OFFICE OF TOXIC SUBSTANCES

REV. 7/27/82

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Doc Title 23

MUTAGENICITY EVALUATION OF TD 8Ø-16 IN THE MOUSE LYMPHOMA FORWARD MUTATION ASSAY WITH MEMO DATED Ø81382

Chemical Name (300 per name) 25	CAS No. (10) 24
ETHYL ACRYLATE	14Ø-88-5
DICYCLOPENTENEOXYETHEL METHACRYLATE	68586-19-6

1A

2/4/85 U.S.

Chemical Name (300 per name) 25	CAS No. (10) 24
2-ETHYHEXYL ^ ACRYLATE	1Ø3-11-7
ACRYLATE ^ MONOMERS	

1B

cc: S.A. Ellis
W.D. Emmons
A.W. Hayes
C.E. Hoey
P.J. McNulty
L.J. Shestack
A.S. West
QAU
TD Central File

TD 82M-593

Spring House, August 13, 1982

To: D.H. Clemens*

From: T. Fedorowski*

Subject: Results, Mammalian Cell point Mutation Assay: QM-657
Lot SW 79/3739; TD No. 80-16; Report No. 81RC-52

QM-657 was tested in the Mouse Lymphoma Mammalian Cell Point Mutation Assay, L51784 TK +/- cells, with and without rat liver S9 activation. No increase in mutation frequency was observed either in the absence or presence of the activation system.

Under the conditions of this test, QM-657 was not mutagenic in the Mammalian Cell Point Mutation Assay.

This study has been audited by the Quality Assurance Unit.

T. Fedorowski

TF/mct
*Memo and Report

QM-657 =
DICYCLOPENTENE OXYETHYL METHACRYLATE
CAS # 68586-19-6

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cc: A.W. Hayes
H.E. Scribner
J.M. Smith
QAU
TD Central File

TD 82M-594

Spring House, August 13, 1982

To: [redacted] T. Pedorowski

From: K.L. McCarthy

Subject: QM-657: Mammalian Cell Point Mutation Assay

QM-657 (TD No. 80-16, lot SW-79/3739) was tested by Litton Bionetics for mutagenic activity in the Mammalian Cell Point Mutation Assay. L5178Y TK +/- Mouse Lymphoma Cells were used both with and without rat liver S9 activation and were selected for mutants at the thymidine kinase (TK) locus. Cells were exposed to the test compound dissolved in DMSO for 4 hours at 37°C in Fisher's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and 10% horse serum. After treatment the cells were grown and subcultured for 2 days and then cloned into agar containing 5-bromo-2'-deoxyuridine to select for TK locus mutants. After incubation for approximately 10 days, the mutant colonies were counted and compared to the results of simultaneous negative controls.

Without activation, concentrations of 0.78 to 100 nl/ml (approx. 65 to 11% relative growth) yielded mutation frequencies similar to the negative control values. With activation mutation frequencies were also similar to those of the negative controls at concentrations of 25 to 250 nl/ml (approx. 118 to 5% relative growth). Consequently QM-657 is inactive (no adverse effect) in the Mouse Lymphoma Point Mutation Assay both in the absence and presence of an activation system.

Attached is a copy of the report from Litton Bionetics. Please contact me if you have any questions concerning the report or test procedures.

Kay L. McCarthy
K.L. McCarthy

KLM/mct
Attachment

000003

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COMPANY COPY

GENETICS ASSAY NO.: 5145

LBI SAFETY NO.: 6035

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MUTAGENICITY EVALUATION OF

TD 80-16 CAS# 68586-19-6

IN THE
MOUSE LYMPHOMA FORWARD
MUTATION ASSAY

ROHM AND HAAS PROTOCOL NO. 80P-255

FINAL REPORT

REPORT NO. 81RC-52

SUBMITTED TO:

ROHM AND HAAS COMPANY
TOXICOLOGY DEPARTMENT
NORRISTOWN AND MCKEAN ROADS
SPRING HOUSE, PA 19477

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO: 20989

REPORT DATE: JULY, 1982

ROHM AND HAAS COMPANY
TOXICOLOGY DEPARTMENT ARCHIVES
RECEIVED: JUL 21 1982
ENTERED BY: EAS

Litton BIONETICS

FORM 4685 REV. 10/78

PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains Items I-IX. Items I-IV provide sponsor and test article identification information, type of assay, and the protocol reference number. Item V provides the initiation and completion dates of the study. Item VI identifies the supervisory personnel. Item VII indicates the tables and/or figures containing the test results. The interpretation of the results is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report describes the study design, which includes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices.

All test and control results presented in this report are supported by raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895.

Copies of the raw data will be supplied to the sponsor upon request.

The described study was performed in accordance with Good Laboratory Practice regulations except if noted to the contrary. To the best of the signer's knowledge there were no significant deviations from the Good Laboratory Practice regulations which affected the quality or integrity of the study.

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I. SPONSOR: Rohm and Haas Company

II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NO.: 5145

A. Identification: TD 80-16

B. Date Received: May 20, 1980 (Returned to Sponsor on November 31, 1980 and received again on January 21, 1981)

C. Physical Description: Clear yellow liquid

III. TYPE OF ASSAY: Mouse Lymphoma Forward Mutation Assay (Rohm and Haas Protocol No. 80P-255)

IV. ASSAY DESIGN NUMBER: 431, Edition 2

V. STUDY DATES:

A. Initiation Date: May 27, 198~~8~~0 8/6/82 KLM

B. Completion Date: February 6, 1981

VI. SUPERVISORY PERSONNEL:

A. Study Director: Brian C. Myhr, Ph.D.

B. Laboratory Supervisor: Jane Fisher

VII. RESULTS:

The data are presented in Tables 1, 2 and 3 on pages 5, 6 and 7.

VIII. INTERPRETATION OF RESULTS:

The test material, TD 80-16, was immiscible at 100 µl/ml with deionized water, but a pale-yellow solution formed easily in dimethylsulfoxide (DMSO) at the same concentration. Stock solutions were therefore prepared in DMSO just prior to each assay, and these solutions were diluted 1:100 into tubes of culture medium containing the cells to initiate the treatments. The test material at 100 µl/ml appeared to interact with the polystyrene cultureware, so glass tubes and pipets were used to prepare and deliver the DMSO stock solutions. No scoring of plastic was observed during the treatments of the cells in polystyrene tubes. A cloudy suspension of test material formed in the culture medium at concentrations above approximately 50 nl/ml under nonactivation conditions.

In the preliminary cytotoxicity test, the 24-hour cell count in the culture exposed to 31.3 nl/ml was similar to the negative controls, but treatment with 62.5 nl/ml was completely lethal. The mutation assay was therefore initiated with a series of concentrations from 100 nl/ml to 0.78 nl/ml, using less than 2-fold dilution steps between 100 nl/ml and 25 nl/ml, in a effort to cover a wide range of toxicity.

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VIII. INTERPRETATION OF RESULTS: (continued)

The results of the first mutation assay are presented in Table 1. Subsequently, five more trials with S9 metabolic activation only were performed. In the second trial, the DMN positive control was lost to mold contamination so that effective activation conditions could not be demonstrated; the test material did not cause significant increases in the mutant frequency for concentrations up to 200 nl/ml (9.8% relative growth). The third and fourth trials were unacceptable due to low DMN-induced mutant frequencies, poor colony growth, and poor suspension growth. The results of the fifth and sixth activation trials are given in Tables 2 and 3.

Under nonactivation conditions, concentrations of test material from 0.78 nl/ml to 100 nl/ml were analyzed for the induction of mutants. As shown in Table 1, moderate toxicity was observed over a 100-fold increase in applied concentrations. At 100 nl/ml, however, high toxicity was obtained (10.9% relative growth). No significant increases in the mutant frequency were obtained for the treated cultures. The minimum criterion for indicating mutagenesis by a given treatment was a mutant frequency greater than 27.0×10^{-6}, and the calculated frequencies remained essentially constant at a level below this criterion. Thus, the test material was considered to be nonmutagenic under nonactivation conditions.

In the presence of the S9 microsomal activation mix, only weak or no toxicity was observed for treatments with concentrations up to 100 nl/ml, which suggested an interaction with the activation system. In the first trial (Table 1), only one treated culture exhibited a mutant frequency that exceeded the minimum criterion of 43.8×10^{-6} used to indicate a significant response. The other cultures exposed to concentrations of test material ranging from 25 nl/ml to 100 nl/ml had mutant frequencies below the minimum criterion. Since the response at 50 nl/ml was isolated and represented only a 2.1-fold increase over the background, it appeared to be spurious and not indicative of any mutagenic activity by the test material. Another trial was necessary to extend the assay to more toxic treatments to provide a conclusive test for the presence of mutagenic substances.

In a subsequent activation trial (Table 2), concentrations of test material up to 200 nl/ml were analyzed. Moderate toxicity was obtained with the relative growth reduced to approximately 28-30% by the two highest doses. Treatment with 225 nl/ml (not shown) was completely lethal to the cells. The mutant frequencies in the treated cultures remained very similar to the negative controls and well below the criterion of 44.4×10^{-6} used to indicate a significant increase. Thus, the small increase observed in Trial 1 at 50 nl/ml was not confirmed and no indication of mutagenesis by the test material or any metabolic products was obtained for concentrations closely approaching the point of excessive lethality.

VIII. INTERPRETATION OF RESULTS: (continued)

An additional activation trial was performed with a number of duplicate and triplicate treatments within the 100 nl/ml to 300 nl/ml concentration range in a further effort to achieve highly toxic treatments resulting in sufficient survivors to perform the assay. The DMN positive control treatment was also performed in duplicate to demonstrate consistency in metabolic activation, and four solvent control cultures were utilized to provide an improved definition of the background mutant frequency. As shown in Table 3, ten treated cultures were analyzed for the mutant frequency. Only one culture exhibited a mutant frequency that just met the minimum criterion 45.7×10^{-6} used to indicate a significant increase, and since this result was isolated at the low end of the assayed concentration range, the increase was considered to be anomalous and not indicative of mutagenic activity by the test material. Triplicate treatments at 200 nl/ml and 225 nl/ml were moderately to highly toxic (18.8% to 6.1% relative growth range) and did not induce increases in the mutant frequency related to the degree of toxicity. Two treatments with 250 nl/ml were extremely toxic and a third treatment (not shown) was too toxic to perform the assay. Treatments with higher concentrations (275 and 300 nl/ml) were either too toxic to perform the assay or completely lethal to the cells. This trial therefore confirmed in a rigorous manner the lack of mutagenic activity by the test material in the presence of the rat liver S9 metabolic activation system.

