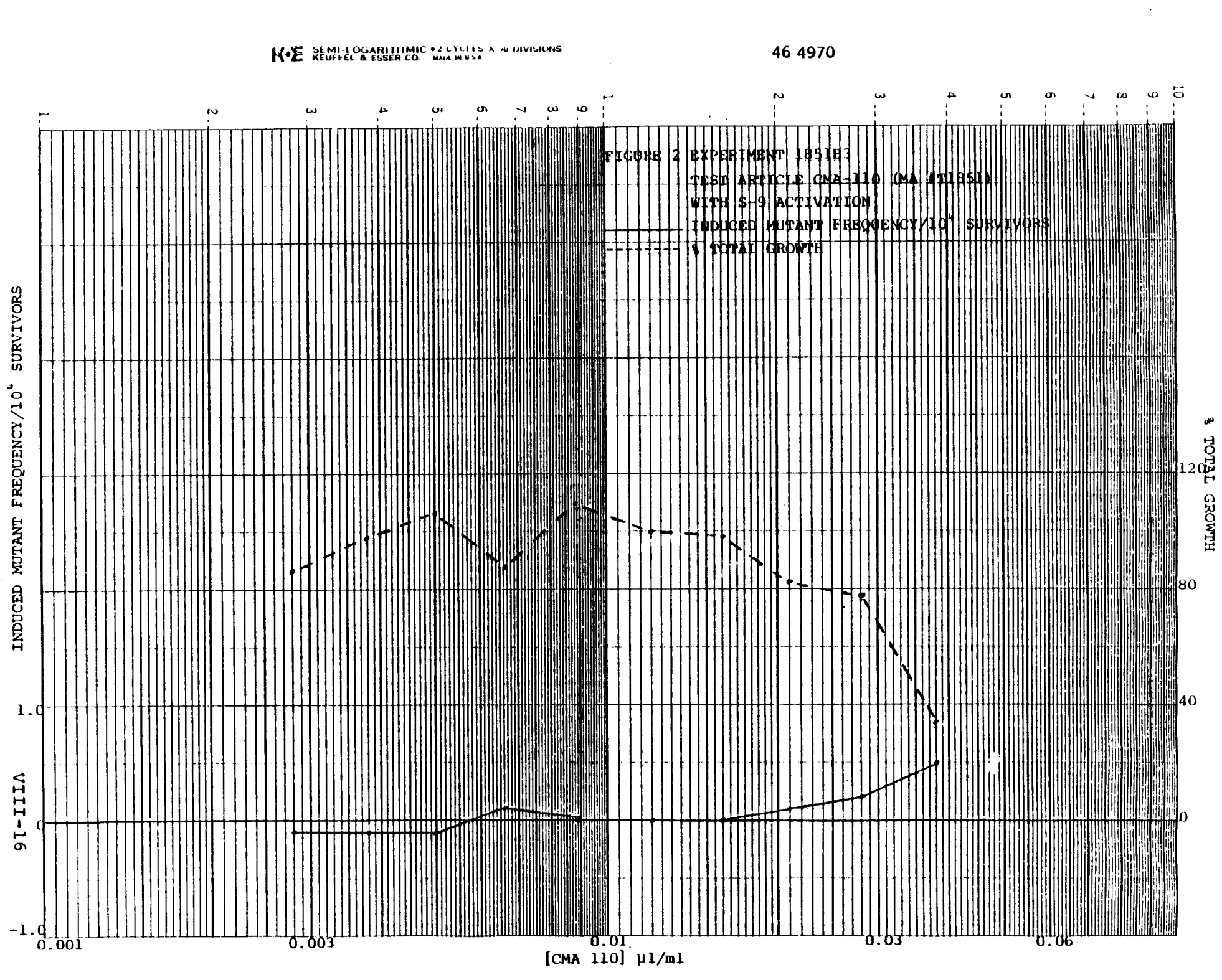
(Signature & Date) 2/22/82

\+ Culture Lost
++ Too Toxic to Clone

Workbook Page No. 83 Report Page No. VIII-15

Study No. T1851.701006

K&E SEMI-LOGARITHMIC 2 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.



Form No. MA-184
2/16/82

Workbook Page No. 1704 Report Page No. VIII-17

Study No. T1851.701006

Table 11
L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1851.701006	Paul E. Kirby	185184
Study Number	Study Director	Experiment Number
CMA 110 (T1851)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Avg. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Avg. #/ Plate	Mutant* Frequency	Induced Mutant* Frequency
Solvent 1	+	+	+	+	160	139	153	151		
Solvent 2	65	69	55	63	+	+	+	+		
0.055 µl/ml A	++	++	++		++	++	++			
0.055 µl/ml B	110	109	+	110	172	153	166	164	1.3	(0.7)
0.050 µl/ml A	158	+	+	158	99	100	+	100	3.2	(2.6)
0.050 µl/ml B	128	115	+	122	141	141	104	129	1.9	(1.3)
0.045 µl/ml A	137	+	+	137	+	+	+	+		
0.045 µl/ml B	102	147	130	126	+	+	+	+		
0.039 µl/ml A	88	+	+	88	159	+	+	159	1.1	(0.5)
0.039 µl/ml B	98	105	+	102	155	+	+	155	1.3	(0.7)
0.035 µl/ml A	80	86	+	83	161	125	+	168	1.0	(0.4)
0.035 µl/ml B	89	+	+	89	130	164	130	141	1.3	(0.7)
0.029 µl/ml A	102	+	+	102	139	165	+	152	1.3	(0.7)
0.029 µl/ml B	81	73	90	81	152	186	131	156	1.0	(0.4)

Plates Counted By: Leah Grunwald 3/29/83 (4/22/83)
(Signature & Date)

Calculations Performed By: Leah Grunwald 3/29/83 (4/22/83) LCG
(Signature & Date)

* Per 10^4 surviving cells
IMF values calculated using solvent control mutant frequencies from the positive control portion of the assay LCG 4/22/83

\+ Culture Lost
++ too toxic to clone

Table 12

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

TK5L 701006 — Study Number

Paul E. Kisby — Study Director

1851B3 — Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced* Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1										
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	223	224	+	224	113	113	+	113	4.0	3.4
5.0 µg/ml	162	153	160	158	100	86	+	93	3.4	2.8
SOLVENT 1	47	52	+	50	142	+	+	142	0.7 ≥ 0.6	
SOLVENT 2	39	+	+	39	140	146	142	143	0.5	

Not Applicable 3-29-83 LLC

Plates Counted By: Leah Griswold 3/29/83 (Signature & Date)

Calculations Performed By: Leah Griswold 3/29/83 (Signature & Date)

*Per 10^5 surviving cells

+ Culture Lost

1851 701006

Workbook Page No. 101 Report Page No. VIII-18

Form No. MA-231

Workbook Page No. 102A

Report Page No. VIII-19

Study No. T1851.701006

TABLE 13

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1851.701006	Paul E. Kirby	1851B4
Study Number	Study Director	Experiment Number
CMA 110 (T1851)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration X 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Avg. V. C.	% Control	% Total Growth
SOLVENT 1	0.784	1.637	14.3		151		
SOLVENT 2	0.857	1.582	+				
0.055 ul/ml A	0.176	0.139	++		++		
0.055 ul/ml B	0.365	1.127	4.6	32%	164	109%	35%
0.050 ul/ml A	0.265	0.707	2.~~1~~4*	*~~15~~6%	100	66%	*11~~0~~%
0.050 ul/ml B	0.311	0.821	2.8	20%	129	85%	17%
0.045 ul/ml A	0.278	0.799	2.~~5~~7*	*19~~17~~%	+		
0.045 ul/ml B	0.267	0.795	2.~~4~~7*	*19~~16~~%	+		
0.039 ul/ml A	0.466	1.502	7.8	54%	159	105%	57%
0.039 ul/ml B	0.485	1.517	8.2	57%	155	103%	59%
0.035 ul/ml A	0.634	1.561	11.0	77%	168	111%	86%
0.035 ul/ml B	0.613	1.560	10.6	74%	141	93%	69%
0.029 ul/ml A	0.665	1.603	11.8	83%	152	101%	83%
0.029 ul/ml B	0.681	1.567	11.9	83%	156	103%	86%
A							
B							
A							
B							

\+ Culture Lost
++ Too Toxic to Clone

* Calculation errors. AMRB 5/5/83

Tables prepared and calculations performed by: Leah Guswold 3/29/83 (4/22/83) LG
(Signature & Date)

Form No. MA-229
2/16/83

Table 14

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1851 701006 — Study Number
Paul E. Kirby — Study Director
1851B4 — Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V.C.	% Control	% Total Growth
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml							
0.5 µl/ml							
SOLVENT 1							
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.663	1.182	8.7	62%	113	79%	49%
5.0 µg/ml	0.684	1.409	10.7	76%	93	65%	49%
SOLVENT 1	0.773	1.618	13.9 } 14.1		142 } 143		
SOLVENT 2	0.842	1.531	14.3		143		

Not Applicable 3-29-83

Tables prepared and calculations performed by Leah Griswold 5-29-83 (4/12/83) [illegible]
(Signature & Date)

+ Culture Lost
++ Too Toxic to Clone

Workbook Page No. 102A Report Page No. VIII-20

Study No. T1851. 701006

K&E 10 X 10 TO THE INCH • 7 X 10 INCHES
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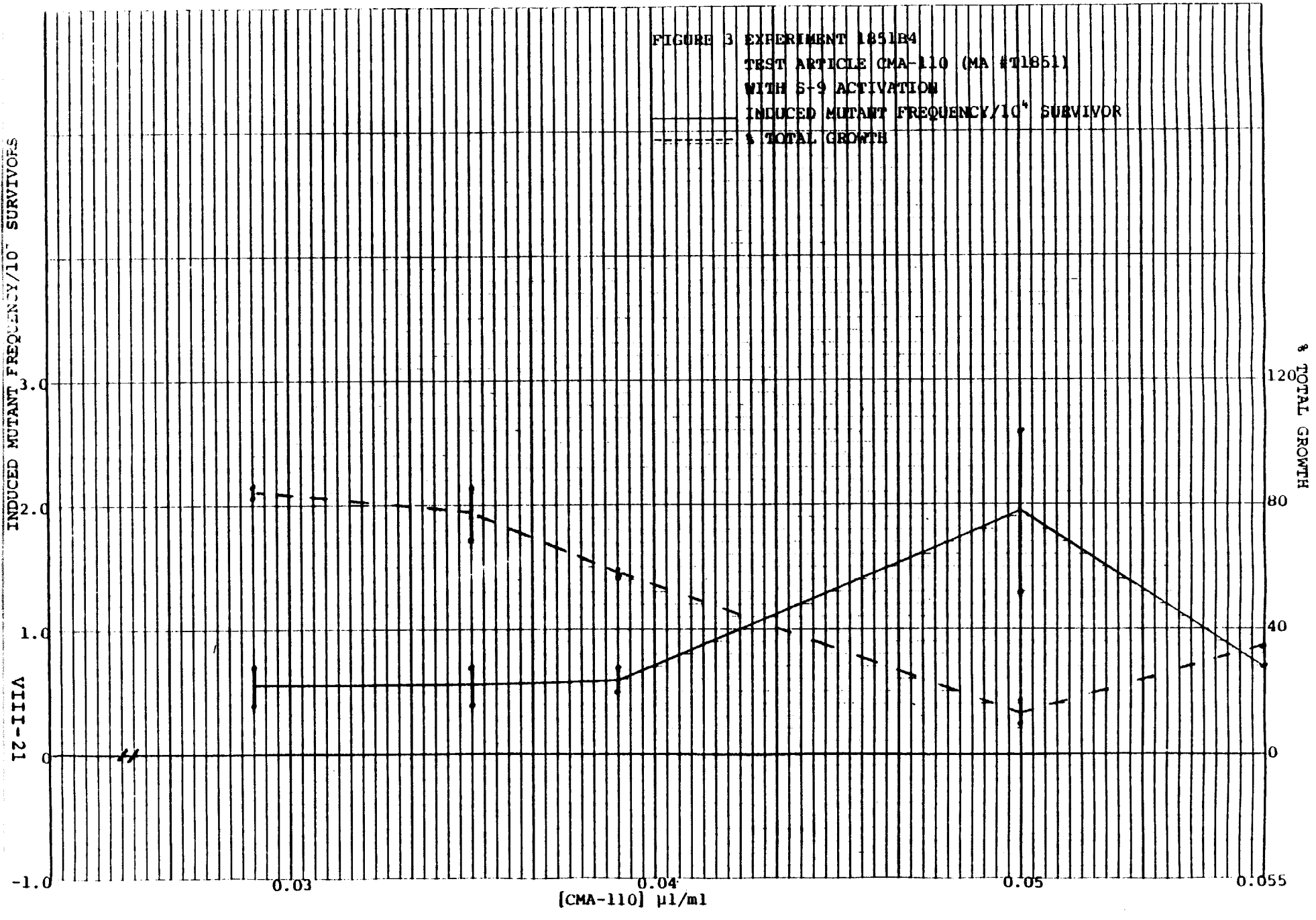

Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-110 (MA #T1851) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The highest concentration cloned in the nonactivated cultures exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant since the Total Growth of this culture was less than 10%. TFT resistance observed at these highly toxic levels may be due to epigenetic events. In the first experiment with S-9 activation, one culture exhibited a mutant frequency which was twice the mean mutant frequency of the solvent controls. There were some technical difficulties associated with the second experiment with S-9 activation. However, the results obtained correlated well with the results of the first experiment. The results indicate that under the conditions of this test, test article CMA-110 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of exogenous metabolic activation.

APPENDIX

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Rec'd by
RA/QA 12/8/82.
N.K./fkc

L5178Y $TK^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-110

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation: $2,650

Total charge for assay with metabolic activation: $2,650

6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-amino-anthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_2O, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0×10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F_0P will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3×10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3×10^5 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2×10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

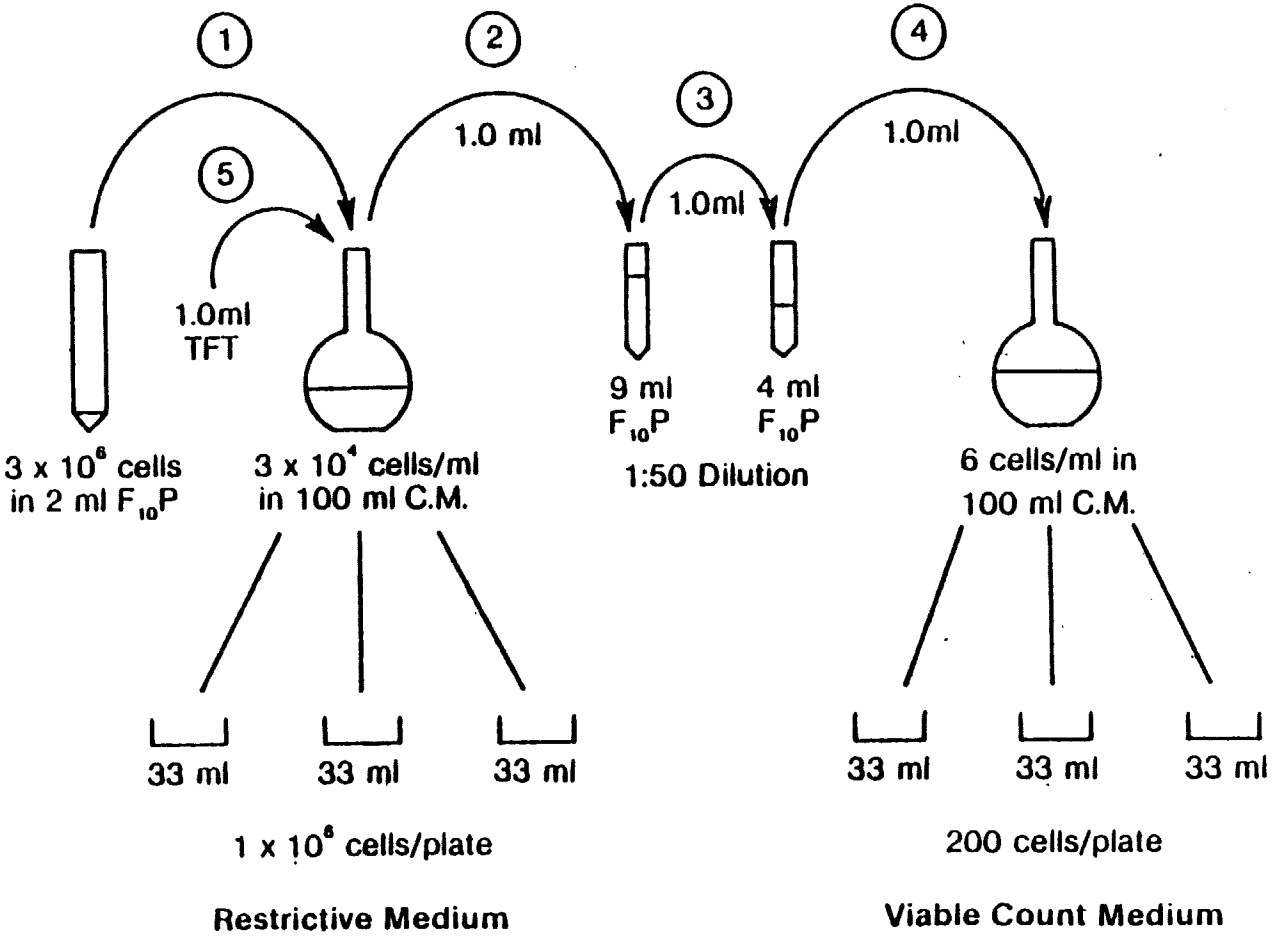

FIGURE 1

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency?_____
If so, to which agency or agencies? _____

Does the sponsor request that samples of the Test Article dosing solutions be returned?_____

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: 11/24/82

13.2 Proposed Initiation Date: December 16, 1982

13.3 Scheduled Completion Date: January 29, 1983

13.4 Report Submission to Sponsor Date: January 29, 1983

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

Carol Stark
SPONSOR'S AUTHORIZED REPRESENTATIVE

11/22/82
DATE PROTOCOL APPROVED BY SPONSOR

Paul E. Kinder 11/24/82
STUDY DIRECTOR DATE

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136) and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \text{Average Counts (corrected for coincidence)} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{cells/ml})\ (20\ \text{ml})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = 20\ \text{ml} - \text{Volume of Cells Retained}$$

---

2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

---

3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138) and L5178Y TK+/- Assay - Daily Counts (Positive Controls) (Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^{6} = \text{Average Counts (corrected for coincidence)} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^6 \text{ Cells/ml})\ (20 \text{ ml}^*)}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20 \text{ ml}^*) - (\text{Volume of Cells Retained})$$

* The final volume is adjusted to 10 ml on the third day of expression.