The average cloning efficiencies for the negative controls (solvent and untreated) varied from 109% without activation and from 71% (Table 3) to 87% (Table 2) with activation, which demonstrated excellent culturing conditions for the assays. The negative control mutant frequencies were in the normal range, and the positive control compounds induced normal mutant frequencies that were greatly in excess of the backgrounds. The average negative control suspension growth was low in the activation trials shown in Tables 1 and 2, presumably due to S9 mix toxicity that was also observed in other concurrent studies, but the growth was considered sufficient to provide reliable evidence for the lack of mutagenic activity by the test material. On October 20, 1980, an absolute criterion of 8.0 for the average negative control suspension growth was established, although the desirable range will remain approximately 12 to 25. In the first two activation assays the growth remained above 8.0, and a high average suspension growth factor of 28 was achieved for the definitive third trial (Table 3). An 8-fold increase in cell number corresponds to three population doublings and is not generally associated with spurious results compared to assays at higher growth rates.

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IX. CONCLUSIONS:

The test material, TD 80-16, did not induce significant increases in the mutant frequency at the TK locus in L5178Y mouse lymphoma cells. Without activation, concentrations up to 100 nl/ml were assayed before reaching excessive toxicity. In the presence of rat liver S9 metabolic activation, concentrations up to 250 nl/ml became extremely toxic but did not induce significant increases in the mutant frequency. Thus, the test material was considered to be inactive in the Mouse Lymphoma Forward Mutation Assay.

SUBMITTED BY:

Study Director:

Brian C. Myhr

Brian C. Myhr, Ph.D.
Section Chief
Mammalian Genetics
Department of Molecular Toxicology

7/20/82
Date

REVIEWED BY:

David J. Brusick

David J. Brusick, Ph.D.
Director
Department of Molecular Toxicology

7/20/82
Date

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6. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: TD 80-16
B. LBI CODE #: 5145
C. SOLVENT: DIMETHYL SULFOXIDE
D. TEST DATE: 06/10/80

TEST	S-9 SOURCE	S-9 TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY**
NONACTIVATION											
SOLVENT CONTROL	---	---	9.4	12.4		100.0	32.0	301.0	100.0	100.0	10.6
SOLVENT CONTROL	---	---	11.2	12.0		100.0	44.0	338.0	100.0	100.0	13.0
UNTREATED CONTROL	---	---	9.4	9.9		74.2	35.0	338.0	105.8	78.5	10.4
EMS .5 UL/ML	---	---	5.7	7.3		33.2	340.0	65.0	20.3	6.7	523.1
TEST COMPOUND											
0.780 NL/ML	---	---	8.7	10.0		69.3	41.0	214.0	67.0	46.4	19.2
37.500 NL/ML	---	---	5.9	11.4		53.6	48.0	226.0	70.7	37.9	21.2
50.000 NL/ML	---	---	8.8	12.4		87.0	42.0	238.0	74.5	64.8	17.6
75.000 NL/ML	---	---	5.5	13.5		59.2	43.0	207.0	64.8	38.3	20.8
100.000 NL/ML	---	---	2.3	7.9		18.9	37.0	184.0	57.6	10.9	20.1
ACTIVATION[S]											
SOLVENT CONTROL	RAT	LIVER	8.7	8.5		100.0	71.0	283.0	100.0	100.0	25.1
SOLVENT CONTROL	RAT	LIVER	6.5	7.3		100.0	59.0	233.0+	100.0	100.0	25.3
UNTREATED CONTROL	RAT	LIVER	10.5	10.5		181.6	39.0	229.0	88.8	161.2	17.0
DMN .3 UL/ML	RAT	LIVER	5.3	6.4		55.9	107.0	44.0	17.1	9.5	243.2
TEST COMPOUND											
25.000 NL/ML	RAT	LIVER	8.2	7.0		94.6	65.0	322.0	124.8	118.0	20.2
37.500 NL/ML	RAT	LIVER	6.9	10.6		120.5	66.0	187.0	72.5	87.3	35.3
50.000 NL/ML	RAT	LIVER	6.0	9.2		90.9	100.0	216.0	83.7	76.1	46.3
75.000 NL/ML	RAT	LIVER	4.6	13.8		104.6	75.0	287.0+	111.2	116.3	26.1
100.000 NL/ML	RAT	LIVER	4.6	12.8		97.0	77.0	213.0	82.6	80.1	36.2

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4. THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-4. THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.
\+ ONE PLATE CONTAMINATED; VALUE BASED ON REMAINING TWO PLATES.
[S]RAT LIVER HOMOGENATE BATCH NO. DD-027

COMPLIANCE DEPT. ... PHILADELPHIA
FORM 465 REV. 10/78
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4. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS TABLE 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: TD 80-16
B. LBI CODE #: 5145
C. SOLVENT: DIMETHYL SULFOXIDE
D. TEST DATE: 09/30/80

TEST	S-9 SOURCE	TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY**
ACTIVATION[5]											
SOLVENT CONTROL	RAT	LIVER	6.5	12.6		100.0	75.0	262.0	100.0	100.0	28.6
SOLVENT CONTROL	RAT	LIVER	7.4	11.0		100.0	43.0	230.0	100.0	100.0	18.7
UNTREATED CONTROL	RAT	LIVER	6.6	8.5		68.7	62.0	291.0	118.3	81.3	21.3
DMN .3 UL/ML	RAT	LIVER	4.8	6.7		39.4	218.0	52.0	21.1	8.3	419.2
TEST COMPOUND											
50.000 NL/ML	RAT	LIVER	4.9	12.5		75.0	70.0	246.0	100.0	75.0	28.5
100.000 NL/ML	RAT	LIVER	4.6	8.1		45.6	85.0	295.0	119.9	54.7	28.8
150.000 NL/ML	RAT	LIVER	3.0	12.9		47.4	81.0	235.0	95.5	45.3	34.5
175.000 NL/ML	RAT	LIVER	3.2	8.5		31.2	77.0	219.0	89.0	27.8	35.2
200.000 NL/ML	RAT	LIVER	1.8	7.9		29.0	68.0	253.0	102.8	29.9	26.9

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4.
THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-4.
THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.
[5] RAT LIVER HOMOGENATE BATCH NO. RDH-088

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SMITHKLINE CORPORATION
SMITHKLINE COMPANY PHILADELPHIA
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4. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

TABLE 3

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: TD 80-16
B. LBI CODE #: 5145
C. SOLVENT: DIMETHYL SULFOXIDE
D. TEST DATE: 01/26/81

TEST	S-9 SOURCE	TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY**
ACTIVATION[S]											
SOLVENT CONTROL	RAT	LIVER	16.4	16.6		100.0	42.0	211.0	100.0	100.0	19.9
SOLVENT CONTROL	RAT	LIVER	16.1	18.4		100.0	50.0	215.0	100.0	100.0	23.3
SOLVENT CONTROL	RAT	LIVER	13.3	15.8		100.0	56.0	209.0	100.0	100.0	26.8
SOLVENT CONTROL	RAT	LIVER	10.9	19.3		100.0	53.0	211.0	100.0	100.0	25.1
DMN .3 UL/ML	RAT	LIVER	9.1	7.5		27.3	112.0	26.0	12.3	3.4	430.8
DMN .3 UL/ML	RAT	LIVER	6.6	7.2		19.0	118.0	27.0	12.8	2.4	437.0
TEST COMPOUND											
100.000 NL/ML	RAT	LIVER	10.1	12.8		48.6	75.0	208.0	98.3	47.8	36.1
150.000 NL/ML	RAT	LIVER	8.5	14.0		47.7	63.0	138.0	65.2	31.1	45.7
200.000 NL/ML	RAT	LIVER	3.7	9.8		11.8	58.0	195.0	92.2	10.9	29.7
200.000 NL/ML	RAT	LIVER	4.6	7.8		14.4	78.0	237.0	112.1	16.1	32.9
200.000 NL/ML	RAT	LIVER	5.5	7.4		16.3	85.0	244.0	115.4	18.8	34.8
225.000 NL/ML	RAT	LIVER	2.0	6.7		8.1	35.0	160.0	75.7	6.1	21.9
225.000 NL/ML	RAT	LIVER	2.7	5.8		11.8	42.0	125.0	59.1	7.0	33.6
225.000 NL/ML	RAT	LIVER	6.3	5.9		14.9	43.0	193.0	91.3	13.6	22.3
250.000 NL/ML	RAT	LIVER	1.4	3.6+++		4.3	33.0	153.0	60.3	2.6	21.6
250.000 NL/ML	RAT	LIVER	2.2	4.3		5.2	72.0	189.0	89.4	4.6	38.1

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** THE RATIO OF CELLS SEEDED FOR MUTANT SELECTION TO CELLS SEEDED FOR CLONING EFFICIENCY IS 10E+4.
THEREFORE THE MUTANT FREQUENCY IS: (TOTAL MUTANT CLONES/TOTAL VIABLE CLONES)*10E-4.
THE MUTANT FREQUENCY IS GIVEN IN UNITS OF 10E-6.

[S] Rat Liver Homogenate Batch No. RDH-088.

+++ Culture not split back to 3.0 x 10^5 cells; the entire culture was assayed and 360 cells seeded for viable clone count. Values for relative cloning efficiency and percent relative growth were adjusted accordingly.

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ASSAY DESIGN (NO. 431)

1. OBJECTIVE

The objective of this study is to evaluate the test material for its ability to induce forward mutation in the L5178Y TK+/- mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU) or 5-trifluorothymidine (TFT).

2. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as BrdU is included in the growth medium, the analog will be phosphorylated via the TK pathway and be incorporated into DNA, eventually resulting in cellular death. Cells which are heterozygous at the TK locus (TK+/-) may undergo a single step forward mutation to the TK -/- genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by de novo synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK-/- mutants is the lack of any ability to utilize toxic analogs of thymidine, which enables only the TK-/- mutants to grow in the presence of BrdU. Cells which grow to form colonies in the presence of BrdU are therefore assumed to have mutated, either spontaneously or by the action of a test substance, to the TK-/- genotype.

3. MATERIALS

A. Indicator Cells

The mouse lymphoma cell line, L5178Y TK+/-, used in this assay is derived from the Fischer L5178Y line of Dr. Donald Clive. Stocks are maintained in liquid nitrogen and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK-/- mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK-/- phenotype (exposure to methotrexate) and are then returned to normal growth medium for three or more days before use.

B. Media

The cells are maintained in Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate, and horse serum (10% by volume). Cloning medium consists of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium is cloning medium containing 100 µg/ml of BrdU or 3 µg/ml of TFT.