---

4. Tables 2, 3, and 4 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^4 \text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left(\text{Mutant Frequency of Treated Cultures}\right) - \left(\text{Average Mutant Frequency of Solvent Controls}\right)$$

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5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than 0.3×10^6 cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

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5. (Cont'd.)

$$\text{\% Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\text{\% Growth} = \frac{(\text{\% Suspension Growth})\ (\text{\% Cloning Growth})}{100}$$

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Form No. MA-228
2/16/83

Table 9

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1867.701006	Paul E. Kirby	186784
Study Number	Study Director	Experiment Number
(T1867)CMA 111	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^4 cells/ml		Suspension Growth		Cloning Growth		% Total Growth *
	Day 1	Day 2	Total	% Control	Ave. V. C.	% Control	
0.032 ml	0.169	0.125	0.0	0%	++		
0.024 ml	0.349	0.962	3.7	22%	157	122%	27%
0.018 ml	0.675	1.460	11.0	64%	114	88%	57%
0.013 ml	0.721	1.519	12.2	71%	78	60%	43%
0.010 ml	0.813	1.409	12.7	74%	114	88%	66%
0.0075 ml	0.923	1.415	14.5	85%	153	119%	101%
0.0056 ml	0.936	1.422	14.8	86%	149	116%	100%
0.0042 ml	0.938	1.394	14.5	85%	157	122%	103%
0.0032 ml	0.922	1.393	14.3	83%	111	86%	72%
0.0024 ml	1.021	1.399	15.9	93%	154	119%	111%
0.0018 ml	0.974	1.419	15.4	90%	160	124%	111%
SOLVENT 1	1.041	1.491	17.2	17.1	123	129	
SOLVENT 2	1.040	1.459	16.9		135		

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: Ave P Bada 4-18-83
(Signature & Date)

Workbook Page No. 95 Report Page No. IX-13

Study No. T1867.701006

Form No. MA-229

Workbook Page No. 69 Report Page No. IX-14

Table 10

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1867.701006 — Study Number

Paul E. Kirby — Study Director

1867BY — Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V.C.	% Control	% Total Growth
Ethyl Methanesulfonate (No Activation)							
1.0 µl/ml	Not Applicable		QNS	4-19-87			
0.5 µl/ml							
SOLVENT 1							
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.509	1.061	6.0	38%	90	69%	26%
5.0 µg/ml	0.805	1.371	12.3	78%	84	66%	51%
SOLVENT 1	0.960	1.431	15.3	15.7	120	131	
SOLVENT 2	1.036	1.392	16.0		141		

Tables prepared and calculations performed by: Anne P Burdick 4-19-87

(Signature & Date)

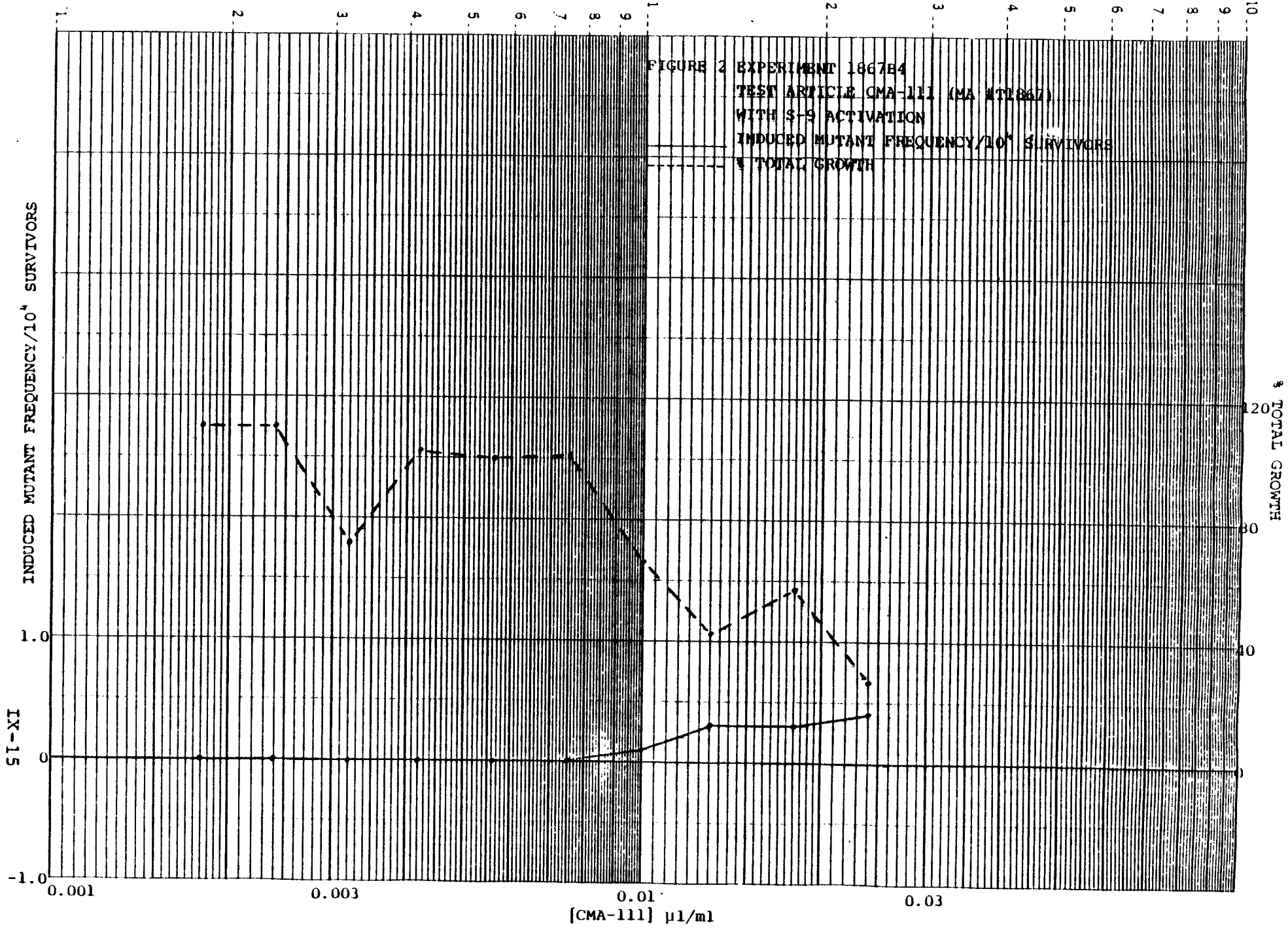
Study No. T1867.701006

\+ Culture Lost

++ Too Toxic to Clone

K&E SEMI-LOGARITHMIC • 2 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO MADE IN U.S.A.

46 4970



Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-111 (MA #T1867) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. One nonactivated culture that was cloned exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant since the Total Growth of this culture was less than 10%. TFT resistance observed at these highly toxic levels may be due to epigenetic events. One S-9 activated culture that was cloned exhibited a mutant frequency which was significantly greater than the mean mutant frequency of the solvent controls. The results indicate that, under the conditions of this test, test article CMA-111 produced a negative response in the absence of exogenous metabolic activation and an equivocal response in the presence of metabolic activation.

APPENDIX

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Rec'd. by
RA/QA 12/15/82.
N.K./jkc

L5178Y TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-111

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:
$2,650

Total charge for assay with metabolic activation:
$2,650

6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-amino-anthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_2O, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0×10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F_0P will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3×10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3×10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2×10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of

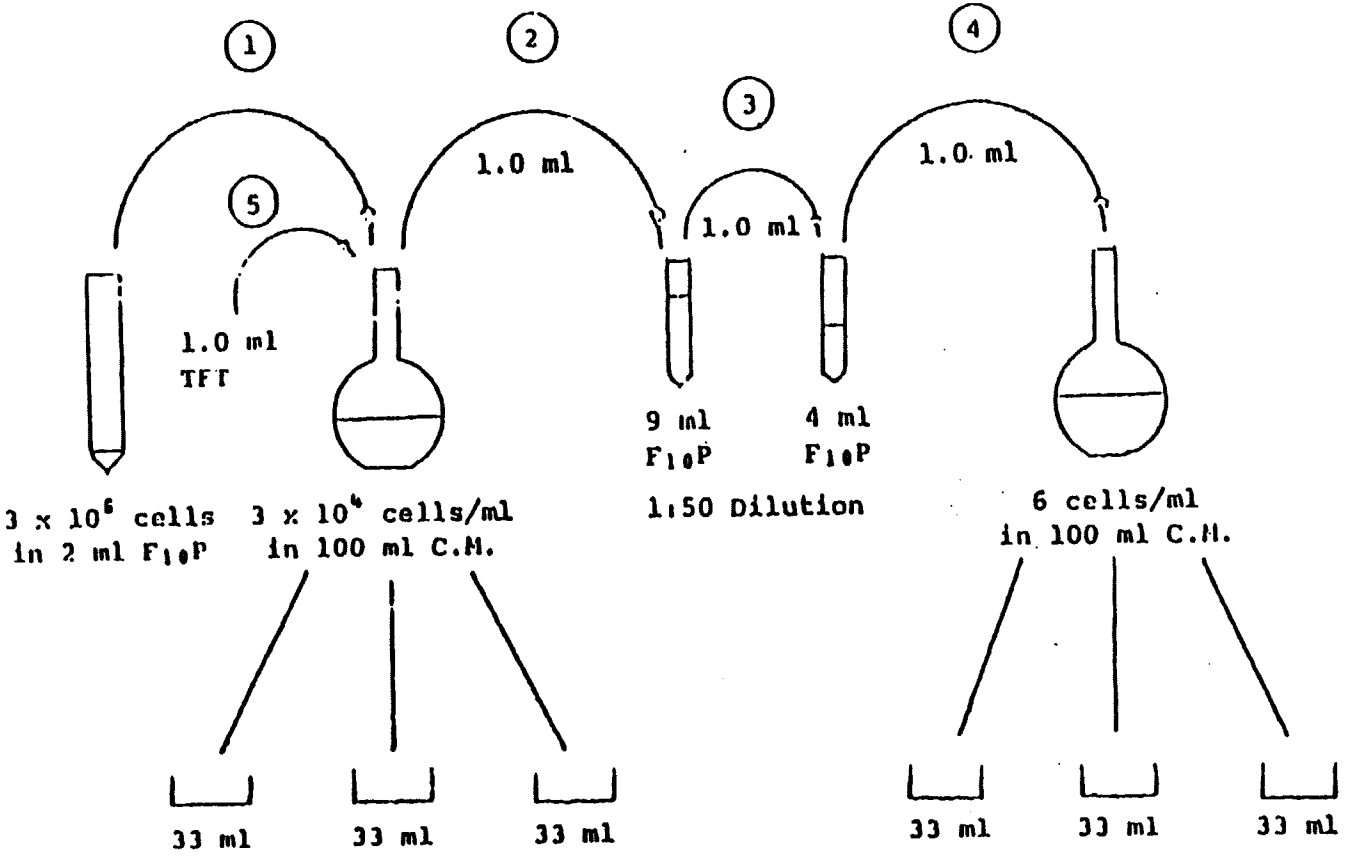

Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

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that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.G. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency?_____
If so, to which agency or agencies? _____

Does the sponsor request that samples of the Test Article dosing solutions be returned?_____

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: 12/10/82

13.2 Proposed Initiation Date: 1/18/83 or 5/5/83

13.3 Scheduled Completion Date: 2/18/83

13.4 Report Submission to Sponsor Date: 2/18/83

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

Carol Stark
SPONSOR'S AUTHORIZED REPRESENTATIVE

11/24/82
DATE PROTOCOL APPROVED BY SPONSOR

Paul E. Kilby — 11/24/82
STUDY DIRECTOR — DATE

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136) and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \text{Average Counts (corrected for coincidence)} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{cells/ml})\ (20\ \text{ml})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = 20\ \text{ml} - \text{Volume of Cells Retained}$$

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2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

---

3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138) and L5178Y TK+/- Assay - Daily Counts (Positive Controls) (Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^{6} = \text{Average Counts (corrected for coincidence)} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{Cells/ml})\ (20\ \text{ml}^{*})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20\ \text{ml}^{*}) - (\text{Volume of Cells Retained})$$

* The final volume is adjusted to 10 ml on the third day of expression.

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4. Tables 2, 3, and 4 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^{6}\text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left(\text{Mutant Frequency of Treated Cultures}\right) - \left(\text{Average Mutant Frequency of Solvent Controls}\right)$$

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5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

$$\text{\% Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\text{\% Growth} = \frac{(\text{\% Suspension Growth})\ (\text{\% Cloning Growth})}{100}$$

Summary

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-112 (MA #T1853) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. The nonactivated cultures were cloned over a range of test article concentrations which produced from 4% to 93% Total Growth. The S-9 activated cultures were cloned over a range of test article concentrations which produced from 22% to 103% Total Growth.

One nonactivated culture having less than 10% Total Growth exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant due to the high toxicity observed. None of the S-9 activated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that under the conditions of this test, test article CMA-112 produced a negative response in the presence and absence of exogenous metabolic activation.

Results

The Initial Toxicity Test (Table 1) performed on test article CMA-112 (MA #T1853) in the absence of S-9 indicated a threshold level of complete toxicity at 0.1 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the absence of S-9 over a range of concentrations from 0.1 µl/ml to 0.0013 µl/ml.

After a two day expression period, eight cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018 or 0.0013 µl/ml test article. These concentrations produced a range in Suspension Growth of 18% to 98%. The Cloning Data are presented in Tables 2 and 3 and the Total Compound Toxicity Data are presented in Tables 4 and 5. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 1.

One culture that was cloned exhibited a mutant frequency which was 2.7 times the mean mutant frequency of the solvent controls. The Total Growth of this culture was 4%. None of the remaining cultures exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 24% to 93%.

An Initial Toxicity Test (Table 6) was conducted in the presence of S-9 on test article CMA-112 (MA #T1853). The results indicated a threshold level of complete toxicity at 0.05 µl/ml. Based on these data, the test article was tested in a mutagenesis assay in the presence of S-9 over a range of concentrations from 0.05 µl/ml to 0.00067 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.016, 0.0089, 0.0067, 0.0050, 0.0038, 0.0028, 0.0021, 0.0016, 0.0012 or 0.00089 µl/ml. These concentrations produced a range in Suspension Growth of 43% to 108%. The Cloning Data are presented in Tables 7 and 8 and the Total Compound Toxicity Data

are presented in Tables 9 and 10. The Cloning Data and Total Compound Toxicity Data are also presented graphically in Figure 2.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 22% to 103%.

Study No. T1853 701005

Table 1

701 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL COMPOUND TOXICITY TEST

T1853.701006 — Study Number
Paul E Kirby — Study Director
100 ul/ml – 0.001 ul/ml — Dose Range
CHA 112 (T1853) — Test Article Identity
Acetone — Solvent

	Test Article Concentration	Cell Concentration (X 10^6) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation	100 ul/ml	1.880**	*		
	10 ul/ml	0.580**	*		
	1.0 ul/ml	0.036	*		
	0.1 ul/ml	0.041	0.055	0.0	0%
	0.01 ul/ml	0.277	0.402	1.3	24%
	0.001 ul/ml	0.574	0.876	5.6	102%
	Solvent 1	0.580	0.819	5.3	5.5
	Solvent 2	0.597	0.848	5.6	
With S-9 Activation					
	Solvent 1				
	Solvent 2				

not applicable AMRB 12/22/82

+ Culture Lost

Table Prepared By: Andrea N Rogers-Bardo — Signature — 12/22/82 — Date

** debris only no viable cells. AMRB 12/22/82

* not counted on 2nd day AMRB 12/22/82

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Form No. WL-232
2/22/82

Form No. ML-41
2/25/82

Workbook Page No. 30

Report Page No. X-5

Study No. T1853.701006

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1853.701006	Paul E. Kirby	1853B1
Study Number	Study Director	Experiment Number
CMA 112 (T853)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced * Mutant Frequency
.010 ml/l	31	57	42	43	58	41	39	46	1.9	1.2
.0075 ml/l	50	42	45	46	108	110	123	114	0.8	0.1
.0056 ml/l	44	51	57	51	175	153	184	171	0.6	-0.1
.0042 ml/l	51	53	31	45	152	135	176	154	0.6	-0.1
.0032 ml/l	52	48	39	46	190	190	177	186	0.5	-0.2
.0024 ml/l	46	57	42	48	186	199	174	186	0.5	-0.2
.0018 ml/l	39	29	42	37	165	164	159	163	0.5	-0.2
.0013 ml/l	35	52	53	47	177	138	162	159	0.6	-0.1
Solvent 1	53	64	68	62	187	188	186	187	0.7	0.2
Solvent 2	78	53	67	66	176	191	206	191	0.7	

Plates Counted By: Eleanor M. Carey 1-31-83 (Signature & Date)

Calculations Performed By: Eleanor M. Carey 1-31-83 (Signature & Date)

* Per 10^6 surviving cells

\+ Culture Lost

+ try Error nc 1-31-83

Form No. WL-128
2/25/82

Workbook Page No. 31 Report Page No. X-6

Study No. T1853.701003

Table 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1853.701006	Paul E. Kirby	1853B1
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced * Mutant Frequency
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml	210	212	198	207	23	19	18	20	20.7	20.2
0.5µl/ml	384	360	358	367	134	116	88	113	6.5	6.0
SOLVENT 1	50	47	61	53	199	219	197	205	0.5 } 0.5	
SOLVENT 2	47	56	44	49	194	211	190	198	0.5 }	
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml										
5.0 µg/ml										
SOLVENT 1										
SOLVENT 2										

Not Applicable - Eleanor M. Carey 1-31-83

Plates Counted By: Eleanor M. Carey 1/31/83 (Signature & Date)

Calculations Performed By: Eleanor M. Carey 1-31-83 (Signature & Date)