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3. MATERIALS (continued)

C. Control Compounds

1. Negative Controls

A negative control consisting of assay procedures performed on untreated cells is performed in all cases. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are also assayed as the solvent negative control to determine any effects on survival or mutation caused by the solvent alone. For test substances assayed with activation, the untreated and solvent negative controls will include the activation mixture.

2. Positive Controls

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and will be used at 0.5 µl/ml as a positive control for nonactivation studies.

Dimethylnitrosamine (DMN) requires metabolic activation by microsomal enzymes to become mutagenic and will be used at 0.3 µl/ml as a positive control for assays performed with activation.

D. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

4. EXPERIMENTAL DESIGN

A. Dosage Selection (Cytotoxicity testing)

The solubility of the test chemical in growth medium and/or DMSO is first determined. Then a wide range of chemical concentrations is tested for cytotoxicity, starting with a maximum applied dose of 10 mg/ml for test chemicals soluble in media or 1 mg/ml for solutions in organic solvents. After an exposure time of four hours, the cells are washed and a viable cell count is obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of untreated cells are used to select seven to ten doses that cover the range from 0 to 50-90% reduction in 24-hour growth. These selected doses are subsequently applied to cell cultures prepared for mutagenicity testing, but only four or five of the doses will be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of four or five doses spaced from 0 to 50-90% reduction in cell growth.

B. Mutagenicity Testing

1. Nonactivation Assay

The procedure used is based on that reported by Clive and Spector (1975) and is summarized as follows. Cultures exposed to the test chemical for four hours at the preselected doses are washed and placed in growth medium for two or three days to allow recovery, growth and expression of the induced TK-/- phenotype. Cell counts are determined daily and appropriate dilutions are made to allow optimal growth rates.

At the end of the expression period, 3×10^6 cells for each selected dose are seeded in soft agar plates with selection medium and resistant (mutant) colonies are counted after 10 days incubation. To determine the actual number of cells capable of forming colonies, a portion of the cell suspension is also cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number is the mutant frequency.

A detailed flow diagram for the mutation assay is provided in Figure 1.

2. Activation Assay

The activation assay can be run concurrently with the nonactivation assay. The only difference is the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the four-hour treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension are: 2.4 mg NADP/ml; 4.5 mg isocitric acid/ml; and 50 µl S9/ml.

3. S9 Homogenate

A 9,000 x g supernatant prepared from Fischer 344 adult male rat liver induced by Aroclor 1254 (described by Ames et al., 1975) is purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. and used in this assay.

5. REPORT

The screened doses, cell counts, and mutant and viable colony counts will be entered into a computer program. The results are analyzed and printed.

The suspension growth of each culture is calculated as (Day 1 Cell Count/3) x (Day 2 Cell Count/3) x (Day 3 Cell Count/3) when the cultures are split back to 3 x 10^5 cells/ml after the daily count. If the cell count is less than 4 x 10^5 cells/ml, the culture is not split back and the cell count is substituted for 3 in the denominator of the next daily count. In most assays, 3-day expressions are not used, so only the first two factors in the preceding calculation are used. The suspension growth is calculated for each solvent control and then averaged. Relative suspension growth values are derived by dividing the suspension growth values by the average solvent control value and multiplying by 100%.

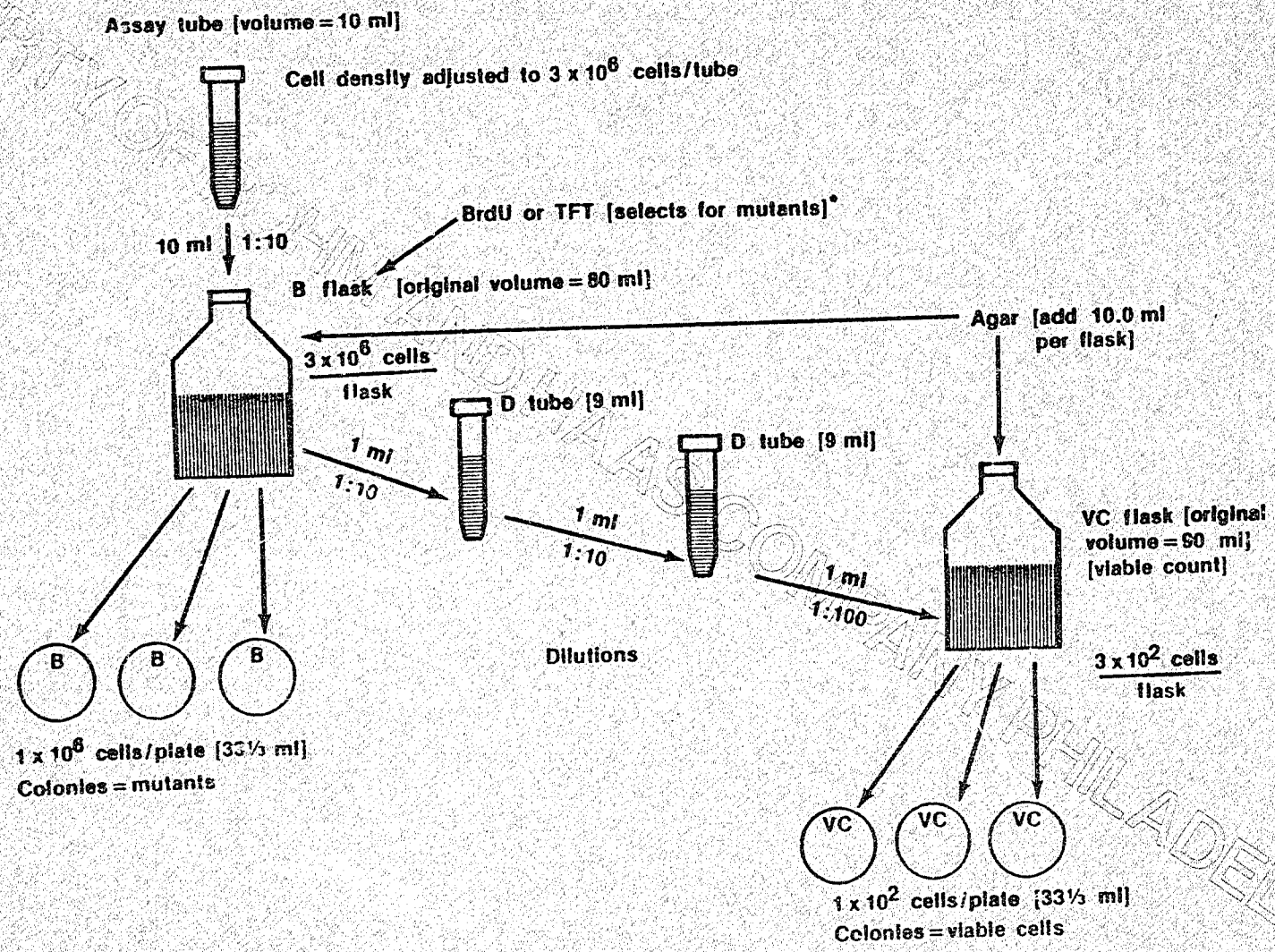
The average cloning efficiency for the negative controls in an assay is the average number of viable colonies for the solvent and untreated controls, divided by 300 and multiplied by 100%. In the computer tables, the cloning efficiency of each culture is expressed relative to the average solvent control cloning efficiency. Whenever the number of cells seeded for viable colony counts differs from 300, the computer calculation of the relative cloning efficiency is adjusted by the factor (300/cells seeded).

A percent relative growth value is calculated as (relative suspension growth) x (relative cloning efficiency/100). Corrected values for the relative cloning efficiency are used in the cases where the number of cells seeded for viable colonies differs from 300.

The mutant frequency is calculated as the ratio of mutant colonies to viable colonies times 10^{-4}. This calculation is unaffected by changes in the number of cells seeded for viable count because the number of cells seeded for mutant selection is changed by the same factor. Thus, as an example, if 250 cells are seeded for viable count, 2.5 x 10^6 cells are seeded for mutant selection; the 10^{-4} factor remains constant.

6. REFERENCE

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.



*Added after removal of 1 ml for viable count dilutions.

FIGURE 1. LYMPHOMA CLONING FLOW CHART

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ASSAY ACCEPTANCE CRITERIA

An assay will normally be considered acceptable for evaluation of the test results only if all of the criteria given below are satisfied. The activation and nonactivation portions of the mutation assays are usually performed concurrently, but each portion is in fact an independent assay with its own positive and negative controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

1) The average absolute cloning efficiency of the negative controls (average of the solvent and untreated controls) should be between 70% and 130%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and cell division during unavoidable delays between the counting and cloning of many cell cultures. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50% to 70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range are conditionally acceptable and dependent on the scientific judgement of the study director. All assays below 50% cloning efficiency are unacceptable.

2) The solvent and untreated negative controls normally have the same growth rates and cloning efficiencies within experimental error. An unusual effect by the solvent therefore indicates an abnormal cell state or excessive amount of solvent in the growth medium. An assay will be unacceptable if the average percent relative growth of the solvent controls is less than about 70% of the untreated control value.

3) The minimum acceptable value for the suspension growth of the average negative control (average of the solvent and untreated control values) for two days is 8.0. Lower values will render an assay unacceptable for evaluation because of the high frequency of unreliable measurements for both the induced mutant frequency and toxicity of a given treatment. The value of 8 corresponds to three population doublings over the 2-day expression period. The most desirable range for the negative control suspension growth is 12 to 25, since the cells are capable of a 5-fold increase in number under optimal growth conditions for 24 hours.

4) The background mutant frequency (average frequency of the solvent and untreated negative controls) is calculated separately for concurrent activation and nonactivation assays, even though the same population of cells is used for each assay. The activation negative controls contain the S9 activation mix and typically have a somewhat higher mutant frequency than the nonactivation negative controls. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is 5×10^{-6} to 50×10^{-6}. Assays with backgrounds outside this range are not necessarily invalid but will not be used as primary evidence for the evaluation of a test material. These assays can provide supporting evidence.

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5) A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. The normal range of mutant frequencies induced by 0.5 µl/ml EMS (nonactivation assay) is 300 to 800 x 10^{-6}; for 0.3 µl/ml DMN (activation assay) the normal range is 200 to 800 x 10^{-6}. The concurrent background frequencies have been subtracted from these values. These ranges are broad primarily because the effective treatment with these agents is variable between assays. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test material clearly shows mutagenic activity as described in the evaluation criteria. If the test material appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency above the lower limits of the normal range. Assays in which the normal range is exceeded may require further interpretation by the study director.

6) For test materials with little or no mutagenic activity, an assay must include applied concentrations that reduce the suspension growth to 5% to 10% of the average solvent control or reach the maximum applied concentrations given in the evaluation criteria. Suspension growth is a combined measure of cell death and reduced growth rates. A 5% relative suspension growth therefore could correspond to 90% killing followed by growth of the survivors at one-half the normal rate for one day and normal growth for the second day. At the other extreme, this condition could be obtained by no killing and complete inhibition of growth for two days. A reasonable limit to testing for the presence of mutagenic action is about 80% to 90% killing of cells. Because of the uncertainty in the actual lethality of treatment in the assay and the fact that mutant frequencies increase as a function of lethality, an acceptable assay for the lack of mutagenic activity must extend to the 5% to 10% relative suspension growth range. There is no maximum toxicity requirement for test materials which clearly show mutagenic activity.

7) An experimental treatment that results in fewer than 2.5 x 10^6 cells by the end of the two-day growth period will not be cloned for mutant analysis.

8) An experimental mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds about 20. These limits avoid problems with the statistical distribution of scoreable colonies among dishes and allows factors no larger than 10 in the adjustment of the observed number of mutant clones to a unit number of cells (10^6) able to form colonies.

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9) Mutant frequencies are normally derived from sets of three dishes for both the mutant colony count and the viable colony count. In order to allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set if the colony numbers in the two dishes differ by no more than about 3-fold.

10) The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions to accept a single assay for evaluation of the test material.

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ASSAY EVALUATION CRITERIA

Mutation assays are initiated by exposing cell cultures to a range of concentrations of test material that is expected, on the basis of preliminary toxicity studies, to span the cellular responses of no observed toxicity to growth to complete lethality within 24 hours of treatment. Then five dose levels are usually selected for completion of the mutation assay. The doses are selected to cover a range of toxicities to growth with emphasis on the most toxic doses. An assay may need to be repeated with different concentrations in order to properly evaluate a test material.

The minimum condition considered necessary to demonstrate mutagenesis for any given treatment is a mutant frequency that exceeds 150% of the concurrent background frequency by at least 10×10^{-6}. The background frequency is defined as the average mutant frequency of the solvent and untreated negative controls. The minimum increase is based on extensive experience which indicates that assay variability increases with higher backgrounds and the calculated minimum increase as defined above is often a repeatable result; statistical analysis for the confidence limits is not yet available.

The observation of a mutant frequency that meets the minimum criterion for a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test material as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions:

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses, but this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears.

- An increase in mutant frequency may be followed by only small or no further increases at higher concentrations or toxicities. However, a decrease in mutant frequency to values below the minimum criterion is not acceptable in a single assay for classifying the test material as a mutagen. If the mutagenic activity at lower concentrations or toxicities was large, a repeat assay will be performed to confirm the mutagenic activity.

- If an increase of about two times the minimum criterion or greater is observed for a single dose near the highest testable toxicity, as defined in the Assay Acceptance Criteria, the test material will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.

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- For some test materials, the correlation between toxicity and applied concentration is poor. The proportion of the applied material that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent relative growth), can be used to establish whether the mutagenic activity is related to an increase in effective treatment. A negative correlation with dose is acceptable only if a positive correlation with toxicity exists. An apparent increase in mutagenic activity as a function of decreasing toxicity is not acceptable evidence for mutagenicity.

A test material will be evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to toxicity causing 5% to 10% relative suspension growth. If the test material is relatively nontoxic, the maximum applied concentrations will normally be 10 mg/ml (or 10 µl/ml) for water-soluble materials or 1 mg/ml (or 1 µl/ml) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response, as discussed above, the test material will be evaluated as nonmutagenic in this assay system.

The ASSAY ACCEPTANCE AND EVALUATION CRITERIA are presented to acquaint the sponsor with the considerations used by the study director to determine assay validity and the mutagenic activity of the test material. This presentation may not encompass all test situations, and the study director may use other criteria, especially when data from several repeat assays are available, to arrive at a conclusion. The report will provide the reasoning involved when departures from the above descriptions occur.

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Rohm and Haas Protocol No. 80P-255

PROTOCOL NO. 431
EDITION 2

5145

L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

This assay evaluates the mutagenic potential of test articles in a specific locus mutation assay using mammalian cells. The test can also be used to confirm, in mammalian cells, presumptive mutagenicity demonstrated in microbial screens.

Time required for completion of the test and submission of the final report is about 12 weeks from receipt of test article. One gram (or equivalent) of test article is requested although smaller samples can be evaluated. Sponsor-prescribed dose levels are acceptable, and two or three replicates per dose level may be specified in special protocols.

1. OBJECTIVE

The objective of this study is to evaluate a test article for its ability to induce forward mutation in the L5178Y TK+/- mouse lymphoma cell line, as assessed by colony growth in the presence of 5-bromo-2'-deoxyuridine (BrdU) or 5-trifluorothymidine (TFT).

2. RATIONALE

Thymidine kinase (TK) is a cellular enzyme that allows cells to salvage thymidine from the surrounding medium for use in DNA synthesis. If a thymidine analog such as BrdU is included in the growth medium, the analog will be phosphorylated via the TK pathway and incorporated into DNA, eventually resulting in cellular death. Cells which are heterozygous at the TK locus (TK+/-) may undergo a single step forward mutation to the TK-/- genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by de novo synthetic pathways that do not involve thymidine as an intermediate. The basis for selection of the TK-/- mutants is the lack of any ability to utilize toxic analogs of thymidine, which enable only the TK-/- mutants to grow in the presence of BrdU. Cells which grow to form colonies in the presence of BrdU are therefore assumed to have mutated, either spontaneously or by the action of a test article, to the TK-/- genotype.

3. MATERIALS

A. Indicator Cells

The mouse lymphoma cell line to be used in this assay, L5178Y TK+/-, is derived from the Fischer L5178Y line by Dr. Donald Clive. Stocks are maintained in liquid nitrogen, and laboratory cultures are periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (spontaneous frequency) of TK-/- mutants to as low level as possible, cell cultures are exposed to conditions which select against the TK-/- phenotype and are then returned to normal growth medium for 3 or more days before use.

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L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

3. MATERIALS (Continued)

B. Media

The cells are maintained in Fischer's mouse leukemia medium supplemented with L-glutamine, sodium pyruvate and horse serum (10% by volume). Cloning medium will consist of the preceding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium will be cloning medium containing 100 µg/ml of BrdU or 3 µg/ml of TFT.

C. Control Articles

1. Negative control articles

A negative control article consisting of assay procedures performed on untreated cells will be utilized in all cases. If the test article is not soluble in growth medium, an organic solvent (normally dimethylsulfoxide) will be used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium will also be assayed as the solvent negative control article to determine any effects on survival or mutation caused by the solvent alone. For test articles assayed with activation, the untreated and solvent negative control articles will include the activation mixture.

2. Positive control articles

Ethylmethane sulfonate (EMS) is highly mutagenic via alkylation of cellular DNA and will be used at 0.5 µliters/ml as a positive control article for nonactivation studies.

Dimethylnitrosamine (DMN) requires metabolic activation by microsomal enzymes to become mutagenic and will be used at 0.3 µliters/ml as a positive control article for assays performed with activation.

D. Sample Forms

All types of test articles can be evaluated in the mouse lymphoma assay. Solid articles will be dissolved in growth medium, if possible, or in dimethylsulfoxide (DMSO), unless another solvent is requested. Liquids will be tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent. Highly volatile liquids which must be tested in the vapor phase will be added to an airtight container of fixed volume and allowed to completely volatilize in the presence of the exposed cell population. Gases will be treated by measuring known volumes of gas into an airtight container of fixed volume.

L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

4. EXPERIMENTAL DESIGN

A. Dose Selection

The solubility of the test article in growth medium and/or DMSO will be determined first. Then a wide range of test article concentrations will be tested for cytotoxicity, starting with a maximum applied dose of 1 to 5 mg/ml for solid test articles or 1 to 5 µliters/ml for liquid test articles and using twofold dilution steps. After an exposure time of 4 hours, the cells will be washed and a viable cell count obtained the next day. Relative cytotoxicities expressed as the reduction in growth compared to the growth of untreated cells will be used to select 7 to 10 doses that cover the range from 0 to 50-90% reduction in 24-hour growth. These selected doses will subsequently be applied to cell cultures prepared for mutagenicity testing, but only 4 or 5 of the doses will be carried through the mutant selection process. This procedure compensates for daily variations in cellular cytotoxicity and ensures the choice of 4 or 5 doses spaced from 0 to 50-90% reduction in cell growth.