*Per 10^6 surviving cells

+ Culture Lost

Form No. WL-227
2/22/82

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1853, 701006	Paul E. Kirby	1853B1
Study Number	Study Director	Experiment Number
CMA 112 (T1853)	Acetone	None
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth *
	Day 1	Day 2	Total	% Control	Ave. V. C.	% Control	
0.018 µl/ml	0.015	0.014			++		
0.013 µl/ml	0.065	0.082			++		
0.010 µl/ml	0.361	0.696	2.8	18%	46	24%	4%
0.0075 µl/ml	0.572	0.971	6.2	39%	114	60%	24%
0.0056 µl/ml	0.733	1.201	9.8	62%	171	90%	56%
0.0042 µl/ml	0.769	1.441	12.3	78%	154	81%	64%
0.0032 µl/ml	0.988	1.353	14.9	94%	186	98%	93%
0.0024 µl/ml	0.928	1.402	14.5	92%	186	98%	90%
0.0018 µl/ml	0.808	1.550	13.9	88%	163	86%	76%
0.0013 µl/ml	0.986	1.414	15.5	98%	159	84%	83%
SOLVENT 1	1.019	1.384	15.7	158	187	189	
SOLVENT 2	1.007	1.409	15.8		191		

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Study No. T1853 70.003

* % Total Growth = $\frac{(\%\text{ Suspension Growth})\ (\%\text{ Cloning Growth})}{100}$

\+ Culture Lost
++ Too Toxic to Clone

Tables prepared and calculations performed by: Elaine M. Casey 1-31-83
(Signature & Date)

Form No. WL-229
2/25/82

Workbook Page No. 22 Report Page No. X-8

Study No. T1853.701003

Table 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

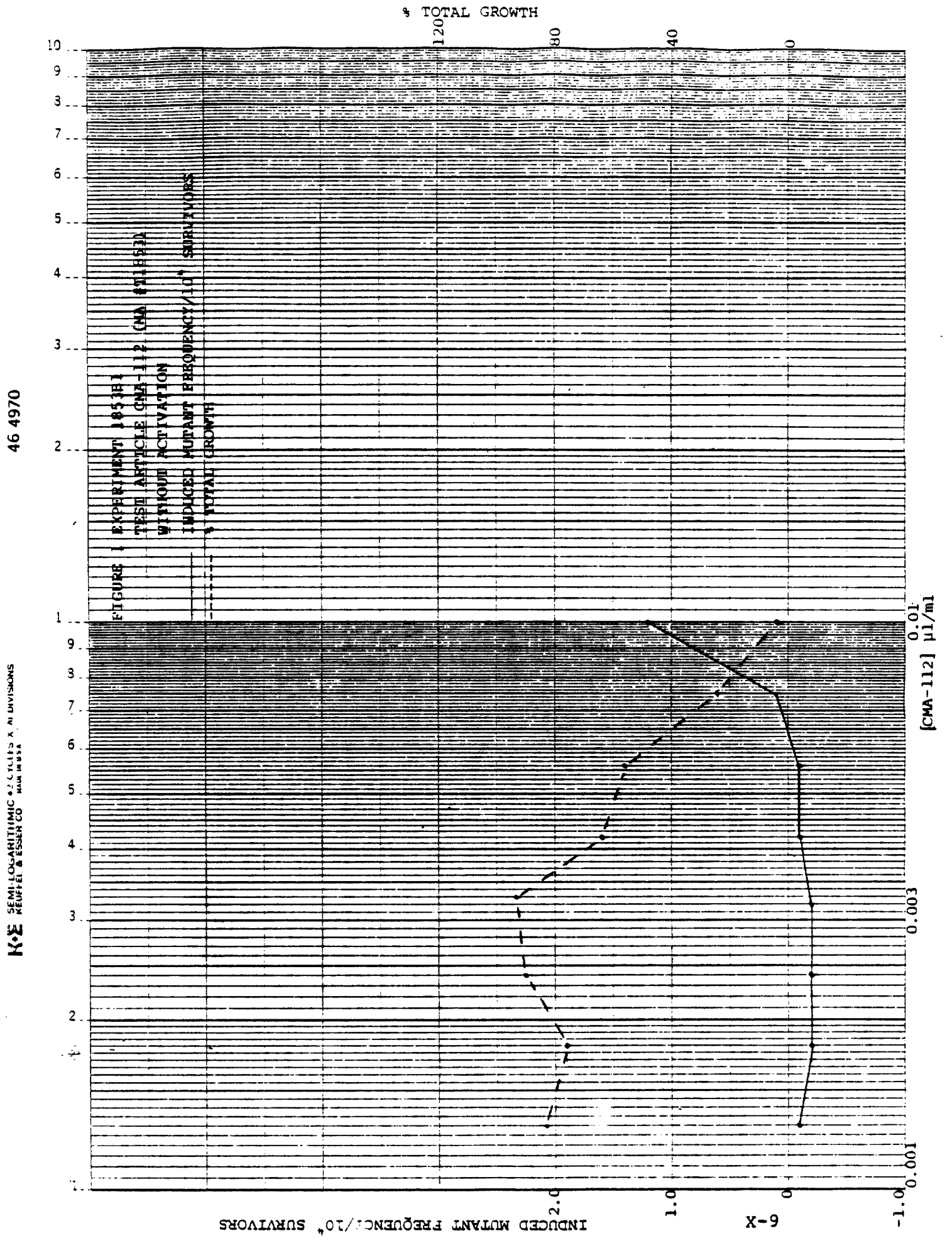
T1853.701006 (Study Number) — Paul E. Kilby (Study Director) — 1853B1 (Experiment No.)

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml	0.524	0.798	4.6	24%	18	9%	2%
0.5 µl/ml	0.826	1.181	10.8	57%	88	45%	26%
SOLVENT 1	1.023	1.442	17.2	91	197	194	
SOLVENT 2	1.330	1.415	20.9		190		
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml			1-31-83				
5.0 µg/ml							
SOLVENT 1							
SOLVENT 2							

Not Applicable Eleanor M. Carey 1-31-83

Tables prepared and calculations performed by: Eleanor M. Carey 1-31-83
(Signature & Date)

\+ Culture Lost
++ Too Toxic to Clone


% TOTAL GROWTH
120
80
40
0
10
9
8
7
6
5
4
3
2
1
FIGURE 1 EXPERIMENT 105 #1
TEST ARTICLE CMA-112 (MA #1163)
WITHOUT ACTIVATION
INDUCED MUTANT FREQUENCY/10⁴ SURVIVORS
% TOTAL GROWTH
46 4970
K+E SEMI-LOGARITHMIC
KEUFFEL & ESSER CO
0.01
[CMA-112] µl/ml
0.003
0.001
INDUCED MUTANT FREQUENCY/10⁴ SURVIVORS
2.0
1.0
0
-1.0
X-9

Study No. T1853 701006

Table 6

702 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL TOXICITY TEST

T1853.701006	Paul E. Kirby	10 µl/ml to 0.001 µl/ml
Study Number	Study Director	Dose Range
CMA-112 (T1853)		Acetone
Test Article Identity		Solvent

	Test Article Concentration	Cell Concentration (x10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
Without Activation					
	Solvent 1				
	Solvent 2				
With S-9 Activation	10 µl/ml	0.205	0.288	0.0	0%
	5.0 µl/ml	0.177	0.233	0.0	0%
	1.0 µl/ml	0.005	0.017	0.0	0%
	0.5 µl/ml	0.009	0.032	0.0	0%
	0.1 µl/ml	0.024	0.039	0.0	0%
	0.05 µl/ml	0.026	0.028	0.0	0%
	0.01 µl/ml	0.826	1.394	12.8	93%
	0.005 µl/ml	0.865	1.374	13.2	96%
	0.001 µl/ml	0.860	1.472	14.1	102%
	Solvent 1	0.862	1.457	14.0 } 13.8	
	Solvent 2	0.861	1.420	13.6 }	

Not applicable JL 2-14-83

+ Culture Lost

* entry error JL 2-14-83

Table Prepared By [signature] Signature 2-14-83 Date

Form No. MA-127
2/16/83

Workbook Page No. 604 Report Page No. X-11

Study No. T1853 701006

Table 7

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

T1853 701006 Study Number	Paul E Kirby Study Director	1853B2 Experiment Number
CMA-112 (T1853) Test Article Identity	Acetone Solvent	Induced Rat Liver S-9 Metabolic Activation

Test Article Conc.	No. of Colonies/TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant * Frequency	Induced Mutant * Frequency
0.016 ul/l	35	38	33	35	87	93	110	97	0.7	0.2
0.0089 ul/l	55	52	47	51	159	163	174	165	0.6	0.1
0.0067 ul/l	42	51	45	46	178	213	185	192	0.5	0.0
0.0050 ul/l	42	51	64	52	165	176	154	165	0.6	0.1
0.0038 ul/l	53	44	49	49	155	200	177	177	0.5	0.0
0.0028 ul/l	68	61	61	63	170	162	161	164	0.8	0.3
0.0021 ul/l	62	54	67	61	183	213	175	190	0.6	0.1
0.0016 ul/l	67	73	65	68	166	162	152	160	0.9	0.4
0.0012 ul/l	57	52	62	57	197	175	166	179	0.6	0.1
0.0089 ul/l	48	58	48	51	179	166	151	165	0.6	0.1
Solvent 1	51	41	45	46	182	166	197	182	0.5	0.5
Solvent 2	40	58	50	49	194	207	179	193	0.5	

Plates Counted By: Leah Griswold 2-28-83 (Signature & Date) (4-15-83)

Calculations Performed By: Leah Griswold 2-28-83 (Signature & Date) (4-15-83)

Table 8

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

T1853.701006	Paul E. Kirby	1853132
Study Number	Study Director	Experiment Number

Test Article Conc.	No. of Colonies/ TFT Plate 1	2	3	Ave. #/ Plate	No. of Colonies/V.C. Plate 1	2	3	Ave. #/ Plate	Mutant* Frequency	Induced Mutant Frequency*
Ethyl Methanesulfonate (No Activation)										
1.0µl/ml										
0.5µl/ml										
SOLVENT 1										
SOLVENT 2										
7,12 Dimethylbenz(a)anthracene (With Activation)										
7.5 µg/ml	178	173	175	175	40	46	45	44	8.0	7.4
5.0 µg/ml	247	201	182	210	125	117	108	117	3.6	3.0
SOLVENT 1	66	54	39	53	177	197	187	187 ~~0.6~~	0.6	0.6
SOLVENT 2	52	50	51	51	149	172	163	161	0.6	

Not applicable LCG 2-28-83

Plates Counted By: Leah Griswold 2-28-83 (Signature & Date)

Calculations Performed By: Leah Griswold 2-28-83 (Signature & Date)

*Per 10^4 surviving cells

+ Culture Lost

* entry [illegible] 2-28-83

Study No. T1853 701005

Workbook Page No. 61 Report Page No. X-12

Form No. WL-128

Form No. MA-228
2/16/83

Workbook Page No. 62A Report Page No. X-13

Study No. T1853 701006

Table 9

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
TOTAL COMPOUND TOXICITY DATA

T1853 701006	Paul E Kirby	1853B2
Study Number	Study Director	Experiment Number
CMA-112 (T1853)	Acetone	Induced Rat Liver S-9
Test Article Identity	Solvent	Metabolic Activation

Test Article Concentration	Cell Concentration x 10^6 cells/ml Day 1	Day 2	Suspension Growth Total	% Control	Cloning Growth Ave. V. C.	% Control	% Total Growth *
0.028 ml/l	0.020	0.025	0.0	0%	++		
0.021 ml/l	0.185	0.130	0.0	0%	++		
0.016 ml/l	0.259	1.699	5.7	43%	97	52%	22%
0.0089 ml/l	0.642	1.685	12.0	92%	165	88%	81%
0.0067 ml/l	0.822	1.441	13.2	100%	192	102%	103%
0.0050 ml/l	0.828	1.388	12.8	97%	165	88%	86%
0.0038 ml/l	0.892	1.331	13.2	101%	177	94%	95%
0.0028 ml/l	0.851	1.354	12.8	98%	164	87%	85%
0.0021 ml/l	0.893	1.333	13.2	101%	190	101%	102%
0.0016 ml/l	0.897	1.378	13.7	105%	160	85%	89%
0.0012 ml/l	0.932	1.270	13.2	100%	179	95%	96%
0.00089 ml/l	0.998	1.281	14.2	108%	165	88%	95%
SOLVENT 1	0.935	1.250	13.0	13.1	182	188	
SOLVENT 2	0.913	1.301	13.2		193		

* % Total Growth = $\frac{(\%\ \text{Suspension Growth})\ (\%\ \text{Cloning Growth})}{100}$

\+ Culture Lost

++ Too Toxic to Clone

Tables prepared and calculations performed by: Leah Ghiswold 2-28-83 (4-15-83)
(Signature & Date)

Table 10

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

T1853.701006	Paul E. Kirby	1853B2
Study Number	Study Director	Experiment No.

Test Article Concentration	Cell Concentration x 10^6 cells/ml		Suspension Growth		Cloning Growth		% Total Growth
	Day 1	Day 2	Total	% Control	Ave. V.C.	% Control	
Ethylmethanesulfonate (No Activation)							
1.0 µl/ml							
0.5 µl/ml							
SOLVENT 1							
SOLVENT 2							
7,12 Dimethylbenz(a)anthracene (With Activation)							
7.5 µg/ml	0.519	0.581	3.4	25%	44	25%	6%
5.0 µg/ml	0.703	1.041	8.1	60%	117	67%	41%
SOLVENT 1	1.027	1.176	13.4		187	174	
SOLVENT 2	0.991	1.237	13.6 (13.5)		161		

Not Applicable 2-28-83 LLG

Tables prepared and calculations performed by: Leah Griswold 2-28-83

(Signature & Date)

\+ Culture Lost

++ Too Toxic to Clone

Study No. T1853 701006

Form No. WL-229

Workbook Page No. 63 Report Page No. X-14

K·E SEMI-LOGARITHMIC • 3 CYCLES X 70 DIVISIONS
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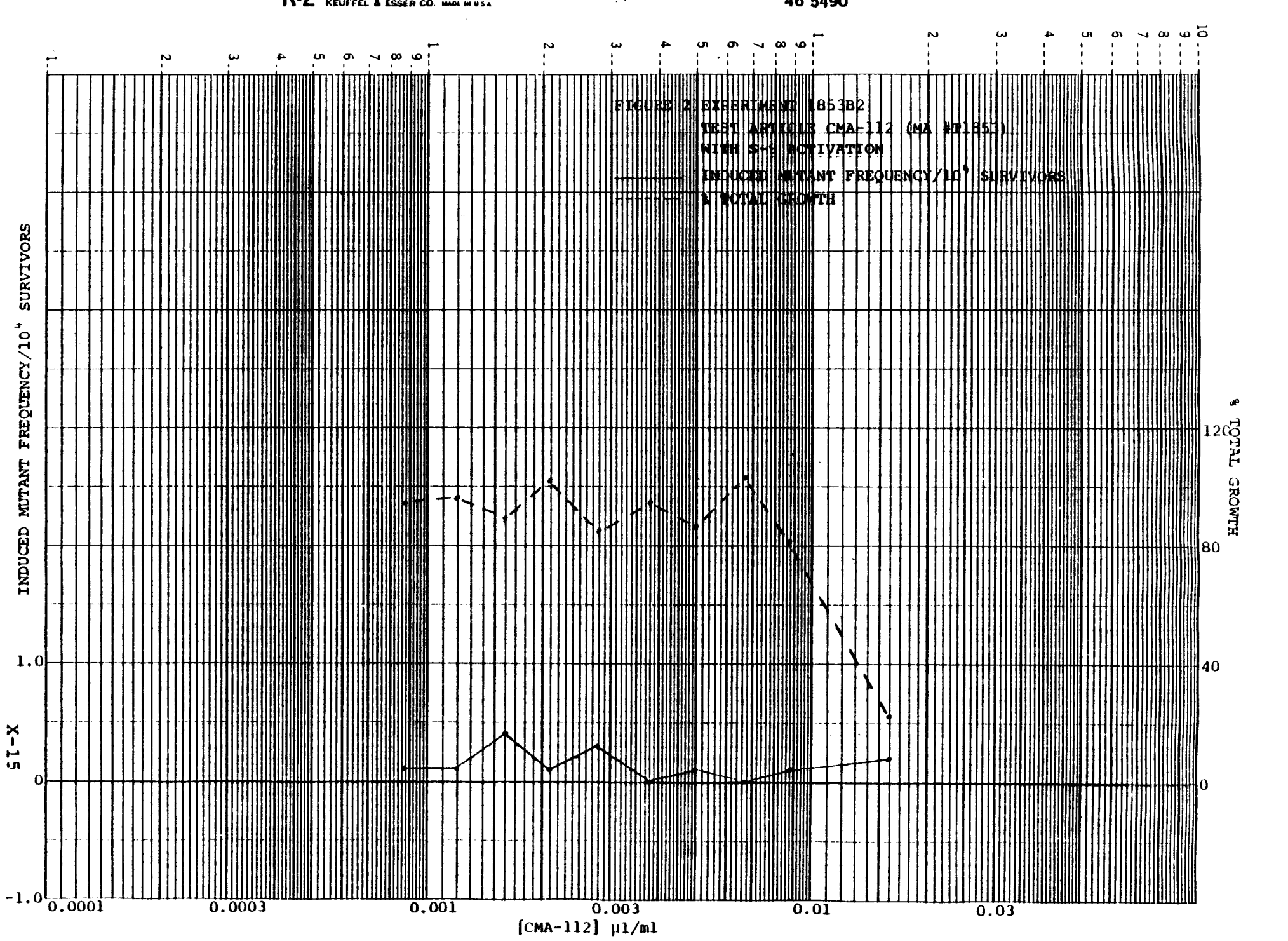

Conclusion

Chemical Manufacturers Association's test article Zinc Dialkyl Dithiophosphate, CMA-112 (MA #T1853) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. One nonactivated culture having less than 10% Total Growth exhibited a mutant frequency which was more than twice the mean mutant frequency of the solvent controls. This result is not considered significant due to the high toxicity observed. None of the S-9 activated cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that under the conditions of this test, test article CMA-112 produced a negative response in the presence and absence of exogenous metabolic activation.

APPENDIX

RECEIVED OCT 0 5 1982

Rec'd. by
RA/QA 12/8/82.
N.K./J.Pc

L5178Y TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article using the L5178Y TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: CMA-112

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M. Street, N.W.
Washington, D.C.

3.3 Authorized Representative: Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted without metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity without metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth. If these test conditions produce positive results as defined herein, no further testing is to be done. If these test conditions produce negative or equivocal results as defined herein, the Sponsor is to be contacted. Upon written instructions from the Sponsor, the test material(s) yielding negative or equivocal results will be tested with metabolic activation. Cloning data with activation must be obtained for a minimum of three dose levels within the range of 90% to 10% total suspension growth, with at least one dose yielding total suspension growth of less than or equal to 30%.