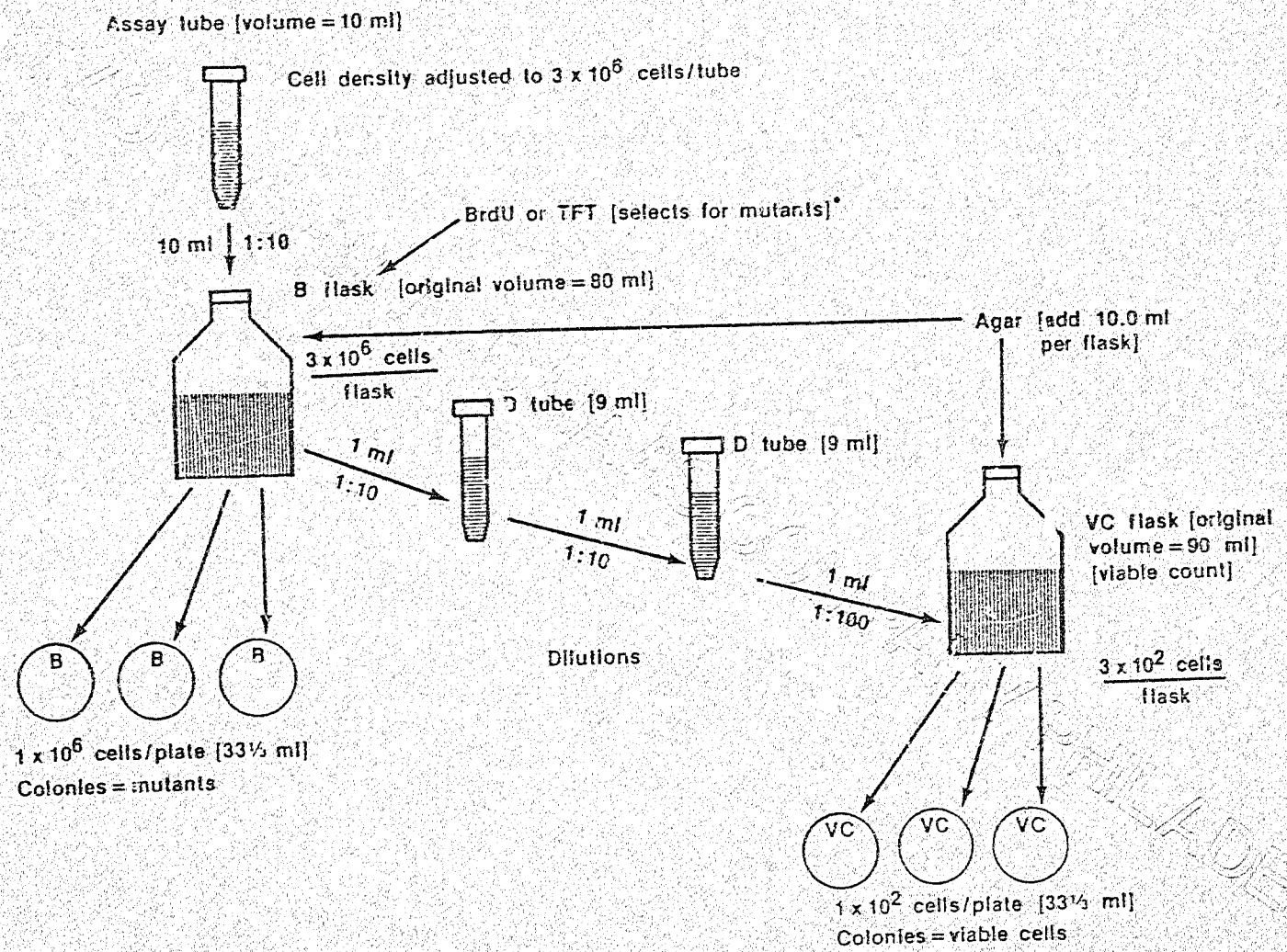
B. Mutagenicity Testing

1. Nonactivation assay

The procedure to be used is based on that reported by Clive and Spector (1975) and is summarized as follows. Cultures exposed to the test article for 4 hours at the preselected doses will be washed and placed in growth medium for 2 or 3 days to allow recovery, growth and expression of the induced TK-/- phenotype. Cell counts will be determined daily and appropriate dilutions made to allow optimal growth rates.

At the end of the expression period, 3×10^6 cells for each selected dose will be seeded in soft agar plates with selection medium; and resistant (mutant) colonies will be counted after 10 days incubation. To determine the actual number of cells capable of forming colonies, a portion of the cell suspension will also be cloned in normal medium (nonselective). The ratio of resistant colonies to total viable cell number will be the mutant frequency.

A detailed flow diagram for the mutation assay is provided in Figure 1.



*Added after removal of 1 ml for viable count dilutions.

FIGUR[illegible] [illegible]PH[illegible] CLONING FLOW CHART

4. EXPERIMENTAL DESIGN (Continued)

B. Mutagenicity Testing

2. Activation assay

The activation assay can be run concurrently with the nonactivation assay. The only difference would be the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the 4-hour treatment period. CORE consists of NADP (sodium salt) and isocitric acid. The final concentrations of the activation system components in the cell suspension will be: 2.4 mg NADP (sodium salt) per milliliter; 4.5 mg isocitric acid per milliliter; and 50 µliters S9 per milliliter.

C. Preparation of 9,000 x g Supernatant (S9)

Fischer 344 male rats will normally be used as the source of hepatic microsomes. Induction with Aroclor 1254, or another agent specified by the Sponsor, will be performed by injections 5 days prior to kill. After decapitation and bleeding, the liver will be immediately dissected from the animal using aseptic technique and placed in ice-cold 0.25M sucrose buffered with ~~tris~~ phosphate at pH 7.4. When an adequate number of livers has been obtained, the collection will be washed twice with fresh buffered sucrose and completely homogenized. The homogenate will be centrifuged for 10 minutes at 9,000 x g in a refrigerated centrifuge, and the supernatant (S9) from this centrifuged sample will be retained and frozen at -80°C until used in the activation system. The S9 fraction may be obtained from induced or noninduced rats or other species, as requested.

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5. ASSAY ACCEPTANCE CRITERIA

An assay normally will be considered acceptable for evaluation of test results only if all of the criteria given on the following page are satisfied. The activation and nonactivation portions of the mutation assays are usually performed concurrently, but each portion is, in fact, an independent assay with its own positive and negative controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

5. ASSAY ACCEPTANCE CRITERIA (Continued)

- The average absolute cloning efficiency of the negative controls (average of the solvent and untreated controls) should be between 70 and 130%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and cell division during unavoidable delays between the counting and cloning of many cell cultures. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50 to 70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range will be conditionally acceptable and dependent on the scientific judgment of the Study Director. All assays below 50% cloning efficiency will be unacceptable.

- The solvent and untreated negative controls normally have the same growth rates and cloning efficiencies within experimental error. An unusual effect by the solvent, therefore, indicates an abnormal cell state or an excessive amount of solvent in the growth medium. An assay will be unacceptable if the average percent relative growth of solvent controls is less than about 70% of the untreated control value.

- The average negative control suspension growth factor should not be less than about 15. The optimal value is 25, which corresponds to fivefold increases in cell number for each of the 2 days following treatment of the experimental cultures.

- The background mutant frequency (average of the solvent and untreated negative controls) is calculated separately for concurrent activation and nonactivation assays, even though the same population of cells is used for each assay. The activation negative controls contain the S9 activation mix and typically have a somewhat higher mutant frequency than the nonactivation negative controls. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is 5×10^{-6} to 50×10^{-6}. Assays with backgrounds outside this range are not necessarily invalid but will not be used as primary evidence for evaluation of a test article. These assays can provide supporting evidence.

L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

5. ASSAY ACCEPTANCE CRITERIA (Continued)

- A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. The normal range of mutant frequencies induced by 0.5 µliters/ml EMS (nonactivation assay) is 300 to 800 x 10^{-6}; for 0.3 µliters/ml DMN (activation assay) the normal range is 200 to 800 x 10^{-6}. The concurrent background frequencies have been subtracted from these values. These ranges are broad primarily because the effective treatment with these agents is variable between assays. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria. If the test article appears to have no, or only weak, mutagenic activity, an acceptable assay must have a positive control mutant frequency above the lower limits of the normal range. Assays in which the normal range is exceeded may require further interpretation by the Study Director.

- For test articles with little or no mutagenic activity, an assay must include applied concentrations that reduce the suspension growth to 5 to 10% of the average solvent control or reach the maximum applied concentrations given in the evaluation criteria. Suspension growth is a combined measure of cell death and reduced growth rates. A 5% relative suspension growth, therefore, could correspond to 90% killing followed by growth of the survivors at one-half the normal rate for 1 day and normal growth for the second day. At the other extreme, this condition could be obtained by no killing and complete inhibition of growth for 2 days. A reasonable limit to testing for the presence of mutagenic action is about 80 to 90% killing of cells. Because of the uncertainty in the actual lethality of treatment in the assay and the fact that mutant frequencies increase as a function of lethality, an acceptable assay for the lack of mutagenic activity must extend to the 5 to 10% relative suspension growth range. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.

- An experimental treatment that results in fewer than 2.5 x 10^6 cells by the end of the 2-day growth period will not be cloned for mutant analysis.

- An experimental mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds about 20. These limits avoid problems with the statistical distribution of scorable colonies among dishes and allow factors no larger than 10 in the adjustment of the observed number of mutant clones to a unit number of cells (10^6) able to form colonies.

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L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

5. ASSAY ACCEPTANCE CRITERIA (Continued)

- Mutant frequencies are normally derived from sets of three dishes for both the mutant colony count and viable colony count. To allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set if the colony numbers in the two dishes differ by no more than about threefold.

- The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions to accept a single assay for evaluation of the test article.

6. ASSAY EVALUATION CRITERIA

Mutation assays are initiated by exposing cell cultures to a range of concentrations of test article that is expected, on the basis of preliminary toxicity studies, to span the cellular responses of no observed toxicity to growth to complete lethality within 24 hours of treatment. Then five dose levels are usually selected for completion of the mutation assay. The doses are selected to cover a range of toxicities to growth with emphasis on the most toxic doses. An assay may need to be repeated with different concentrations to properly evaluate a test article.

The minimum condition considered necessary to demonstrate mutagenesis for any given treatment will be a mutant frequency that exceeds 150% of the concurrent background frequency by at least 10×10^{-6}. The background frequency is defined as the average mutant frequency of the solvent and untreated negative controls. The minimum increase is based on extensive experience which indicates that assay variability increases with higher backgrounds, and this calculated minimum increase is often a repeatable result. Statistical analysis for the confidence limits is not yet available.

The observation of a mutant frequency that meets the minimum criterion for a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test article as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions:

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses, but this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears.

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L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

6. ASSAY EVALUATION CRITERIA (Continued)

- An increase in mutant frequency may be followed by only small or no further increases at higher concentrations or toxicities. However, a decrease in mutant frequency to values below the minimum criterion is not acceptable in a single assay to classify the test article as a mutagen. If the mutagenic activity at lower concentrations or toxicities is large, a repeat assay will be performed to confirm the mutagenic activity.

- If an increase of about two times the minimum criterion or greater is observed for a single dose near the highest testable toxicity, as defined in Section 5 (Assay Acceptance Criteria), the test article will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.

- For some test articles the correlation between toxicity and applied concentration is poor. The proportion of the applied article that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent relative growth), can be used to establish whether the mutagenic activity is related to an increse in effective treatment. A negative correlation with dose is acceptable only if a positive correlation with toxicity exists. An apparent increase in mutagenic activity as a function of decreasing toxicity is not acceptable evidence for mutagenicity.

A test article will be evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to toxicity causing 5 to 10% relative suspension growth. If the test article is relatively nontoxic, the maximum applied concentrations will normally be 10 mg/ml (or 10 µliters/ml) for water-soluble articles or 1 mg/ml (or 1 µliter/ml) for articles in organic solvents. If a repeat assay does not confirm an earlier, minimal response as discussed above, the test article will be evaluated as nonmutagenic in this assay system.

The Assay Acceptance and Evaluation Criteria are presented to acquaint the Sponsor with the considerations used by the Study Director to determine assay validity and mutagenic activity of the test article. This presentation may not encompass all test situations, and the Study Director may use other criteria to arrive at a conclusion, especially when data from several repeat assays are available. The report will provide the reasoning involved when departures from the above descriptions occur.

5145

L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

7. REFERENCE

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.

8. RECORDS TO BE MAINTAINED

All raw data, protocol, modifications, test article weight and dispensation records and correspondence between LBI and the Sponsor will be maintained in a central file within the Department of Genetics and Cell Biology. These records will be filed under Departmental assay number and held up to 2 years following submission of the final report to the Sponsor. After 2 years they will be transferred to the LBI Archives for permanent storage.

9. TEST ARTICLE

The test article to be used in this study is TD 80-16
The identity, strength, quality and purity of each batch, with verifying documentation:

___ will be determined by LBI (at additional cost).
✓ will be determined by the Sponsor.

10. PERSONNEL

The Study Director will be Brian C. Myhr, Ph.D. Resumes for personnel are maintained and available on request.

11. TESTING FACILITIES AND SPONSOR

This study will be conducted at Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, MD 20795. The Sponsor of this study is

12. STUDY DATES

The anticipated date of study initiation is within 3 weeks from receipt of test article and letter of authorization.
The anticipated date of study completion is within 6 weeks after study initiation.
The anticipated date for submission of the final report to the Sponsor is within 30 days of study completion.

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L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY

13. CHANGES OR REVISIONS

Any changes or revisions of this approved protocol will be documented, signed by the Study Director, dated and maintained with this protocol. The Sponsor will be notified prior to any change or revision.

14. APPROVAL OF PROTOCOL

Date: May 1, 1980 Sponsor: Kay L McCarthy

Date: April 21, 1980 Study Director: Brian C. Myhr

ROHM AND HAAS COMPANY PHILADELPHIA

PROJECT CHANGE SHEET

LBI Project No.: 20989	Change No.: 1
Assay No.: 5145	Date: 1/22/81
LBI Safety No.: 6035	Compound: TD 80-16

Sponsor:	Study Director:	Departments Involved:
Rohm & Haas Company	Brian C. Myhr Sign. Brian C. Myhr	Genetics

1. Title and Description of Study: MLA Protocol No. 431

2. Description of Change with Effective Date: 1/22/81

According to discussion with client on January 2, 1981, by telephone, this study will be supplementary to an earlier study. No solubility or toxicity tests will be performed - refer to earlier study for compound handling.

Perform with S9 activation only for following conditions:

4 solvent controls

2 positive controls (EMN)

Doses: 100, 150, 175 (dupl.), 200, 225, 250, 275 (triplicate), nl/ml, 300 nl/ml (dupl.)

Plan to done approx. 10-12 test doses - see ne for selection.

3. Justification for Change:

Attempt to define the response to highly toxic treatments for a compound that has a sharp survival curve. Protocol change meets Sponsor's approval.

DISTRIBUTION: White, Client File; Yellow, Principal Investigator; Pink, Report Folder.

COMPANY CONFIDENTIAL

Q.A. Inspection Statement
(reference 21 CFR 58.35(b)(7))

PROJECT 20989 LBI Assay No. 5145

TYPE of STUDY Mouse Lymphoma Forward Mutation Assay

This final study report was reviewed by the LBI Quality Assurance Unit on Nov 14, 1986 + March 19, 1987. A report of findings was submitted to the Study Director and to Management on March 20, 1987.

The short-term nature of this study precluded inspection while it was in process. The Quality Assurance Unit inspects an in-process study of this type approximately once per month to assure that no significant problems exist that are likely to affect the integrity of this type of study.

Patrice M. Cuccaro
Auditor, Quality Assurance Unit

DEPOSITION EXHIBIT AND PRODUCTION COPY PHILADELPHIA

Copy of Original Data for 81RC-52

KLM 10/23/84

MOUSE LYMPHOMA SOLUBILITY TEST

CLIENT: Rohm & Haas LBI ASSAY NO. 5145 TECH: JJ

CLIENT'S COMPOUND CODE: TD 80-16 DATE: 5-27-80

DESCRIPTION OF TEST COMPOUND: Clear yellow liquid

LBI SAFETY NO. 6035 PROTOCOL NO. 431

INFORMATION PROVIDED BY SPONSOR:

I. TEST: H_2O 100 (UL) MG TEST COMPOUND + 0.9 ML H_2O = 100.0 (UL) MG/ML

APPEARANCE OF THIS STOCK (solubility, clarity, color, precipitate, viscosity, corrosiveness, etc.):

Globules in suspension.

FOR SOLID: WEIGHT OF TEST COMPOUND + PAPER = N.A.

WEIGHT OF PAPER = "

WEIGHT OF TEST COMPOUND = "

II. TEST: DMSO 100 (UL) MG TEST COMPOUND + 0.9 ML DMSO = 100.0 (UL) MG/ML

APPEARANCE OF THIS STOCK (describe as in I.):

Clear pale-yellow colored liquid solution formed quickly.

FOR SOLID: WEIGHT OF TEST COMPOUND + PAPER = N.A.

WEIGHT OF PAPER = "

WEIGHT OF TEST COMPOUND = "

III. FURTHER TESTING (DILUTIONS OF ABOVE STOCKS, ALTERNATE SOLVENTS: ETHANOL, ACETONE, MEDIUM, ETC.)

Note: #5145 appeared to interact with plastic in tubes. Suggest using glass tubes + pipets for handling the compound.

IV. SOLVENT AND CONCENTRATION OF STOCK RECOMMENDED FOR MOUSE LYMPHOMA FORWARD MUTATION ASSAY BASED ON ABOVE OBSERVATIONS:

SOLVENT: DMSO STOCK: 100 ul/ml TECH: JJ DATE: 5-27-80

PROPERTY OF ROHM AND HAAS COMPANY PHILADELPHIA

FORM 4685 REV. 10/78

MOUSE LYMPHOMA CYTOTOXICITY TEST

CLIENT: Rohm + Haas LBI ASSAY NO. 5145 TECH: MEH

CLIENT'S COMPOUND CODE: TD-80-16 DATE: 5-28-80

DESCRIPTION OF TEST COMPOUND: Clear yellow liquid TRIAL NO. I

LBI SAFETY NO. 6035 PROTOCOL NO. 4311

SOLUBILITY TEST: DATE 5-28-80 SOLVENT CHOSEN DMSO SOLUBILITY Excellent

STOCK CONCENTRATION CHOSEN 100.0 ul/ml

PREPARATION OF STOCK SOLUTION/SUSPENSION: 100.0 (UL) ADDED TO 0.9 ml DMSO

FOR SOLID: WEIGHT OF TEST COMPOUND + PAPER = N/A

WEIGHT OF PAPER = N/A

WEIGHT OF TEST COMPOUND = N/A

APPEARANCE OF STOCK (color, clarity, viscosity, etc.):

Clear pale-yellow liquid solution formed quickly. Jf 5-28-80

0.1 ml stock + 9.9 ml total volume = ~~100.0~~ 1.0 ~~ug~~ (ul)/ml final concentration Arith.

LBI/BY MOUSE LYMPHOMA STOCK CELLS USED: LBI-2 STOCK CELL DENSITY: 1 x 10

SEEDED: 3.8 ml/TUBE TO GIVE 3 x 10^6 CELLS/TUBE

GROWTH MEDIUM BATCH NO. N-192 SHAKER 10066 CENTRIFUGE 9163

TREATMENT TIMES (EXPOSURE TO TEST COMPOUND): 4 HOURS, TIMES: 5

DOSED 9:20 am WASHED 1:20 pm

TUBE		DOSE mmol/l	APPEARANCE OF DOSED TUBES: DURING TREATMENT	APPEARANCE OF DOSED TUBES: NEXT DAY (BEFORE COUNT)	VIABLE CELL COUNT: NO. OF CELLS COUNTED *	VIABLE CELL COUNT: CALCULATED DENSITY**
621	1.	1.95 "	normal color no ppt	Normal Color No ppt.		
622	2.	3.901 "				
623	3.	7.801 "				
624	4.	15.6 "				
625	5.	31.3 "			61	6.1
626	6.	62.5 "	normal color small amt. ppt	Pink color No ppt	0	0
627	7.	125 "	cloudy color small amt ppt			
628	8.	250 "	cloudy color large amt ppt			
629	9.	500 "	cloudy color ppt			
630	10.	1000 "				
631	11.	Negative Control	normal color no ppt	Normal color No ppt.	76	7.6
632	12.	Solvent Control		"	73	7.3
			TECH: MEH DATE: 5-28-80 TIME: 9:30 am	TECH: Jf DATE: 5-29-80 TIME: N.R.	TECH: Jf DATE: 5-29-80 TIME: N.R.	TECH: Jf DATE: 5-29-80 TIME: N.R.

rounding error BCM 10/17/80

Not counted - Visual inspection of growth BCM 11-17-80

By visual inspection.

*IN 5 SQUARES OF HEMACYTOMETER

**NO. x 10^6 cells/tube

Jf 5-29-80

Note: #5145 appeared to interact with plastic in tubes. Glass tubes and pipets were used for handling stocks. MEH 5/28/80. From solubility test

There was no interaction seen with closed tubes. Jf 5-28-80.

000037

N.R. = Not recorded on 5-29-80. should have been 9:30am ± 2 hrs. Jf 11-17-80.

FORM 4685 REV. 10/78

REPEAT TEST AUTHORIZATION
after Trial II

Type of Test MLA Protocol No. 431

Assay # 5145

This authorizes a repeat of ~~all or~~ part of the above study based on the criteria in SOP No. 528 +59 only.

REASON FOR REPEAT: (SOP item No. or written explanation)

Loss of DMN positive control. Pos. Control necessary for apparently ⊖ test material.

COMMENTS:

Doses: 300, 250, 225, 200, 175, 150, 100, 50 ml/kd

B.C. Myh
Study Director
or Designate

8/14/80
Date

000038

FORM 4685 REV. 10/78

PROPERTY OF PORTIM AND HAS BEEN PHILADELPHIA

REPEAT TEST AUTHORIZATION
after Trial III

Type of Test MLA Protocol 431

Assay # 5145

This authorizes a repeat ~~of all or~~ part of the above study based on the criteria in SOP No. 528. +59 rly

REASON FOR REPEAT: (SOP item No. or written explanation)

DMN positive control yielded frequency below 200 x 10^6.

COMMENTS:

Use same dose levels as last trial.
Use new batch of 59 (other than DD027)

B C Myhr 9/9/80
Study Director Date
or Designate

000039

FORM 4685 REV. 10/78

REPEAT TEST AUTHORIZATION
after Trial IV

Type of Test MLA Protocol 431

Assay # 5145

This authorizes a repeat ~~of all or~~ part of the above study based on the criteria in SOP No. 528. ±S9 only

REASON FOR REPEAT: (SOP item No. or written explanation)

Minimum DMN-induced frequency of 200×10^{-6} was not achieved. Results of 9/10/80 assay showed erratic toxicity and generally elevated mutant freq. difficult to interpret.

COMMENTS:

Use same dose levels. Take every care to handle S9 and DMN to get the positive control up!!