Total charge for assay without metabolic activation:
$2,650

Total charge for assay with metabolic activation:
$2,650

6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of

the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intra-peritoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-amino-anthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_2O, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with and/or without S-9 activation will be conducted.

Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture or 4 ml of medium will be added to the tubes depending on whether or not they receive activation. Each tube will be gassed with 5% CO_2 in air and placed on a Bellco roller drum apparatus at 25 rpm for a 4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0×10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and NA or S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with or without S-9 activation. Either four ml of S-9 activation mixture or 4 ml of F_0P will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of EMS and 7,12-DMBA will be used as positive controls for direct acting mutagens and promutagens, respectively. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily

cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.35%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and whether or not they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3×10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3×10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2×10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of

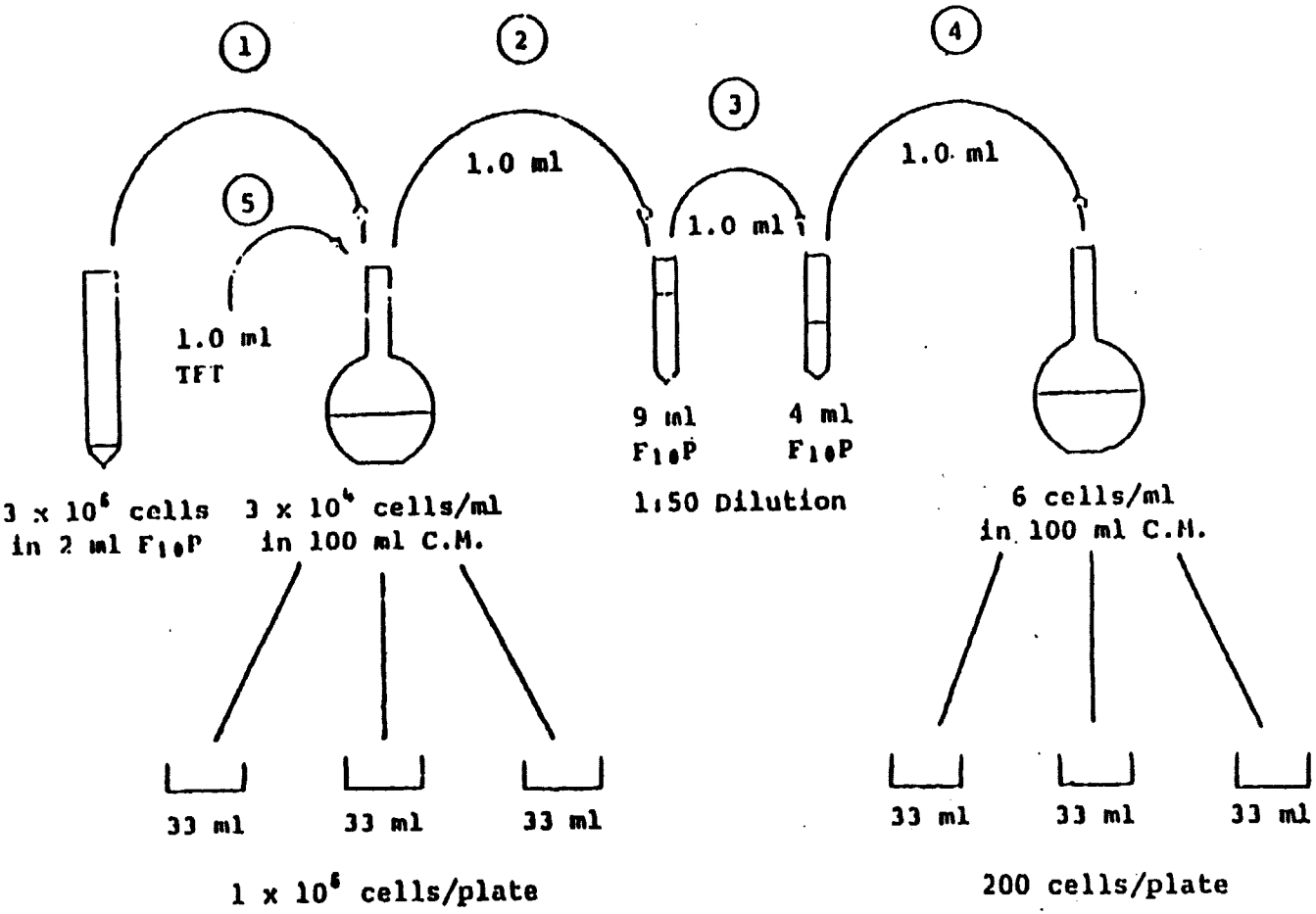

Figure 1

ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

MICROBIOLOGICAL ASSOCIATES

that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test article and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test article and the controls.

10.2.2.4 The mutation frequency of each dose level of the test article and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test article and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A Test Article sample will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency?_____
If so, to which agency or agencies? _____

Does the sponsor request that samples of the Test Article dosing solutions be returned?_____

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: 11/24/82

13.2 Proposed Initiation Date: December 20, 1982

13.3 Scheduled Completion Date: February 11, 1983

13.4 Report Submission to Sponsor Date: February 11, 1983

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

Carol Stark
SPONSOR'S AUTHORIZED REPRESENTATIVE

11/22/82
DATE PROTOCOL APPROVED BY SPONSOR

Paul E. Kirby — 11/24/82
STUDY DIRECTOR — DATE

APPENDIX I

Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

APPENDIX II

FORMULAS AND CALCULATIONS FOR THE L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts (Form No. WL-136) and Final Counts (Form No. WL-137).

$$\text{No. of Cells/ml} = \text{Average Counts (corrected for coincidence)} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^6 \text{ cells/ml})\ (20 \text{ ml})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = 20 \text{ ml} - \text{Volume of Cells Retained}$$

---

2. Table 1 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Initial Compound Toxicity Test (Form No. WL-232).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% of Control Suspension Growth} = \frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^6 cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

---

3. L5178Y TK+/- Assay - Daily Counts (Test Article) (Form No. 138) and L5178Y TK+/- Assay - Daily Counts (Positive Controls) (Form No. WL-139).

$$\text{No. of Cells/ml} \times 10^6 = \text{Average Counts (corrected for coincidence)} \times 20$$

3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^{6}\ \text{Cells/ml})\ (20\ \text{ml}^{*})}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20\ \text{ml}^{*}) - (\text{Volume of Cells Retained})$$

* The final volume is adjusted to 10 ml on the third day of expression.

---

4. Tables 2, 3, and 4 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Cloning Data (Form No. WL-41, WL-127 and WL-128).

$$\text{Mutant Frequency per } 10^{6}\text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left(\begin{array}{c}\text{Mutant Frequency}\\ \text{of Treated Cultures}\end{array}\right) - \left(\begin{array}{c}\text{Average Mutant Frequency}\\ \text{of Solvent Controls}\end{array}\right)$$

---

5. Tables 5, 6, and 7 - L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data (Form No. WL-227, WL-228 and WL-229).

$$\text{Total Suspension Growth} = \frac{\text{Day 1 Cell Concentration}}{0.3\times10^{6}\ \text{Cells/ml}} \times \frac{\text{Day 2 Cell Concentration}}{\text{Day 1 Adjusted Cell Concentration}} \times \frac{\text{Day 3 Cell Concentration}}{\text{Day 2 Adjusted Cell Concentration}}$$

$$\text{\% Control Suspension Growth} = \frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than 0.3×10^{6} cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

5. (Cont'd.)

$$\text{\% Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\text{\% Growth} = \frac{(\text{\% Suspension Growth})\ (\text{\% Cloning Growth})}{100}$$

Results

The Initial Toxicity Test (Table 1) conducted on test article Calcium Dialkyl Dithiophosphate (MA #T2253) in the presence of S-9, indicated complete toxicity at 0.1 µl/ml.* Based on these data, the test article was tested in a mutagenesis assay over a range of concentrations from 0.1 µl/ml to 0.0013 µl/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.018, 0.013, 0.010, 0.0075, 0.0056, 0.0042, 0.0032, 0.0024, 0.0018 or 0.0013 µl/ml. These concentrations produced a range in Suspension Growth of 36% to 96%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 2 and 3. These data are also presented graphically in Figure 1. The Cloning Data and Total Compound Toxicity Data for the positive control are presented in Table 4.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 43% to 119%.

A repeat assay was conducted in an attempt to generate cultures having between 10% and 40% Total Growth. The cultures were treated in triplicate in the presence of S-9 with a range of concentrations from 0.025 µl/ml to 0.012 µl/ml. After a two day expression period, twenty-one cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 0.025, 0.024, 0.023, 0.022, 0.021, 0.020 or 0.019 µl/ml. These concentrations produced a range in Suspension Growth of 19% to 79%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 5 and 6. These data are also presented graphically in Figure 2. The Cloning Data and Total Compound Toxicity Data for the positive control are presented in Table 7.

*The culture treated with 10 µl/ml appeared to have cell growth. Upon microscopic examination of the culture, there were few viable cells present and a large quantity of compound precipitate and cell debris.

CALCIUM DIALKYL
DITHIOPHOSPHATE

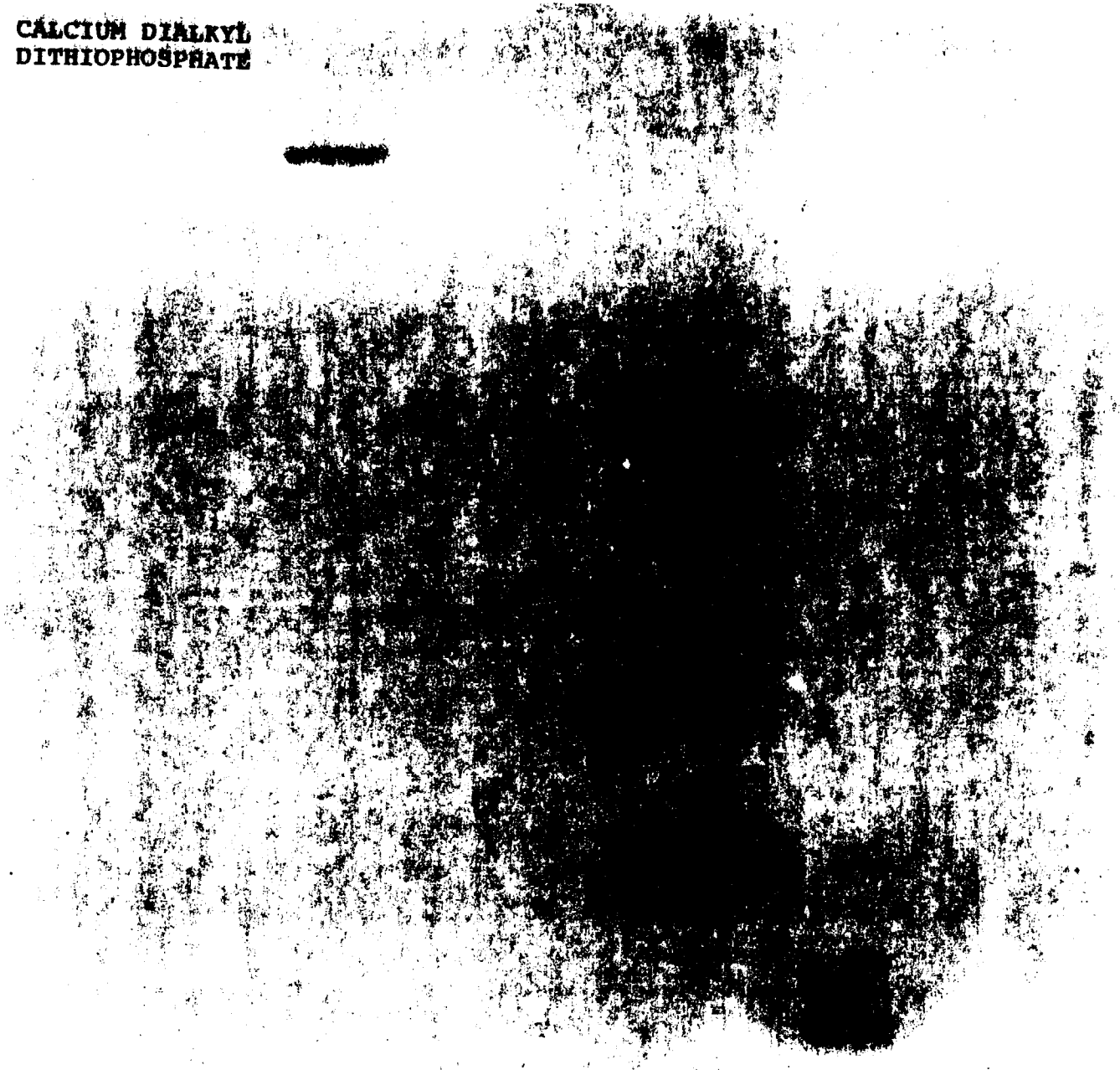

TABLE 1

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL COMPOUND TOXICITY TEST

Study Number : T2253.701012
Test Article : CALCIUM DIALKYL DITHIOPHOSPHATE
Activation : Induced Rat Liver S-9
Trial Number : 1
Dose Range : 10 - .001 MICROLITERS/ML
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 01/27/84 2:18 pm

Test Article Concentration (µl/ml)	Cell Concentration (X 10^4) Day 1	Day 2	Suspension Growth Total	% of Control
WITH S-9 ACTIVATION				
10	0.742	0.488	4.0	25
5	0.216	0.084	0.0	0
1	0.015	0.036	0.0	0
.5	0.013	0.030	0.0	0
.1	0.063	0.029	0.0	0
.05	0.171	0.664	2.2	14
.01	0.935	1.501	15.6	97
.005	0.939	1.382	14.4	89
.001	0.962	1.409	15.1	94
Solvent 1	0.969	1.494	16.1	
Solvent 2	0.979	1.482	16.1	

+ Culture Lost

Table Prepared By: Terrence R. O'Keefe 1/27/84
Signature Date

Workbook No. 15 Report Page No. XI-4

Summary

Two mutagenesis assays in the presence of S-9 were conducted on test article Calcium Dialkyl Dithiophosphate (MA #T2253). In the first assay, the cultures that were cloned were treated with a range of test article concentrations which produced from 43% to 119% Total Growth. None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. A repeat study was conducted to generate cultures having between 10% and 40% Total Growth. In the repeat study, conducted over a narrower dose range and with triplicate cultures per dose, the cultures that were cloned exhibited 17% to 74% Total Growth. None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that, under the conditions of these tests, test article Calcium Dialkyl Dithiophosphate produced a negative response in the presence of exogenous metabolic activation.

TABLE 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

Study Number : T2253.701012
Test Article : CALCIUM DIALKYL DITHIOPHOSPHATE
Activation : Induced Rat Liver S-9
Trial Number : 1
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 1:02 pm

Test Art Concentr (µl/ml)	Cell Concentr (X 10⁴) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
.032	0.027	0.059	0.0	0	++		
.024	0.079	0.148	0.0	0	++		
.018	0.383	1.241	5.3	36	156	120	43
.013	0.648	1.465	10.5	72	128	98	71
.01	0.807	1.375	12.3	84	154	118	99
.0075	0.796	1.510	13.4	92	133	102	94
.0056	0.849	1.379	13.0	89	134	103	92
.0042	0.840	1.399	13.1	90	150	115	104
.0032	0.886	1.424	14.0	96	162	124	119
.0024	0.876	1.372	13.4	92	144	110	101
.0018	0.815	1.473	13.3	91	140	107	97
.0013	0.836	1.495	13.9	95	127	97	92
Solvent 1	.868	1.464	14.1		132		
Solvent 2	.846	1.599	15.0		129		

+ Culture Lost ++ Too Toxic To Clone

Table Prepared By: Terrence R. O'Keefe 2/21/84
Signature Date

Workbook No. 45 Report Page No. XI-6

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 17% to 74%.