B. C. Myhr
Study Director
or Designate

9/26/80
Date

000040

FORM 4685 REV. 10/78

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay#: 5145 Initiation Date: 6-10-80

Client's Compound Code: TD 80-16 LBI Safety#: 6035 Trial#: I

Description: Clear yellow liquid Technician: JF

Solvent: DMSO Solubility: Excellent Protocol#: 431

Tube#	[Dose] µl/ml	Day 0 Viable Cell Count*	Day 0 Calculated Density**	Day 1 Viable Cell Count*	Day 1 Calculated Density**	Day 2 Tubes Cloned [L+]
521 –	0.780	87	8.7	100	10.0	✓
522 –	1.56	84	8.4	85	8.5	T
523 –	3.13	68	6.8	112	11.2	T
524 –	6.25	83	8.3	106	10.6	T
525 –	12.5	89	8.9	101	10.1	T
526 –	25.0	73	7.3	74	7.4	T
527 –	37.5	59	5.9	114	11.4	✓
528 –	50.0	88	8.8	124	12.4	✓
529 –	75.0	55	5.5	135	13.5	✓
530 –	100	23	2.3 x	79	7.9	✓
541 –	Negative Control	94	9.4	99	9.9	✓
543 –	Solvent Control	94	9.4	124	12.4	✓
544 –	Solvent Control	112	11.2	120	12.0	✓
547 –	Positive Control	62	6.2	92	9.2	T
548 –	Positive Control	57	5.7	73	7.3	✓
Nonactivation	Tech:	JF		JF		JF
	Date:	6-11-80		6-12-80		6-12-80
	Time:	9:30 am Split 10:30 am		9:30 am Split 10:30 am		

* Viable Cell Count = actual number of viable cells counted in 5 squares of hemacytometer.

**Calculated cell density = calculated number of cells x 10^5 cells/ml; diluted to 3 x 10^5 cells/ml after count.

x Not diluted [split] to 3 x 10^5 cells/ml

Positive Controls: -Li = EMS 0.5 µl/ml [ethyl methanesulfonate]

-Li = Without activation
C = Contaminated
T = Terminated [due to too much or too little observed toxicity]
L = Lost [due to technical error]

LBI-0319 R 2/79

000041

Page 1

FORM 4685 REV. 10/78

MOUSE LYMPHOMA ASSAY

Client: Rohm + Haas LBI Assay# 5145 Initiation Date: 6-10-80

Client's Compound Code: TD 80-16 LBI Safety# 6035 Trial# I

Description: Clear yellow liquid Technician: Jf

Solvent: DMSO Solubility: Excellent Protocol#: 431

#Tube	[Dose] µl/ml	Day 0	Day 1 Viable Cell Count*	Day 1 Calculated Density**	Day 2 Viable Cell Count*	Day 2 Calculated Density**	Tubes Cloned (✓)
531 +	0.780		58	5.8	129	12.9	T
532 +	1.56		64	6.4	118	11.8	T
533 +	3.13		56	5.6	92	9.2	T
534 +	6.25		72	7.2	119	11.9	T
535 +	12.5		64	6.4	142	14.2	T
536 +	25.0		82	8.2	70	7.0	✓
537 +	37.5		69	6.9	106	10.6	✓
538 +	50.0		60	6.0	92	9.2	✓
539 +	75.0		46	4.6	138	13.8	✓
540 +	100		46	4.6	128	12.8	✓
542 +	Negative Control		10.5	10.5	105	10.5	✓
545 +	Solvent Control		87	8.7	85	8.5	✓
546 +	Solvent Control		65	6.5	73	7.3	✓
549 +	Positive Control		57	5.7	70	7.0	T
550 +	Positive Control		53	5.3	64	6.4	✓
Activation: Arocior-Induced Fisher 344 Rat Liver S-9	Tech:		Jf		Jf		Jf
	Date:		6-11-80		6-12-80		6-12-80
	Time:		9:30am	Spl/+10:30am	9:30am	Spl/+10:30am	

*Viable Cell Count = actual no. of viable cells counted in 5 squares of hemacytometer

**Calculated cell density = calculated number of cells x 10^5 cells/ml; diluted to 3 x 10^5 cells/ml after count.

✗ Not diluted [split] to 3 x 10^5 cells/ml

Positive Controls: +LI = DMN 0.3 µl/ml [Dimethylnitrosamine]

+LI = Activation mixture added to tubes: S-9 liver homogenate and cofs.
C = Contaminated
T = Terminated [due to too much or too little observed toxicity]
L = Lost [due to technical error]

000042

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 6-10-80

Tech.: Jef

Stock Cells Used: LBI-2 date cleansed of mutants 6-4-80 THMG batch# 5

Stock Cells Viable Density 0.696 x10^6 cells/ml; 4.3 ml/tube = 3.0 x 10^6 cells/tube

Compound: Description Clear yellow liquid

Stock Solution: 100 µl ~~mg~~* of #5145 + 9.9 ml of DMSO =

Description: Clear colorless liquid formed quickly. [solubility, appearance, etc.]

Final Concentration of 10 µl/ml /~~mg~~* = A.

Used to dose tube nos. 521-526, 528, 530; 531-536, 538, 540. Clarification by BCM 7-15-82

Serial 1:2 dilutions were made from A.

B = 0.6 ml A + 0.2 ml DMSO = 7.5 µl/ml. 1:2 dilution made from B

Used to dose tube nos. 527, 529, 537 and 539. Clarification by BCM 7-15-82

Appearance of dosed tubes:

#521-528 had normal color & no precipitate

#529 & 530 had a cloudy color

#531-540 had normal cloudy color from S-9 mix; no detectable ppt.

Note: All tubes received 0.1 ml of compound + solvent except:

All dilutions made 1:2 in same solvent except:

Stock B.

Negative Controls received F_0 medium with and without S-9, core.

Solvent Controls received 0.1 ml of DMSO w/wo S-9 and core.

Positive Controls received:

EMS: 0.1ml dissolved in 9.9ml of H_2O, ~~filter-sterilized~~*, dosed with 0.5 ml/tube without activation.

DMN: 0.1ml dissolved in 9.9ml of H_2O, ~~filter-sterilized~~*, dosed with 0.3 ml/tube with activation.

Selective Agent: BrdU [5-Bromo-2'-deoxyuridine] 100 µg/ml final concentration.

S-9 liver homogenate, batch# 00027, dose 0.5 ml/tube final concentration.

Core, batch# 40, dose 1.0 ml/tube final concentration.

Activation added: Core and S-9 mixed immediately before addition to tubes.

Note: 10-ml volume includes the volume of S-9 and Core.

4 hour exposure: tubes dosed 8:45 am, washed 12:45 pm

controls dosed 8:45 am, washed 12:45 pm

Centrifuge LBI# 9163

Shaker LBI# 10056

000043

LBI-0321 R 2/79 N.A. Jef 6-10-80

FORM 4005 REV. 10/78

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 6-10-80

Tech.: [illegible]

Lot Numbers:

1. Growth Medium, Batch Number N-195
   - F_0 Medium [HEM Research] # 18916
   - Horse Serum [KC Biologicals] # 200002
   - L-Glutamine [GIBCO] # 11K6002
   - Sodium Pyruvate Solution # N-19 4-15-80
   - Pluronic Solution # N-12 2-1-80
   - Pen/Strep [GIBCO]# R195102
2. Cloning Medium, Batch Number N-121
   - F_0 Medium [HEM Research] # 18916
   - Horse Serum [KC Biologicals] # 200002
   - L-Glutamine [GIBCO] # 11K6002
   - Sodium Pyruvate Solution # N-19 4-15-80
   - Pen/Strep [GIBCO]# R195102
   - Gentamycin [Schering]# 96RB1
3. DMSO [Eastman] # BSA
4. Agar Noble [Difco] # N-36 665718
5. BrdU [Sigma] # 89C-0159
6. Tissue culture plates used in cloning
   - Supplier: Costar Lot# L06A111
7. EMS Eastman A6G
8. DMN Aldrich 62-75-9

Notes: Glass tubes and pipets were used to handle the test material and solutions in DMSO.
Note added by BCM 7-15-82

PHILADELPHIA COMPANY

5-29-80

Rohm + Haas # 5145 Trial I ± DMSO

Doses:

1 0.780 nl/ml
2 1.56
3 3.13
4 6.25
5 12.5
6 25.0
7 37.5
8 50.0
9 75.0
10 100

Stock: 10,000 nl/ml = 10 ul/ml = 100 ul/10 ml = 0.1 ml # 5145 + 9.9 ml DMSO

Dilutions: 100 $\xrightarrow[(0.5\,ml + 0.5\,ml\ DMSO)]{1:2}$ 50 $\xrightarrow[etc.]{1:2}$ 25 → 12.5 → 6.25 → 3.13 → 1.56 → 0.78

100 $\xrightarrow[(0.6\,ml + 0.2\,ml\ DMSO)]{3:4}$ 75 $\xrightarrow[(0.5\,ml + 0.5\,ml\ DMSO)]{1:2}$ 37.5

Dose: 0.1 ml / tube

Notes: 1. Use glass tubes + pipets for handling the stocks.
2. Clone a range of doses.
3. Carefully describe dosed tubes.

ROHM AND HAAS COMPANY PHILADELPHIA

FORM 4685 REV. 10/78

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas Cloning Efficiency* [C.E.] of Assay 98.8 ~~95.7~~ % JH 6-23-80

LBI Assay No. 5145 C.E.* Nonactivation 108.6 %C.E.* Activation ~~89~~ 82.8 % JH 6-23-80

Agar Plate Colony Counts:

µl/ml Dose	Plate#	B Plate Counts	B Total	VC Plate Counts	VC Total	B/VC
0.780 –	521	14, 11, 16	41	49, 51, 114	214	0.19
37.5 –	527	12, 20, 16	48	35, 94, 97	226	0.21
50.0 –	528	13, 17, 12	42	60, 70, 108	238	0.18
75.0 –	529	14, 12, 17	43	49, 65, 93	207	0.21
100 –	530	13, 9, 15	37	51, 58, 75	184	0.20
25.0 +	536	19, 15, 31	65	83, 95, 144	322	0.20
37.5 +	537	21, 14, 31	66	51, 70, 66	187	0.35
50.0 +	538	28, 32, 40	100	~~36~~, 60, 120	216	0.46
75.0 +	539	19, 27, 29	75	76, 115, ~~(95)~~ (96)c	~~286~~ 287	0.26 JH 6-23-80
100 +	540	16, 22, 39	77	41, 62, 110	213	0.36
N –	541	11, 11, 13	35	60, 149, 129	338	0.10
N +	542	13, 9, 17	39	47, 94, 88	229	0.17
S –	543	10, 10, 12	32	45, 112, 144	301	0.11
S –	544	29, 6, 9	44	57, 127, 154	338	0.13
S +	545	20, 23, 28	71	62, 98, 123	283	0.25
S +	546	8, 23, 28	59	57, 98, ~~(74)~~ (78)c	~~289~~ 233	~~0.20~~ 0.25 JH 6-23-80
P –	548	66, 106, 168	340	15, 23, 27	65	5.23
P +	550	35, 33, 39	107	8, 15, 21	44	2.43

Plates Cloned: Dilutions Tech. JH 11 am Incubator Wedco LBI# 9259 Top [serial#]

Date: 6-12-80 Pouring Tech. JH 12 pm Colony Counter Biotran LBI No. 8764

Plates Removed from Incubator: Date: 6-23-80 Settings: Size: 0.2

Plates Counted By: Tech.: JH Sensitivity: 8.75

Date: 6-23-80 Area: 5100

Calculations by: Tech: V.B. *C.E. based on average of solvent and untreated negative controls divided by 3.