FIGURE 1
Study No. T2253.701012 (Trial 1)
Test Article CALCIUM DIALKYL DITHIOPHOSPHATE
With S-9 Activation

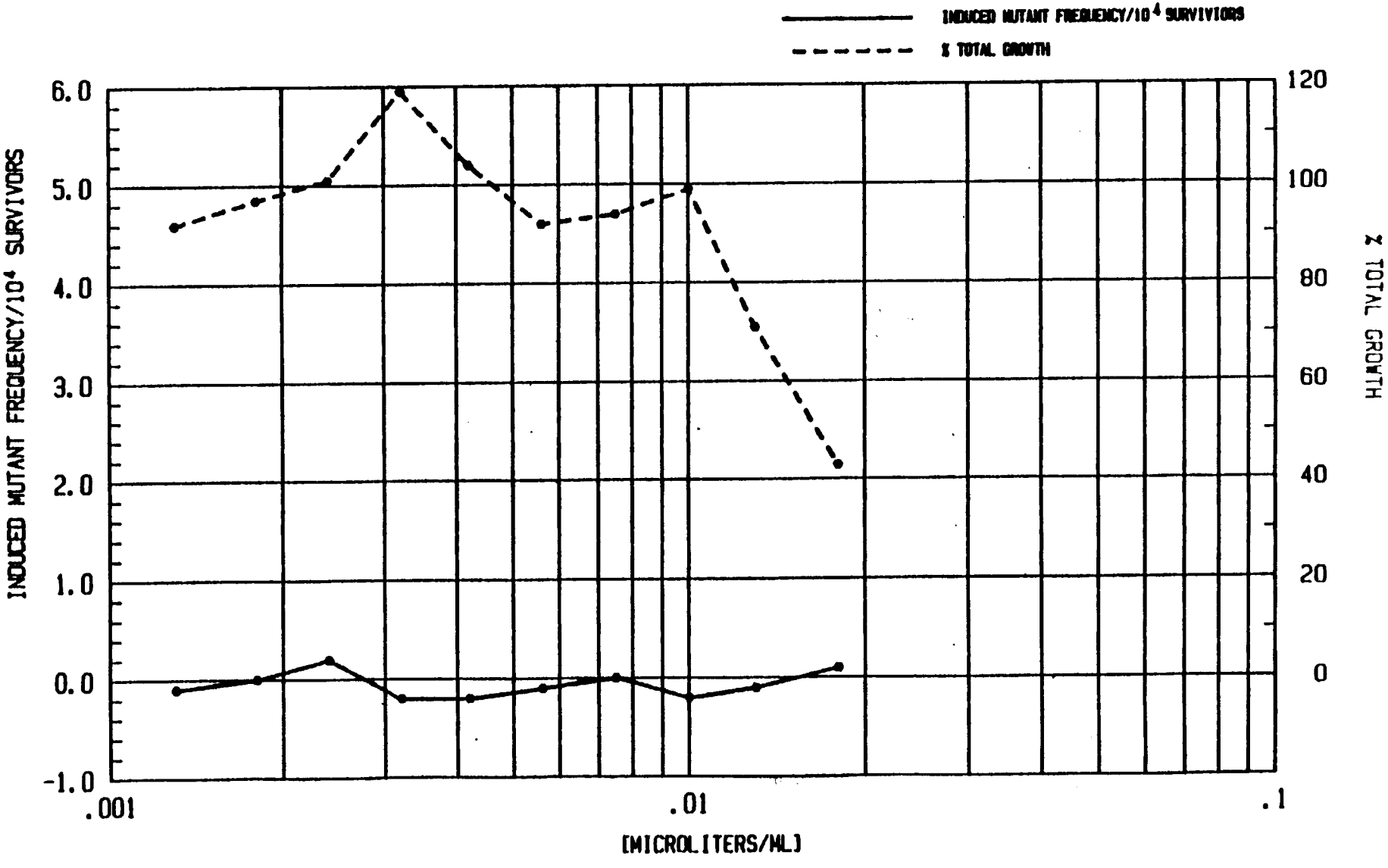

TABLE 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

Study Number : T2253.701012
Test Article : CALCIUM DIALKYL DITHIOPHOSPHATE
Activation : Induced Rat Liver S-9
Trial Number : 1
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 1:00 pm

Test Art Concentr (µl/ml)	Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
.018	85	± 1	156	±10	1.1	0.1	43
.013	58	± 1	128	± 1	0.9	-0.1	71
.01	62	± 2	154	±19	0.8	-0.2	99
.0075	64	± 2	133	± 6	1.0	0.0	94
.0056	57	± 2	134	± 1	0.9	-0.1	92
.0042	63	± 9	150	± 3	0.8	-0.2	104
.0032	64	± 6	162	± 9	0.8	-0.2	119
.0024	83	± 3	144	± 7	1.2	0.2	101
.0018	68	± 3	140	± 9	1.0	0.0	97
.0013	59	±11	127	±15	0.9	-0.1	92
Solvent 1	58	± 3	132	± 6	0.9		
Solvent 2	65	± 6	129	± 1	1.0		

+ Culture Lost * Per 10^4 surviving cells

Table Prepared By: Terrence R. O'Keefe 2/21/84
Signature Date

Workbook No. 44 Report Page No. XI-5

TABLE 6

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

Study Number : T2253.701012
Test Article : CALCIUM DIALKYL DITHIOPHOSPHATE
Activation : Induced Rat Liver S-9
Trial Number : 2
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 04/09/84 3:48 pm

Test Art Concentr (µl/ml)		Cell Concentr (X 10⁴) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
.025	A	0.447	1.334	6.6	43	158	93	40
.025	B	0.223	0.889	3.0	19	148	88	17
.025	C	0.406	1.235	5.6	36	158	93	33
.024	A	0.409	1.333	6.1	39	147	87	34
.024	B	0.324	1.130	4.1	26	141	83	22
.024	C	0.322	1.127	4.0	26	153	91	24
.023	A	0.629	1.301	9.1	59	181	107	63
.023	B	0.455	1.358	6.9	45	162	96	43
.023	C	0.461	1.305	6.7	43	142	84	36
.022	A	0.673	1.407	10.5	68	146	86	58
.022	B	0.335	1.385	5.2	34	131	78	27
.022	C	0.458	1.254	6.4	41	149	88	36
.021	A	0.592	1.296	8.5	55	165	98	54
.021	B	0.609	1.362	9.2	59	162	96	57
.021	C	0.583	1.386	9.0	58	156	92	53
.02	A	0.710	1.345	10.6	68	160	95	65
.02	B	0.763	1.291	10.9	70	166	98	69
.02	C	0.759	0.601	5.1	33	+		
.019	A	0.735	1.503	12.3	79	147	87	69
.019	B	0.796	1.090	9.6	62	202	120	74
.019	C	0.620	1.287	8.9	57	+		
Solvent 1		.985	1.442	15.8		+		
Solvent 2		1.011	1.321	14.8		169		
Solvent 3		1.051	1.373	16.0		+		

+ Culture Lost ++ Too Toxic To Clone

Table Prepared By: Terrence R. O'Keefe 4/9/8
Signature Date

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TABLE 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
POSITIVE CONTROL - WITH ACTIVATION

Study Number : T2253.701012
Test Article : 7,12 Dimethylbenz(a)anthracene
Activation : With activation
Trial Number : 1
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 1:04 pm

CLONING DATA

Test Art Concentr (µg/ml)	Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
7.5	282	± 9	61	± 4	9.2	8.3	7
5	265	± 9	89	±11	6.0	5.1	38
Solvent 1	73	± 4	147	±14	1.0		
Solvent 2	64	± 4	153	±10	0.8		

TOTAL COMPOUND TOXICITY DATA

Test Art Concentr (µg/ml)	Cell Concentr (X 10*) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
7.5	0.335	0.677	2.5	18	61	41	7
5	0.638	1.283	9.1	64	89	59	38
Solvent 1	.923	1.379	14.1		147		
Solvent 2	.916	1.399	14.2		153		

+ Culture Lost * Per 10^4 surviving cells

Table Prepared By: Terrence R. O'Keefe 2/21/8
Signature Date

Workbook No. 16 Report Page No. XI-7

FIGURE 2
Study No. T2253.701012 (Trial 2)
Test Article CALCIUM DIALKYL DITHIOPHOSPHATE
With S-9 Activation

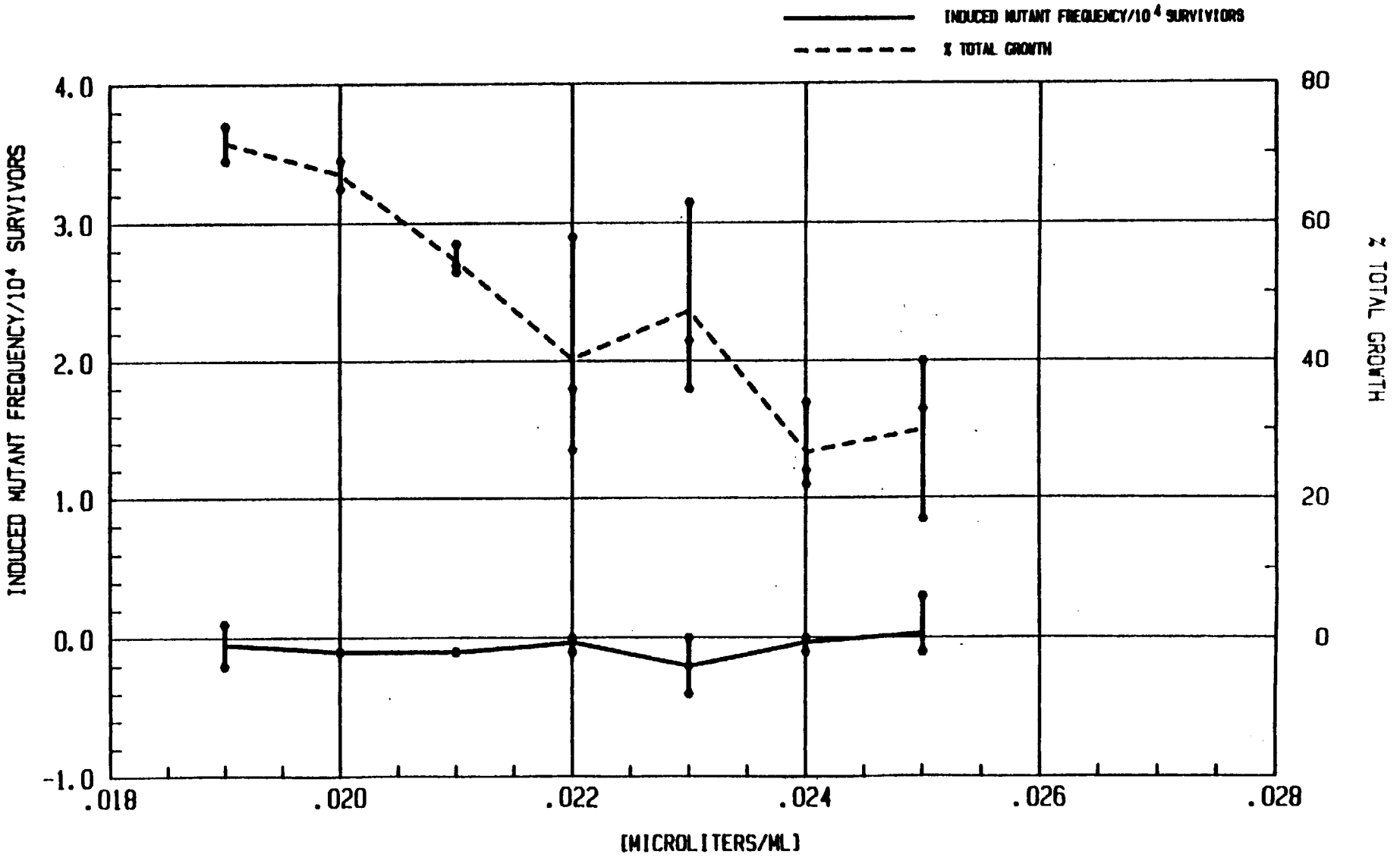

TABLE 5

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

CLONING DATA

Study Number : T2253.701012
Test Article : CALCIUM DIALKYL DITHIOPHOSPHATE
Activation : Induced Rat Liver S-9
Trial Number : 2
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 04/09/84 3:45 pm

Test Art Concentr (µl/ml)		Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
.025	A	60	± 3	158	±11	0.8	-0.1	40
.025	B	87	± 4	148	±13	1.2	0.3	17
.025	C	61	± 9	158	±20	0.8	-0.1	33
.024	A	61	± 2	147	±10	0.8	-0.1	34
.024	B	63	± 5	141	± 8	0.9	0.0	22
.024	C	70	± 7	153	±19	0.9	0.0	24
.023	A	65	± 5	181	± 7	0.7	-0.2	63
.023	B	43	± 4	162	±10	0.5	-0.4	43
.023	C	64	± 8	142	±15	0.9	0.0	36
.022	A	58	± 1	146	±19	0.8	-0.1	58
.022	B	57	± 2	131	± 8	0.9	0.0	27
.022	C	67	± 8	149	±18	0.9	0.0	36
.021	A	64	± 5	165	± 6	0.8	-0.1	54
.021	B	64	± 1	162	±10	0.8	-0.1	57
.021	C	64	± 8	156	±13	0.8	-0.1	53
.02	A	67	± 4	160	± 9	0.8	-0.1	65
.02	B	67	± 6	166	± 4	0.8	-0.1	69
.02	C	+		+				
.019	A	71	± 4	147	± 9	1.0	0.1	69
.019	B	75	±10	202	±19	0.7	-0.2	74
.019	C	+		+				
Solvent 1		+		+				
Solvent 2		73	± 9	169	± 4	0.9		
Solvent 3		+		+				

+ Culture Lost * Per 10^4 surviving cells

Table Prepared By: Terrence R O'Keefe 4/9/84
Signature Date

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Study No. T2254 701012
Study No. T2253 701012

Received by RA/QA 1/13/84
N.K./jtc

L5178Y TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY (with S-9 activation)

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test articles using the L5178Y TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: Zinc Dialkyl Dithiophosphate (CMA 102B) and Calcium Dialkyl Dithiophosphate

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test articles (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M Street, N.W.
Washington, D.C. 20037

3.3 Authorized Representative: Dr. Carol Stack

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.

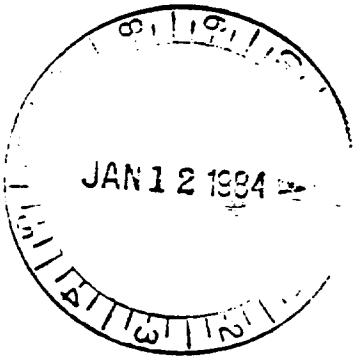

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TABLE 7

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
POSITIVE CONTROL - WITH ACTIVATION

Study Number : T2253.701012
Test Article : 7,12 Dimethylbenz(a)anthracene
Activation : With activation
Trial Number : 2
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 04/09/84 3:50 pm

CLONING DATA

Test Art Concentr (µg/ml)	Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
7.5	262	±11	61	± 8	8.6	7.8	12
5	287	± 8	108	± 9	5.3	4.5	50
Solvent 1	61	±11	152	± 5	0.8		
Solvent 2	53	± 1	150	± 8	0.7		

TOTAL COMPOUND TOXICITY DATA

Test Art Concentr (µg/ml)	Cell Concentr (X 10^6) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
7.5	0.475	0.806	4.3	29	61	40	12
5	0.740	1.241	10.2	69	108	72	50
Solvent 1	1.012	1.412	15.9		152		
Solvent 2	1.003	1.203	13.4		150		

+ Culture Lost * Per 10^4 surviving cells

Table Prepared By: Terrence R. O'Keefe 4/9/84
Signature Date

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6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, in the presence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

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Conclusion

Two mutagenesis assays were conducted on test article Calcium Dialkyl Dithiophosphate (MA #T2253). None of the cultures that were cloned in the first assay exhibited mutant frequencies which were more than twice the mean mutant frequency of the solvent controls. A repeat assay was conducted in an attempt to produce cultures having between 10% and 40% Total Growth. Cultures were treated in triplicate and none of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that, under the conditions of these tests, test article Calcium Dialkyl Dithiophosphate produced a negative response in the presence of exogenous metabolic activation.

4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0 x 10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

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5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted with metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity with metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth.

Total charge for assay with metabolic activation:
$2,950 per test article

6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, or additional information concerning the test article indicates alternative doses would be better, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and that they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3×10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3×10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2×10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of

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6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_2O, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with S-9 activation will be conducted. Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1 x 10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture will be added to the tubes. Each tube will be gassed with 5% CO_2 in air and placed on a roller drum apparatus at 25 rpm for a

colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

7.2.2 Test Article Preparation

The test article will be added to each tube labeled with the test article number, test concentration and S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with S-9 activation. Four ml of S-9 activation mixture will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of 7,12-DMBA will be used as a positive control. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time

After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.2.4 Cloning

At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.34%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? YES
If so, to which agency or agencies? OSHA, EPA, NIOSH, NCI, NIEHS, CPSC, FDA
Does the Sponsor request that samples of the Test Article dosing solutions be returned? NO

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: December 12, 1983

13.2 Proposed Initiation Date: January 23, 1984 3PK 5/24/84

13.3 Scheduled Completion Date: February 24, 1984

13.4 Report Submission to Sponsor Date: March 2, 1984

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

Carol D. Stamp
SPONSOR'S AUTHORIZED REPRESENTATIVE

12/7/83
DATE PROTOCOL APPROVED BY SPONSOR

Paul E. Kirby 11/28/83
STUDY DIRECTOR DATE

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10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test articles and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test articles and the controls.

10.2.2.4 The mutation frequency of each dose level of the test articles and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test articles and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
Test Article samples will be held in storage in accordance with the Terms and Conditions.

FORMULAS AND CALCULATIONS FOR THE L5178Y $TK^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts and Final Counts

$$\text{No. of Cells/ml} = \frac{\text{Average Counts (corrected for coincidence)}}{} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^6 \text{ cells/ml})\ (20 \text{ ml})}{\text{(No. of Cells/ml)}}$$

$$\text{Volume of Media to Add} = 20 \text{ ml} - \text{Volume of Cells Retained}$$

---

2. Table 1 - L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay
Initial Compound Toxicity Test

Total Suspension Growth =

$$\frac{\text{Day 1 Cell Conc.}}{0.3\times10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Conc.}}{\text{Day 1 Adjusted Cell Conc.}}$$

% of Control Suspension Growth =

$$\frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^6 cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

---

3. L5178Y $TK^{+/-}$ Assay - Daily Counts (Test Article) and L5178Y $TK^{+/-}$ Assay - Daily Counts (Positive Controls)

$$\text{No. of Cells/ml} \times 10^6 = \text{Average Counts (corrected for coincidence)} \times 20$$

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Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

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5. (Cont'd.)

$$\% \text{ Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\% \text{ Growth} = \frac{(\% \text{ Suspension Growth})\ (\text{Cloning Growth})}{100}$$

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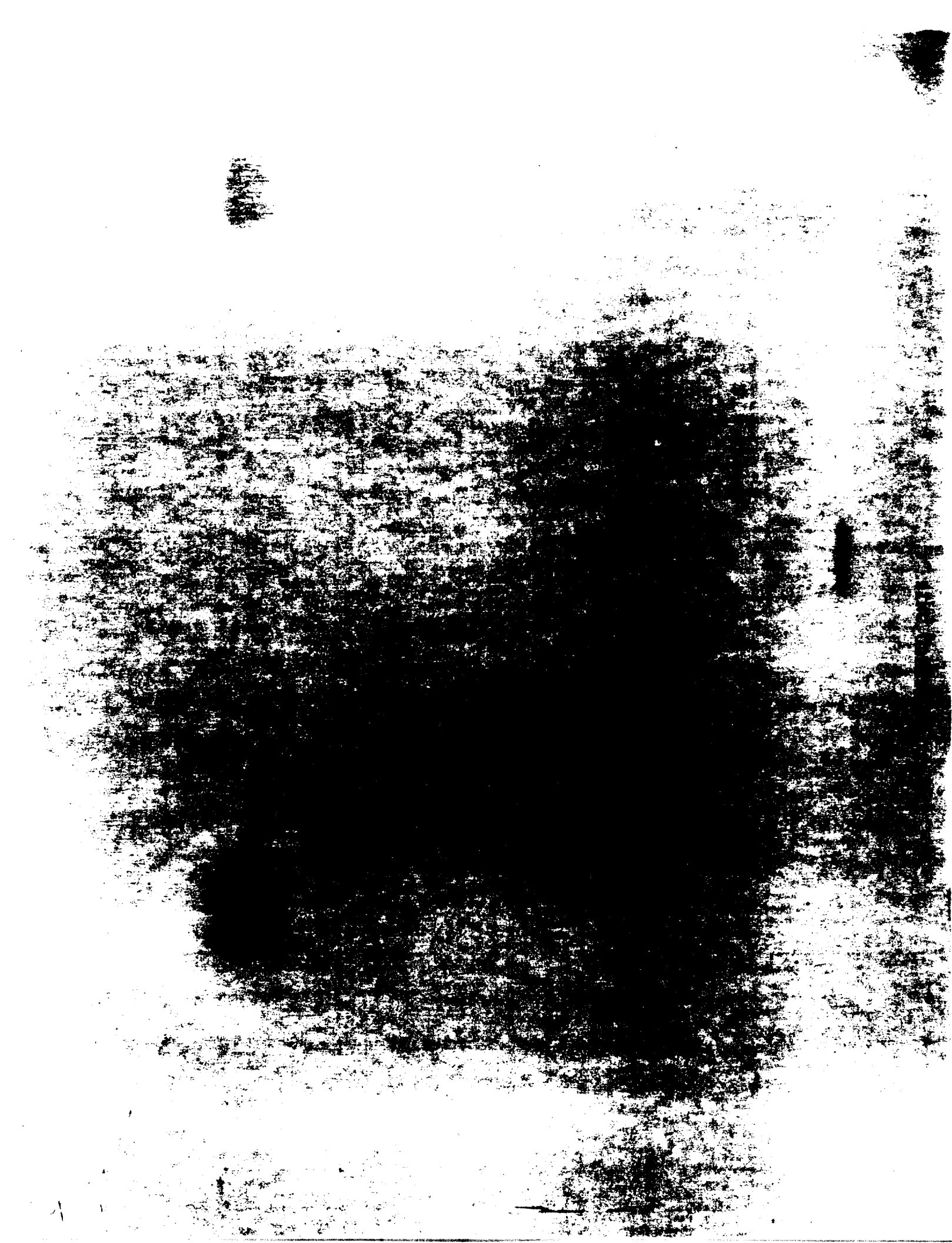
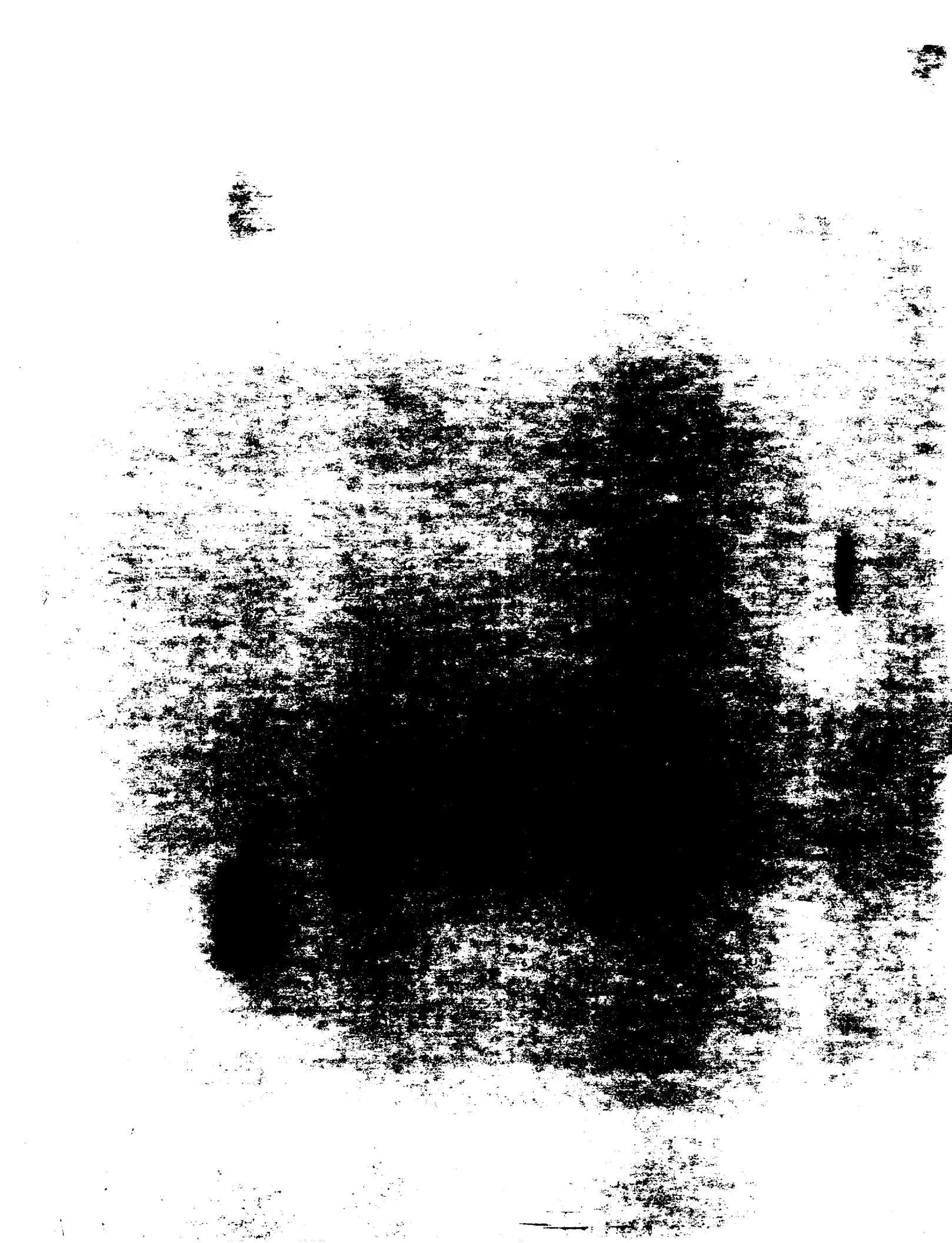




Summary

Chemical Manufacturers Association's test article Zinc Oleate, Lot No. 34495-10 (MA #T2256) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence of Aroclor induced rat liver S-9. Due to solubility problems with the test article, the cultures that were cloned exhibited a range in Total Growth of 74% to 107%. None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that, under the conditions of this test, test article Zinc Oleate produced a negative response in the presence of exogenous metabolic activation.

Results

The Initial Toxicity Test (Table 1) conducted on test article Zinc Oleate, Lot No. 34495-10 (MA #T2256) in the presence of S-9, indicated complete toxicity at 100 µg/ml. Based on these data, the test article was tested in the mutagenesis assay over a range of concentrations from 100 µg/ml to 1.3 µg/ml.

After a two day expression period, the first ten cultures were cloned. Due to solubility problems with the test article, the toxic response demonstrated in the Initial Toxicity Test was not reproduced. The cultures that were cloned were treated with 100, 75, 56, 42, 32, 24, 18, 13, 10 or 7.5 µg/ml test article. These concentrations produced a range in Suspension Growth of 85% to 109%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 2 and 3. These data are also presented graphically in Figure 1. The data for the positive controls are presented in Table 4.

None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of the cultures ranged from 74% to 107%.

TABLE 1

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL COMPOUND TOXICITY TEST

Study Number : T2256.701012
Test Article : ZINC OLEATE
Activation : Induced Rat Liver S-9
Trial Number : 1
Dose Range : 1000 UG/ML TO 0.05 UG/ML
Solvent : Acetone
Operator : Terrence R. O'Keefe
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 01/27/84 3:40 pm

Test Article Concentration (µg/ml)	Cell Concentration (X 10⁴) Day 1	Day 2	Suspension Growth Total	% of Control
WITH S-9 ACTIVATION				
1000	0.069	0.017	0.0	0
500	0.038	0.042	0.0	0
100	0.087	0.200	0.0	0
50	0.676	1.624	12.2	69
10	0.877	1.659	16.2	92
5	0.962	1.559	16.7	95
1	0.969	1.548	16.7	95
.5	1.006	1.615	18.1	103
.1	0.875	1.522	14.8	84
.05	1.040	1.618	18.7	106
Solvent 1	0.987	1.585	17.4	
Solvent 2	1.049	1.531	17.8	

+ Culture Lost

Table Prepared By: Terrence R. O'Keefe 1/27/84
Signature Date

Workbook No. 15 Report Page No. XII-3

Table 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

Study Number : T2256.701012
Test Article : ZINC OLEATE
Activation : Induced Rat Liver S-9
Trial Number : 1
Solvent : Acetone
Operator : Janine L. Johnson
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 5:28 pm

Test Art Concentr (µg/ml)	Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
100	75	± 3	141	± 1	1.1	0.3	74
75	70	± 4	134	± 3	1.0	0.2	79
56	65	± 3	155	±10	0.8	0.0	91
42	68	± 6	153	±15	0.9	0.1	85
32	69	± 3	148	±11	0.9	0.1	88
24	70	± 2	136	± 1	1.0	0.2	75
18	67	±13	161	±15	0.8	0.0	93
13	72	± 3	149	± 9	1.0	0.2	84
10	54	± 3	132	±11	0.8	0.0	78
7.5	53	± 6	158	±15	0.7	-0.1	107
Solvent 1	69	± 6	177	± 3	0.8		
Solvent 2	61	± 2	147	± 1	0.8		

+ Culture Lost * Per 10^4 surviving cells

Table Prepared By: Janine L. Johnson 2-21-84
Signature Date

TABLE 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

Study Number : T2256.701012
Test Article : ZINC OLEATE
Activation : Induced Rat Liver S-9
Trial Number : 1
Solvent : Acetone
Operator : Janine L. Johnson
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 5:39 pm

Test Art Concentr (µg/ml)	Cell Concentr (X 10⁴) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
100	0.798	1.486	13.2	85	141	87	74
75	0.875	1.515	14.7	95	134	83	79
50	0.917	1.455	14.8	95	155	96	91
40	0.870	1.433	13.9	90	153	94	85
32	0.924	1.468	15.1	97	148	91	88
24	0.926	1.337	13.8	89	136	84	75
18	0.931	1.399	14.5	94	161	99	93
17	1.079	1.222	14.1	91	149	92	84
16	0.959	1.396	14.9	96	132	81	78
7.5	0.996	1.526	16.9	109	158	98	107
Solvent 1	.986	1.444	15.8		177		
Solvent 2	.994	1.376	15.2		147		

* Culture Lost ** Too Toxic To Clone

Table Prepared By: Janine L. Johnson 2-21-84
Signature Date

Workbook No. 45 Report Page No. XII-5

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
POSITIVE CONTROL - WITH ACTIVATION

Study Number : T2256.701012
Test Article : 7,12 Dimethylbenz(a)anthracene
Activation : With activation
Trial Number : 1
Solvent : Acetone
Operator : Janine L. Johnson
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 5:32 pm

CLONING DATA

Test Art Concentr (µg/ml)	Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
7.5	272	± 7	62	± 9	8.8	7.9	8
5	242	±10	111	± 2	4.4	3.5	52
Solvent 1	67	± 4	173	±11	0.8		
Solvent 2	77	± 4	162	±11	1.0		

TOTAL COMPOUND TOXICITY DATA

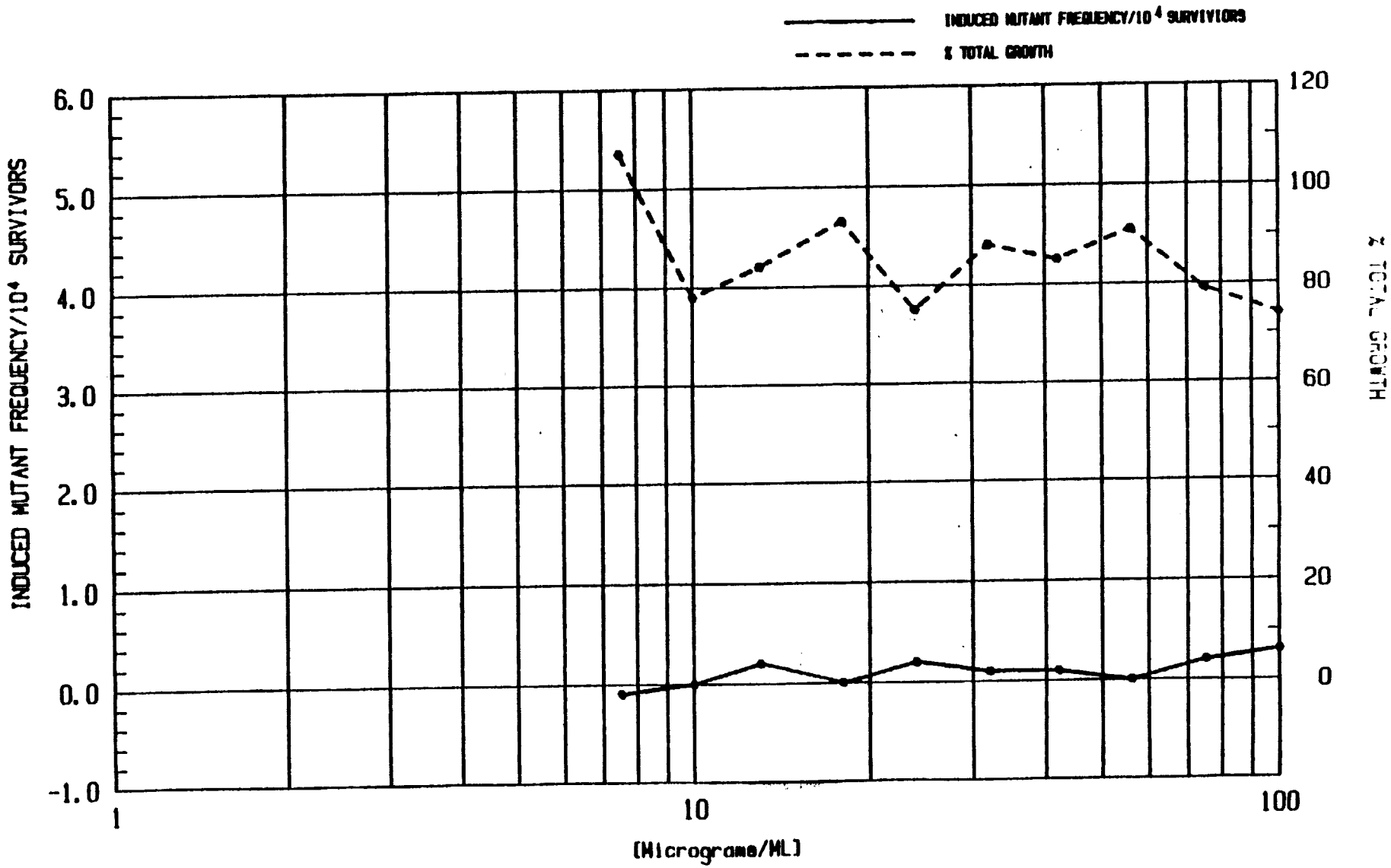
Test Art Concentr (µg/ml)	Cell Concentr (X 10*) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
7.5	0.384	0.721	3.1	22	62	37	8
5	0.753	1.301	10.9	79	111	66	52
Solvent 1	.924	1.295	13.3		173		
Solvent 2	.974	1.313	14.2		162		

* Culture Lost ‡ Per 10^4 surviving cells

Table Prepared By: Janine L. Johnson 2-21-84
Signature Date

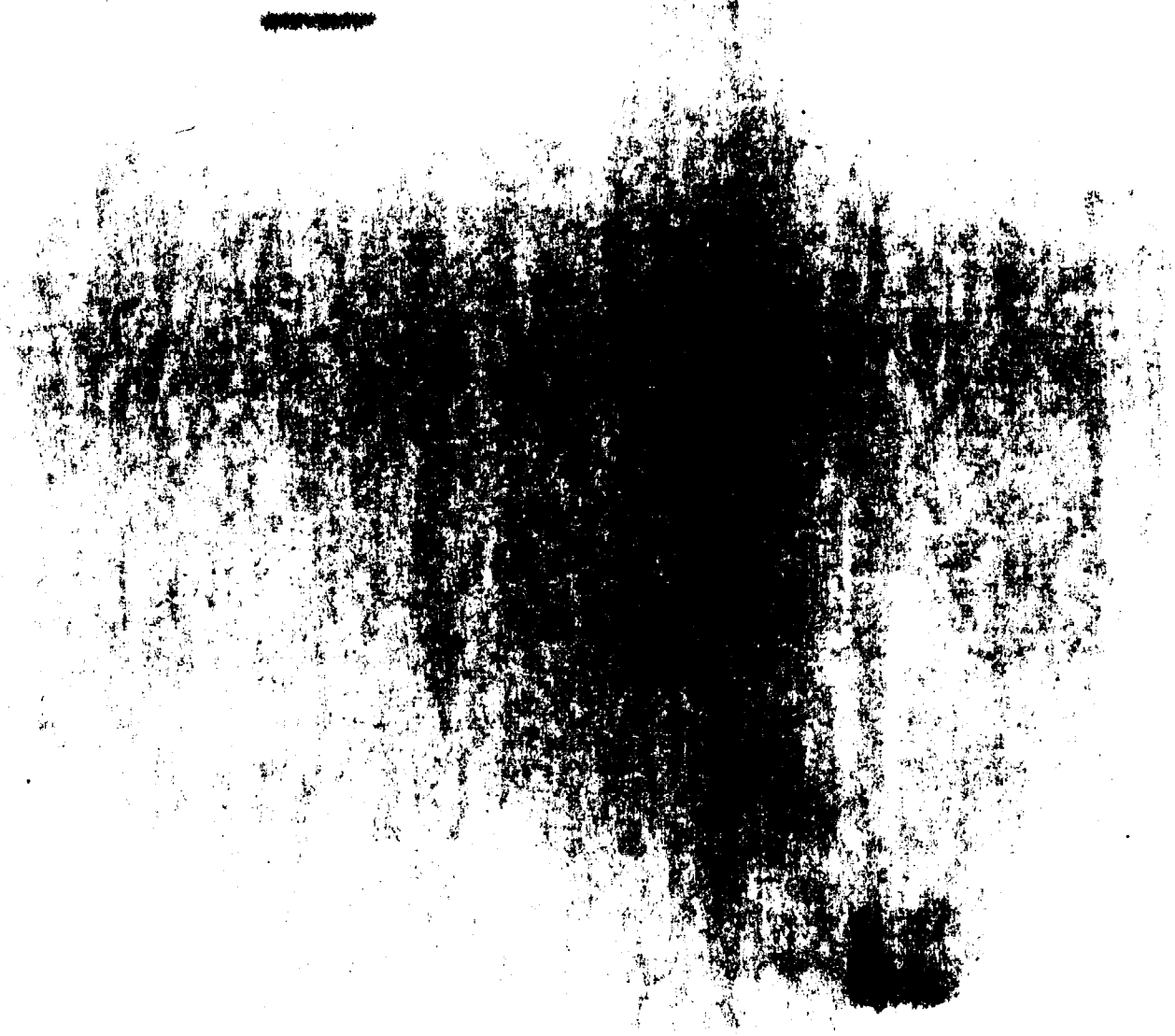
Workbook No. 46 Report Page No. XII-6

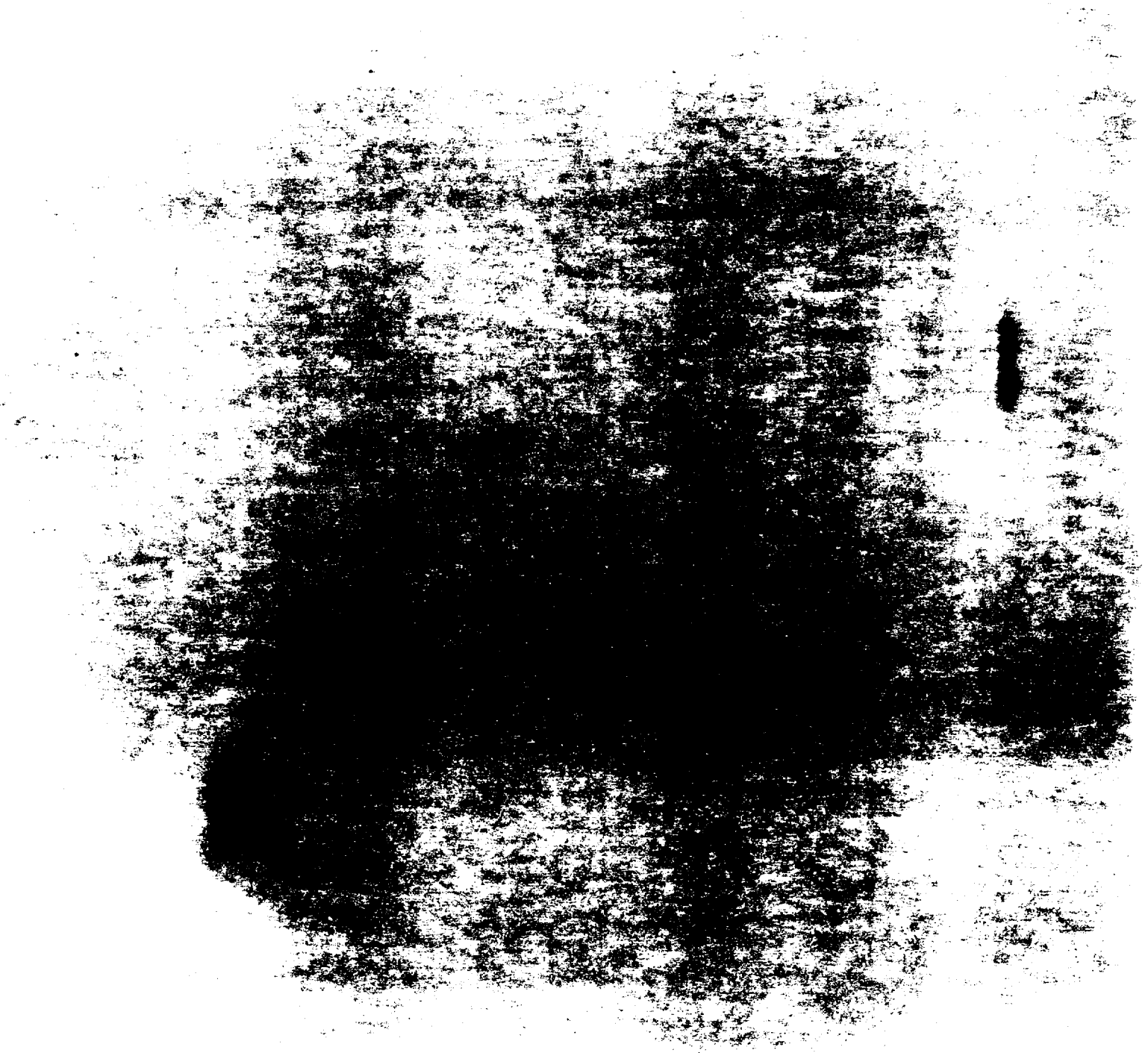
FIGURE 1
Study No. T2258.701012 (Trial 1)
Test Article ZINC OLEATE
With S-9 Activation



Conclusion

Chemical Manufacturers Association's test article Zinc Oleate, Lot No. 34495-10 (MA #T2256) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence of Aroclor induced rat liver S-9. Solubility difficulties were experienced with the test article and a Total Growth range between 10% and 50% was not achieved in the assay. None of the cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that, under the conditions of this test, test article Zinc Oleate produced a negative response in the presence of exogenous metabolic activation.





Summary

Chemical Manufacturers Association's test article Zinc Chloride, Lot No. KTJY (MA #T2255) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence of Aroclor induced rat liver S-9. The cultures that were cloned were treated with a range of test article concentrations which produced from 2% to 77% Total Growth. Seven of ten cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls.

The results indicate that, under the conditions of this test, test article Zinc Chloride produced a positive response in the presence of exogenous metabolic activation.

Results

The Initial Toxicity Test (Table 1) conducted on test article Zinc Chloride, Lot No. KTJY (MA #T2255) indicated complete toxicity at 50 µg/ml. The cultures were treated in the presence of S-9. Based on these data, the test article was tested in the mutagenesis assay over a range of concentrations from 75 µg/ml to 1.0 µg/ml.

After a two day expression period, ten cultures were cloned based on their degree of toxicity. The cultures that were cloned were treated with 56, 42, 32, 24, 18, 13, 10, 7.5, 5.6 or 4.2 µg/ml. These concentrations produced a range in Suspension Growth of 10% to 95%. The Cloning Data and Total Compound Toxicity Data for the test article are presented in Tables 2 and 3. These data are also presented graphically in Figure 1. The Cloning Data and Total Compound Toxicity Data for the positive control are presented in Table 4.

Seven cultures that were cloned exhibited mutant frequencies which ranged from 19.2 to 2.0 times the mean mutant frequency of the solvent controls. The Total Growth of these cultures ranged from 2% to 61%. The three remaining cultures that were cloned (7.5, 5.6 and 4.2 µg/ml) exhibited mutant frequencies which were not significantly greater than the mean mutant frequency of the solvent controls. The Total Growth of these cultures was 62%, 77% and 57%, respectively.

TABLE 1

L5178Y TK +/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
INITIAL COMPOUND TOXICITY TEST

Study Number : T2255.701012
Test Article : ZINC CHLORIDE
Activation : Induced Rat Liver S-9
Trial Number : 1
Dose Range : 5000 UG/ML TO 0.05 UG/ML
Solvent : Deionized,Distilled Water
Operator : Geri Breidenthal
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 05/25/84 9:45 am

Test Article Concentration (µg/ml)	Cell Concentration (X 10⁶) Day 1	Day 2	Suspension Growth Total	% of Control
WITH S-9 ACTIVATION				
5000	0.253	0.248	0.0	0
1000	0.142	0.073	0.0	0
500	0.212	0.189	0.0	0
100	0.028	0.043	0.0	0
50	0.205	0.194	0.0	0
10	0.789	1.991	17.5	79
5	0.863	1.732	16.6	75
1	0.972	1.862	20.1	91
.5	1.061	1.865	22.0	99
.1	1.132	1.605	20.2	91
.05	1.119	1.765	21.9	99
Solvent 1	1.118	1.751	21.9	
Solvent 2	1.129	1.798	22.6	

+ Culture Lost

Table Prepared By: Geri Breidenthal 5/25/84
Signature Date

Workbook No. 15C Report Page No. XIII-3

TABLE 2

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
CLONING DATA

Study Number : T2255.701012
Test Article : ZINC CHLORIDE
Activation : Induced Rat Liver S-9
Trial Number : 1
Solvent : Deionized, Distilled Water
Operator : Janine L. Johnson
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 5:38 pm

Test Art Concentr (µg/ml)	Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
56	231	± 4	48	±10	9.6	9.1	2
42	299	±10	64	± 4	9.3	8.8	7
32	287	± 7	76	± 6	7.6	7.1	16
24	160	± 2	109	± 5	2.9	2.4	37
18	88	± 8	127	± 2	1.4	0.9	44
13	83	± 9	160	± 5	1.0	0.5	61
10	85	± 3	151	±12	1.1	0.6	59
7.5	69	± 4	168	±18	0.8	0.3	62
5.6	69	± 3	177	±27	0.8	0.3	77
4.2	62	± 6	148	± 1	0.8	0.3	57
Solvent 1	58	± 8	282	±27	0.4		
Solvent 2	49	± 5	156	±13	0.6		

+ Culture Lost * Per 10^4 surviving cells

Table Prepared By: Janine L. Johnson 2-21-84
Signature Date

Workbook No. 44 Report Page No. XIII-4

TABLE 3

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

TOTAL COMPOUND TOXICITY DATA

Study Number : T2255.701012
Test Article : ZINC CHLORIDE
Activation : Induced Rat Liver S-9
Trial Number : 1
Solvent : Deionized, Distilled Water
Operator : Janine L. Johnson
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 5:40 pm

Test Art Concentr (µg/ml)	Cell Concentr (X 10⁴) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
75	0.223	0.120	0.0	0	++		
56	0.266	0.457	1.5	10	48	22	2
42	0.390	0.899	3.9	25	64	29	7
32	0.526	1.192	7.0	46	76	35	16
24	0.706	1.410	11.1	73	109	50	37
18	0.732	1.419	11.5	75	127	58	44
13	0.849	1.365	12.9	84	160	73	61
10	0.862	1.367	13.1	86	151	69	59
7.5	0.790	1.389	12.2	80	168	77	62
5.6	0.898	1.449	14.5	95	177	81	77
4.2	0.850	1.368	12.9	84	148	68	57
Solvent 1	.978	1.393	15.1		282		
Solvent 2	1.006	1.381	15.4		156		

+ Culture Lost ++ Too Toxic To Clone

Table Prepared By: Janine L. Johnson 2-21-84
Signature Date

Workbook No. 45 Report Page No. XIII-5

Table 4

L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY
POSITIVE CONTROL - WITH ACTIVATION

Study Number : T2255.701012
Test Article : 7,12 Dimethylbenz(a)anthracene
Activation : With activation
Trial Number : 1
Solvent : Acetone
Operator : Janine L. Johnson
Study Director : Paul E. Kirby, Ph.D.
Date & Time : 02/21/84 5:42 pm

CLONING DATA

Test Art Concentr (µg/ml)	Ave #/ TFT Plate	TFT Stand Dev	Ave #/ V.C. Plate	V.C. Stand Dev	Mutant* Frequency	Induced* Mutant Frequency	% Total Growth
7.5	268	±12	58	± 4	9.2	8.4	8
5	259	±11	132	± 8	3.9	3.1	55
Solvent 1	63	± 5	168	± 4	0.8		
Solvent 2	68	± 5	163	± 8	0.8		

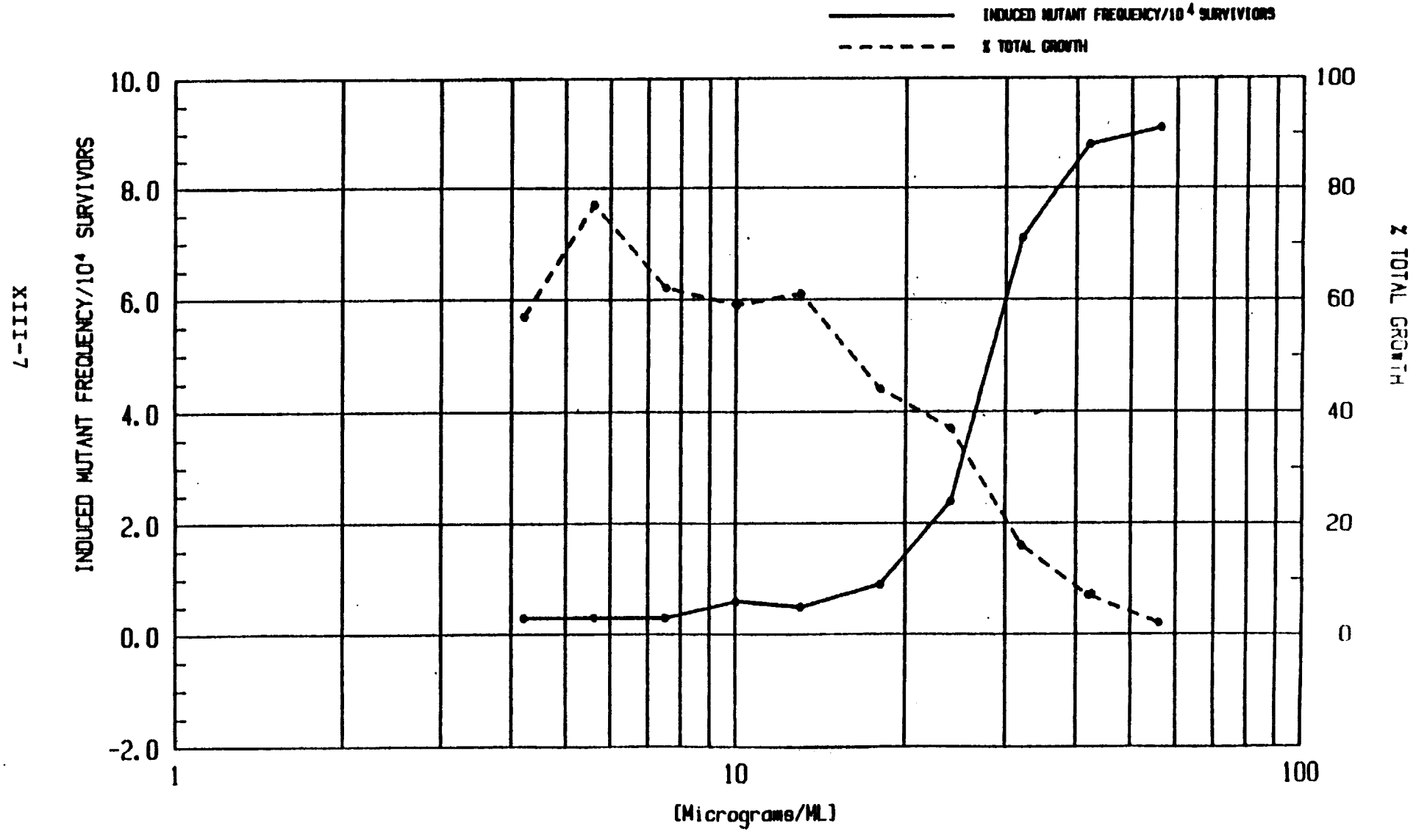
TOTAL COMPOUND TOXICITY DATA

Test Art Concentr (µg/ml)	Cell Concentr (X 10⁴) Day 1	Day 2	Susp Growth Total	%Cntl	Cloning Growth Ave VC	%Cntl	% Total Growth
7.5	0.427	0.671	3.2	22	58	35	8
5	0.730	1.263	10.2	69	132	80	55
Solvent 1	.945	1.415	14.9		168		
Solvent 2	.943	1.374	14.4		163		

+ Culture Lost * Per 10^4 surviving cells

Table Prepared By: Janine L. Johnson 2-21-84
Signature Date

FIGURE 1
Study No. T2255.701012 (Trial 1)
Test Article ZINC CHLORIDE
With S-9 Activation



Conclusion

Chemical Manufacturers Association's test article Zinc Chloride, Lot No. KTJY (MA #T2255) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence of Aroclor induced rat liver S-9. Seven of ten cultures that were cloned exhibited mutant frequencies which were significantly greater than the mean mutant frequency of the solvent controls. The results indicate that, under the conditions of this test, test article Zinc Chloride produced a positive response in the presence of exogenous metabolic activation.

Study No. T2356 701012
T2355 701012

Received by RA/QA 1/13/94
N.K./jbc

L5178Y $TK^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY (with S-9 activation)

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test articles using the L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

2.0 TEST ARTICLE

2.1 Identification: Zinc Chloride and Zinc Oleate

2.2 Analysis:
The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test articles (see attached Test Article Characterization form) and the stability and strength of the dosing solutions.

3.0 SPONSOR

3.1 Name: Chemical Manufacturers Association

3.2 Address: 2501 M Street, N.W.
Washington, D.C. 20037

3.3 Authorized Representative: Dr. Carol Stack

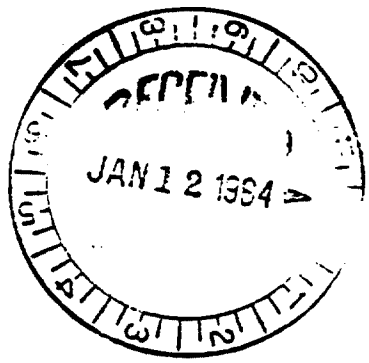
4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Paul E. Kirby, Ph.D.



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5.0 TEST SYSTEM

L5178Y $TK^{+/-}$ mouse lymphoma cells are used because they are sensitive indicators of mutagenic activity of a broad range of chemical classes.

5.1 Source

The L5178Y $TK^{+/-}$ mouse lymphoma cells Clone 3.7.2C used in the mutagenesis assay were originally obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. The cells were cryopreserved and stock cultures are prepared from reconstituted cells.

6.0 EXPERIMENTAL DESIGN

Preliminary cytotoxicity experiments will be conducted with metabolic activation to determine optimal dose levels. Test material will then be tested for mutagenic activity with metabolic activation over a concentration range yielding no more than 90% and no less than 10% total suspension growth; cloning data must be obtained for a minimum of three dose levels within the above range of growth inhibition, with at least one dose yielding less than or equal to 30% total suspension growth.

Total charge for assay with metabolic activation:
$2,950 per test article

6.1 Dosages

For the initial toxicity test, unless solubility limits are exceeded, or additional information concerning the test article indicates alternative doses would be better, solid test articles will be tested at 5,000, 1,000, 500, 100, 50, 10, 5.0, 1.0, 0.5, 0.1 and 0.05 ug/ml, and liquid test articles will be tested at 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 ul/ml. Test article toxicity will be determined by comparing the cell population growth at each dose level with that of the solvent controls. Based on the data derived from the toxicity test, the test article will be prepared for the mutagenesis assay so that the highest concentration is 100% toxic and the lowest concentration is nontoxic. The test article will be solubilized and, depending on the breadth of the dose range over which 0% to 100% toxicity is observed, either 16 doses will be selected for treatment of 16 individual cultures or 8 doses will be selected for treating 8 sets of duplicate cultures.

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6.2 Route and Frequency of Administration
Cell cultures will be treated once by way of a vehicle compatible with the system, in the presence of metabolic activation. This route of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Species, Strain, Sex and Inducer
Liver homogenate will be prepared from male Sprague-Dawley rats that have been injected with a 2:1 mixture of Aroclor 1242 and Aroclor 1254. The Aroclors will be diluted in corn oil to a concentration of 200 mg/ml. Each animal will be inoculated intraperitoneally with 500 mg/kg. Five days post injection the rats will be decapitated and the livers excised.

6.3.1.2 Homogenate Preparation
Preparation of the microsomal enzyme fraction will be carried out using sterile glassware and solutions maintained in an ice bath. Excised livers will be placed in pre-weighed beakers containing a 0.25 M sucrose solution. After determining the weight of the livers in each beaker they will be rinsed three times in 0.25 M sucrose and then resuspended in 3 ml of 0.25 M sucrose per gram of liver. The livers will be minced with sterile scissors, homogenized, and centrifuged at 9000 x g for 10 minutes. The supernatant (S-9) will be collected and stored at or below -70°C.

6.3.1.3 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by de Serres (de Serres, et. al., Science 203:563-565, 1979).

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6.3.2 S-9 Mix
The S-9 mix will be prepared immediately before its use in each assay.

The following amounts of homogenate and cofactors will be combined per ml of S-9 mix prepared.

Isocitric acid	11.25 mg
NADP	6.0 mg
F_0P	0.75 ml
S-9 homogenate	0.25 ml

The isocitric acid and NADP will be dissolved in cold F_0P and this solution will be adjusted to a pH of approximately 7.0. Prior to adding the S-9 homogenate, the cofactor mix will be sterilized by passage through a 0.45 um filter.

6.4 Controls

6.4.1 Negative Control
The solvent vehicle for the test article will be used as the negative control. Solvents compatible with this test system in order of preference include, but are not limited to, deionized distilled H_2O, ethanol, acetone, and DMSO.

6.4.2 Positive Controls
Two concentrations of ethylmethanesulfonate (EMS) will be used as a positive control for direct acting mutagens, and two concentrations of 7,12-dimethylbenz(a)anthracene (DMBA) will be used for promutagens.

7.0 METHODS

7.1 Determination of Toxicity
In order to determine the optimal dose levels at which to assay test articles, a preliminary toxicity test with S-9 activation will be conducted. Cultures will be initiated by seeding Corning polypropylene centrifuge tubes with 6 ml of a cell suspension from a common pool containing 1×10^6 cells/ml. The test article will be solubilized and diluted in an appropriate solvent and added to each appropriately labeled centrifuge tube in amounts at which the final solvent concentration is nontoxic to the cell suspension. Four ml of S-9 activation mixture will be added to the tubes. Each tube will be gassed with 5% CO_2 in air and placed on a roller drum apparatus at 25 rpm for a

4-hour exposure period. To avoid inactivation of light sensitive compounds, the test solutions will be prepared under amber lights and kept in darkness during the entire exposure period.

After 4 hours the test article will be removed by centrifuging the cells at 1000 x g for 10 minutes and decanting the supernatant. The cells will be washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml of $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus.

Cell population density will be determined 24 and 48 hours after the initial exposure to the test article by removing 1 ml samples from each centrifuge tube, making 1:10 dilutions in 0.1% trypsin, incubating at 37°C for 10 minutes to disperse the cells, and counting the samples with an electronic cell counter.

7.2 Testing for Mutagenic Activity

7.2.1 Cell Preparation

Prior to use in the assay, L5178Y $TK^{+/-}$ cells which are actively growing in culture will be cleansed as described by Clive (Clive, et al., Mutation Research 31:17-29, 1975) to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Three ml of THMG stock solution will be added to a 100 ml cell suspension containing 0.1 x 10^6 cells/ml. The culture will be gassed with 5% CO_2 in air and placed on an environmental incubator shaker at 125 rpm and 37°C. After 24 hours, the THMG will be removed by pelletizing the cells and decanting the supernatant. The cells will be rinsed in 20 ml of $F_{10}P$ and reinstated in culture at 3 x 10^4 cells per ml in 100 ml of $F_{10}P$ plus 1 ml of THG stock solution.

The cells will be ready for use after 72 hours incubation. Fresh cultures will be periodically started from the cryopreserved stock.

The cell population density of the prepared cultures will be determined by adding a 1 ml sample of cells to 9.0 ml of 0.1% trypsin, incubating at 37°C for 10 minutes, and making three counts per sample with an electronic cell counter. Based on the determination of the number of cells per ml, a cell suspension containing 1.0 x 10^6 cells per ml will be prepared, and 6 ml aliquots will be dispensed into centrifuge tubes.

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7.2.2 Test Article Preparation
The test article will be added to each tube labeled with the test article number, test concentration and S-9, in amounts at which the final solvent concentration is nontoxic to the cell suspension. The compound will be tested with S-9 activation. Four ml of S-9 activation mixture will be added to the tubes. This will yield a final cell suspension of 0.6×10^6 cells per ml.

To establish the background level of $TK^{-/-}$ colonies, two control tubes will receive solvent only. Two concentrations of 7,12-DMBA will be used as a positive control. All tubes will be gassed with 5% CO_2 in air and placed on a roller drum apparatus for 4 hours at 37°C. The preparation and addition of the test article will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

At the end of the exposure period, the cells will be washed twice in 10 ml of $F_{10}P$ by centrifuging at 1000 x g for 10 minutes and decanting the supernatant. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5% CO_2 in air, and replaced on the roller drum apparatus at 37°C.

7.2.3 Expression Time
After the initial exposure to the test article, the cells will be incubated for two days with a cell population adjustment at 24 and 48 hours. The adjustment will be made by taking daily cell counts and then replacing a volume of cells with fresh medium totaling 20 ml at 24 hours and 10 ml at 48 hours which will yield a cell population density of 0.3×10^6 cells/ml.

7.2.4 Cloning
At the end of the expression period, the cells will be placed in a restrictive medium which will allow only the $TK^{-/-}$ cells to grow. The restrictive medium used is cloning medium (C.M.) with Trifluorothymidine (TFT) (2-4 ug/ml). The C.M. will contain agar (approximately 0.34%) which maintains the cells in suspension and allows them to form discrete colonies of $TK^{-/-}$ cells. Those cultures exhibiting approximately 5% to 90% growth inhibition will be cloned.

Two florence flasks per culture to be cloned and two per control tube will be labeled to indicate the test article concentration and that they received S-9 activation. For each pair of flasks one will be labeled TFT and one will be labeled V.C. (viable count). Each flask will be prewarmed to 37°C, filled with 100 ml of C.M. and placed on an incubator shaker at 37°C until used.

Six 100 mm petri plates per test article concentration will be labeled to indicate the concentration, whether or not activation was used, and the experiment number. Three of the six will be labeled TFT and three will be labeled V.C.

Cell counts will be made for each tube to determine the volume of each cell population which will yield 3×10^6 cells. This volume will be removed, the remainder of the cells discarded, and the 3×10^6 cells replaced in the centrifuge tube. The cells will be centrifuged at 1000 x g for 10 minutes, and the supernatant, except for 2 ml, will be removed by pipetting. The cells will be resuspended in the remaining 2 ml of medium and placed in a TFT flask labeled with the corresponding concentration of the test article (see Figure 1).

A 2×10^{-4} dilution will be carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml $F_{10}P$, and adding 1.0 ml of that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1 ml of stock solution of the restrictive agent will be added to the TFT flask, and both this flask and the V.C. flask will be placed on the shaker at 125 rpm and 37°C.

After 15 minutes the flasks will be removed one at a time, and 33 ml of the cell suspension will be pipetted into each of three appropriately labeled petri plates. To accelerate the gelling process, the plates will be placed in cold storage (4°C) for 20 minutes. The plates will be removed and incubated at 37°C in a humidified 5% CO_2 atmosphere for 10-12 days.

7.3 Accumulation of Data

After the incubation period, both the TFT plates and the V.C. plates will be scored for the total number of

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ILLUSTRATION OF CELL DILUTION AND PLATING IN THE CLONING PROCESS

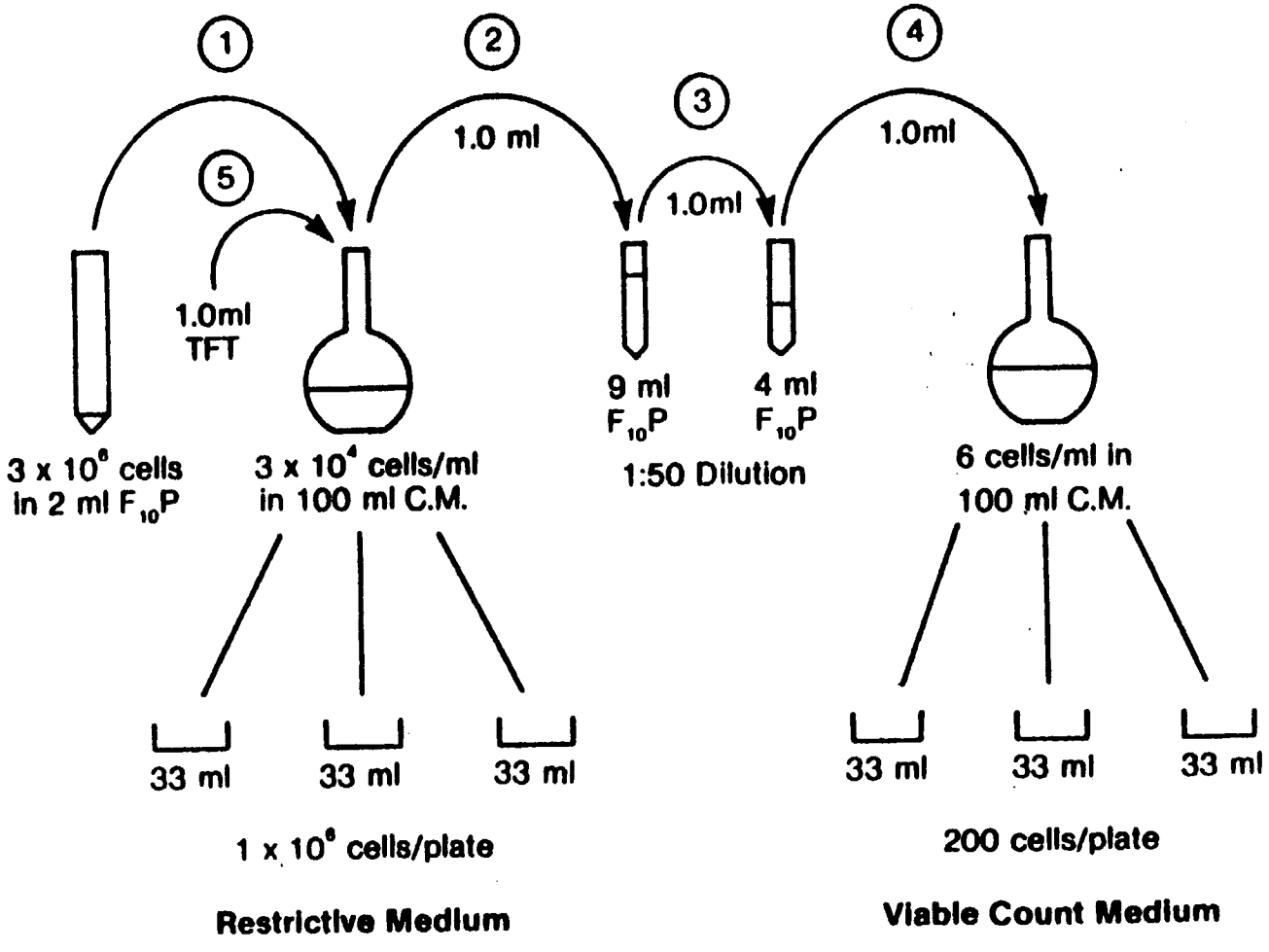

FIGURE 1

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colonies per plate. Three counts per plate will be made on an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the plates will be counted by hand. The mutation frequency will be determined by dividing the average number of colonies in the three TFT plates by the average number of colonies x 10^4 in the three corresponding V.C. plates and multiplying the quotient by two. By comparing the mutation frequency of the treated plates to that of the control plates, the presence of a significant level of mutagenic activity can be detected.

8.0 EVALUATION OF TEST RESULTS

The following criteria will be used as guidelines in judging the significance of the activity of a test article in this system. In evaluating the results, it is considered that increases in mutant frequencies, which occur only at highly toxic concentrations, may be due to epigenetic events. Unfortunately, it is impossible to formulate criteria which would apply to all types of data which may be generated and therefore the conclusion of the study will be based on the scientist's evaluation.

8.1 Positive - if there is a positive dose response and one or more of the three highest doses exhibit a mutant frequency which is two-fold greater than the background level.

8.2 Equivocal - if there is no dose response but any one or more doses exhibit a two-fold increase in mutant frequency over background.

8.3 Negative - if there is no dose response and none of the test cultures exhibit mutant frequencies which are two-fold greater than background.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

9.1 The mutation frequency of the positive controls must be at least twice that of the appropriate solvent control cultures.

9.2 The spontaneous mutation frequency of the solvent control cultures must be between 0.2 and 1.0 per 10^4 surviving cells.

9.3 The plating efficiency of the solvent controls must be greater than 50%.

10.0 FINAL REPORT

10.1 ____ copies of the draft final report (with ____ copies of all raw data) will be sent to the Study Monitor within one month of termination. ____ copies of the final report will be sent after the draft report has been reviewed by the Sponsor.

10.2 A report of the results of this study will be prepared by the Testing Laboratory and will include the following information:

10.2.1 The data from the toxicity test.

10.2.2 The data generated by the mutagenesis assay which includes:

10.2.2.1 The % total growth at each dose level which reflects test article toxicity.

10.2.2.2 The number of $TK^{-/-}$ colonies per TFT plate for the test articles and the controls.

10.2.2.3 The number of colonies per V.C. plate for the test articles and the controls.

10.2.2.4 The mutation frequency of each dose level of the test articles and the controls.

10.2.2.5 The induced mutation frequency of each dose level of the test articles and the positive controls.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
Test Article samples will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be conducted in accordance with FDA Good Laboratory Practice Regulations. If requested, copies of relevant Standard Operating Procedures will be made available to the Sponsor for inspection on site. At least 10% of the in-process phases of this study are to be monitored by the Quality Assurance Unit personnel.

Will this study be submitted to a regulatory agency? YES
If so, to which agency or agencies? OSHA, EPA, NIOSH, NCI, NIEHS, CPSC, FDA
Does the Sponsor request that samples of the Test Article dosing solutions be returned? NO

13.0 SCHEDULE OF EVENTS

13.1 Test Material Received Date: December 12, 1983 PK 5/24/84

13.2 Proposed Initiation Date: January 23, 1984

13.3 Scheduled Completion Date: February 24, 1984

13.4 Report Submission to Sponsor Date: March 2, 1984

14.0 REFERENCES

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

de Serres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

Carol A. Hauk
SPONSOR'S AUTHORIZED REPRESENTATIVE

12/19/83
DATE PROTOCOL APPROVED BY SPONSOR

Paul E. Kerby 11/28/83
STUDY DIRECTOR DATE

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Abbreviations Used in the L5178Y Mouse Lymphoma Mutagenesis Assay

C.M.	Cloning Medium
DMBA	7,12-Dimethylbenz(a)anthracene
EMS	Ethyl Methanesulfonate
F_0P	Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronic
$F_{10}P$	F_0P with 10% heat inactivated horse serum
NADP	Nicotinimide Adenine Dinucleotide Phosphate
S-9	1254, 1242 Aroclor-induced rat liver S-9
TFT	Trifluorothymidine
THMG	Thymidine, Hypoxanthine, Methotrexate and Glycine
V.C.	Viable Count

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FORMULAS AND CALCULATIONS FOR THE L5178Y $TK^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1. Initial Toxicity Daily Counts and Final Counts

$$\text{No. of Cells/ml} = \text{Average Counts (corrected for coincidence)} \times 20$$

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^6 \text{ cells/ml})\ (20 \text{ ml})}{\text{(No. of Cells/ml)}}$$

$$\text{Volume of Media to Add} = 20 \text{ ml} - \text{Volume of Cells Retained}$$

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2. Table 1 - L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay
Initial Compound Toxicity Test

Total Suspension Growth =

$$\frac{\text{Day 1 Cell Conc.}}{0.3\times10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Conc.}}{\text{Day 1 Adjusted Cell Conc.}}$$

% of Control Suspension Growth =

$$\frac{\text{Total Suspension Growth (Test Culture)}}{\text{Average Solvent Control Total Suspension Growth}} \times 100$$

Cultures containing less than 0.3×10^6 cells/ml on day 1 and day 2 will be considered as having 0% Total Suspension Growth.

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3. L5178Y $TK^{+/-}$ Assay - Daily Counts (Test Article)
and L5178Y $TK^{+/-}$ Assay - Daily Counts (Positive Controls)

$$\text{No. of Cells/ml} \times 10^6 = \text{Average Counts (corrected for coincidence)} \times 20$$

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3. (Cont'd.)

$$\text{Volume of Cells to Retain} = \frac{(0.3\times10^6 \text{ Cells/ml})\ (20 \text{ ml}^*)}{(\text{No. of Cells/ml})}$$

$$\text{Volume of Media to Add} = (20 \text{ ml}^*) - (\text{Volume of Cells Retained})$$

* The final volume is adjusted to 10 ml on the second day of expression.

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4. Tables 2, 4, 6 and 7 - L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay Cloning Data

$$\text{Mutant Frequency per } 10^4 \text{ Survivors} = \frac{\text{Average No. of TFT Colonies}}{\text{Average No. of V.C. Colonies}} \times 2$$

$$\text{Induced Mutant Frequency} = \left(\text{Mutant Frequency of Treated Cultures}\right) - \left(\text{Average Mutant Frequency of Solvent Controls}\right)$$

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5. Tables 3, 5, 6 and 7 - L5178Y $TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay Total Compound Toxicity Data

Total Suspension Growth =

$$\frac{\text{Day 1 Cell Conc.}}{0.3\times10^6 \text{ Cells/ml}} \times \frac{\text{Day 2 Cell Conc.}}{\text{Day 1 Adjusted Cell Conc.}}$$

% Control Suspension Growth =

$$\frac{\text{Treated Culture of Suspension Growth}}{\text{Average Suspension Growth of Controls}} \times 100$$

Cultures containing less than 0.3×10^6 cells/ml on day 1 and on day 2 will be considered as having 0% Total Suspension Growth.

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5. (Cont'd.)

$$\% \text{ Control Cloning Growth} = \frac{\text{Average V.C. of Treated Cultures}}{\text{Average V.C. of Solvent Controls}} \times 100$$

$$\% \text{ Growth} = \frac{(\% \text{ Suspension Growth})\ (\text{Cloning Growth})}{100}$$

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