Calculations Reviewed by: JH

000046

LBI-0323 R 2/70 ( )c = 1 contaminated plate; Ave. of 2. JH 6-23-80.

FORM 4685 REV. 10/70 Page 5

MOUSE LYMPHOMA ASSAY

Client: Rohm + Haas LBI Assay#: 5145 Initiation Date: 7/21/80

Client's Compound Code: JD 80-16 LBI Safety#: 6035 Trial#: II*

Description: Clear yellow liquid Technician: MEH

Solvent: DMSO Solubility: Excellent Protocol#: 431

#Tube	[Dose] ul/ml	Day 0 Viable Cell Count*	Day 1 Calculated Density**	Day 2 Viable Cell Count*	Day 2 Calculated Density**	Tubes Cloned ✓
661 +	50.0 "	41	4.1	129	12.9	✓
662 +	100 "	70	7.0	130	13.0	✓
663 +	150 "	30	3.0 *	90	9.0	✓
664 +	200 "	25	2.5 *	33	3.3 *	✓
665 +	250 "	0	0 *	0	0 *	T
666 +	300 "	0	0 *	0	0 *	T
667 +	400 "	0	0 *	0	0 *	T
668 +	500 "	0	0 *	0	0 *	T
669 +	600 "	0	0 *	0	0 *	T
— +						
670 +	Negative Control	53	5.3	165	16.5	✓
671 +	Solvent Control	69	6.9	161	16.1	✓
672 +	Solvent Control	60	6.0	185	18.5	✓
673 +	Positive Control	60	6.0	68	6.8	✓
674 +	Positive Control	50	5.0	91	9.1	T
Activation: Aroclor-induced Fisher 344 Rat Liver S-9	Tech:	J. Malin		MEH		MEH
	Date:	7/22/80		7/23/80		7/23/80
	Time:	10:30 A.M.		11:30 AM	split: 11:45 AM	

*Viable Cell Count = actual no. of viable cells counted in 5 squares of hemacytometer

**Calculated cell density = calculated number of cells x 10^5 cells/ml; diluted to 3 x 10^5 cells/ml after count.

* Not diluted [split] to 3 x 10^5 cells/ml

Positive Controls: +Li = DMN 0.3 µl/ml [Dimethylnitrosamine]

+Li = Activation mixture added to tubes: S-9 liver homogenate and core.
C = Contaminated
T = Terminated [due to too much or too little observed toxicity]
L = Lost [due to technical error]

000047

LBI-0320 R 2/79

MOUSE LYMPHOMA ASSAY

Client: Rohm + Haas LBI Assay#: 5145 Date: 7/21/80

Tech.: MEH

Stock Cells Used: LBI-2 date cleansed of mutants 7/16/80 THMG batch#: 5

Stock Cells Viable Density 0.73 x10^6 cells/ml; 4.1 ml/tube = 3.0 x 10^5 cells/tube

Compound: Description Clear yellow liquid

Stock Solution: 120.0 (µl) ~~mg~~* of #5.15 + 1.88 ml of DMSO =

Description: [solubility, appearance, etc.] Clear colorless liquid forms quickly.

Final Concentration of 60.0 (µl)/ml ~~mg~~*

Appearance of dosed tubes: All tubes had a normal color with no sign of precipitate.

Note: All tubes received 0.1 ml of compound + solvent except:

All dilutions made 1:2 in same solvent except:

600 → (0.5ml + 0.5ml DMSO) 300 —1:2→ 150
(0.5ml + 0.1ml DMSO) 500 —5:6→ 250 —1:2→
(0.4ml + 0.2ml DMSO) 400 —4:6→ 200 —1:2→ 100 —1:2→ 50

Negative Controls received F$_0$ medium with and without S-9, core.

Solvent Controls received 0.1 ml of DMSO w/wo S-9 and core.

Positive Controls received:

* ~~EMS: 0.1ml dissolved in 9.9ml of _____, filter-sterilized, dosed with _____ml/tube without activation.~~

DMN: 0.1ml dissolved in 9.9ml of H_2O, ~~filter-sterilized,~~* dosed with 0.5 ml/tube with activation.

Selective Agent: BrdU [5-Bromo-2-deoxyuridine] 100 µg/ml final concentration.

S-9 liver homogenate, batch# DD627, dose 0.5 ml/tube final concentration.

Core, batch# 40, dose 1.0 ml/tube final concentration.

Activation added: Core and S-9 mixed immediately before addition to tubes.

Note: 10-ml volume includes the volume of S-9 and Core.

6 hour exposure: tubes dosed 9:50 AM, washed 1:50 PM

controls dosed 9:50 AM, washed 1:50 PM

Centrifuge LBI# 9163

Shaker LBI# 10066

* N/A MEH 7/21/80

000048

LBI-0321 R 2/79

FORM 4685 REV. 10/78

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 7/21/80

Tech.: MEH

Lot Numbers:

1. Growth Medium, Batch Number N-206
   - F_0 Medium [HEM Research] # 19003
   - Horse Serum [KC Biologicals] # 268094
   - L-Glutamine [GIBCO] # 11K6002
   - Sodium Pyruvate Solution # N-20 5-19-80
   - Pluronic Solution # N-13 7-11-80
   - Pen/Strep [GIBCO] # 16N6100
2. Cloning Medium, Batch Number N-126
   - F_0 Medium [HEM Research] # 19003
   - Horse Serum [KC Biologicals] # 580007
   - L-Glutamine [GIBCO] # 11K6002
   - Sodium Pyruvate Solution # N-20 5-19-80
   - Pen/Strep [GIBCO] # 16N6100
   - Gentamycin [Schering] # 9GRB1
3. DMSO [Eastman] # B8B
4. Agar Noble [Difco] # N-38 665718
5. BrdU [Sigma] # 89C-0159
6. Tissue culture plates used in cloning
   - Supplier: Costar Lot# 508B042
7. EMS * N/A
8. DMN Aldrich 62-75-9

Notes: 6) Glass tubes & pipettes were used for handling stock MEH 7/21/80

* N/A MEH 7/21/80

000049

LBI-0322 R 2/79

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FORM 4685 REV. 10/78

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 7/21/80

Tech.: MEH

Lot Numbers:

1. Growth Medium, Batch Number CN-206
   - F_0 Medium [HEM Research] # 19003
   - Horse Serum [KC Biologicals] # 268097
   - L-Glutamine [GIBCO] # 11K6002
   - Sodium Pyruvate Solution # CN-20 5-19-80
   - Pluronic Solution # CN-13 7-11-80
   - Pen/Strep [GIBCO] # 16N6100
2. Cloning Medium, Batch Number CN-126
   - F_0 Medium [HEM Research] # 19003
   - Horse Serum [KC Biologicals] # 580007
   - L-Glutamine [GIBCO] # 11K6002
   - Sodium Pyruvate Solution # CN-20 5-19-80
   - Pen/Strep [GIBCO] # 16N6100
   - Gentamycin [Schering] # 9GRB1
3. DMSO [Eastman] # B8B
4. Agar Noble [Difco] # CN-38 665718
5. BrdU [Sigma] # 89C-0159
6. Tissue culture plates used in cloning
   - Supplier: Costar Lot# 108B042
7. EMS * N/A
8. DMN Aldrich 62-75-9

Notes: (1) Glass tubes & pipettes were used for handling stock MEH 7/21/80

* N/A MEH 7/21/80

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LBI-0322 R 2/79

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MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 7/21/80

Tech.: MEH

Lot Numbers:

1. Growth Medium, Batch Number CN-206
   - F_0 Medium [HEM Research] # 19003
   - Horse Serum [KC Biologicals] # 268097
   - L-Glutamine [GIBCO] # 11K6002
   - Sodium Pyruvate Solution # CN-20 5-19-80
   - Pluronic Solution # CN-13 7-11-80
   - Pen/Strep [GIBCO] # 16N6100
2. Cloning Medium, Batch Number CN-126
   - F_0 Medium [HEM Research] # 19003
   - Horse Serum [KC Biologicals] # 280007
   - L-Glutamine [GIBCO] # 11K6002
   - Sodium Pyruvate Solution # CN-20 5-19-80
   - Pen/Strep [GIBCO] # 16N6100
   - Gentamycin [Schering] # 9GRB1
3. DMSO [Eastman] # B8B
4. Agar Noble [Difco] # CN-38 665718
5. BrdU [Sigma] # 89C-0159
6. Tissue culture plates used in cloning
   - Supplier: Costar Lot# 108B042
7. EMS * N/A
8. DMN Aldrich 62-75-9

Notes: (1) Glass tube & pipettes were used for handling stock MEH 7/21/80

* N/A MEH 7/21/80

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MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas Cloning Efficiency* [C.E.] of Assay 68.3 %

LBI Assay No. 5145 † C.E.* Nonactivation MEH 8/1/80 68.3 % C.E.* Activation 68.3 %

Agar Plate Colony Counts:

µl/ml Dose	Plate#	B Plate Counts	B Total	VC Plate Counts	VC Total	B/VC
+ *						
+ *						
+ *						
+ *						
+ *						
50.0 +	661	24, 24, 26,	74	100, 93, 50,	243	0.30
100 +	662	16, 14, 16,	46	71, 70, 96,	237	0.19
150 +	663	13, 6, (10)c	29	60, 60, 50,	170	0.17
200 +	664	17, 8, 12,	37	76, 76, 112,	264	0.14
* *						
N + *						
N +	670	8, (c) (c)	—	50, 70, 60,	180	—
S + *						
S + *						
S +	671	13, 8, 13,	34	60, 78, 82,	220	0.15
S +	672	10, 6, 20,	36	80, 47, 88,	215	0.17
P + *						
P +	673	(c) (c) (c)	—	17, 19, 11,	47	—

Dosing Tech. MEH 7/29/80

Plates Cloned: Dilutions Tech. MEH 7/29/80 12:00pm Incubator Wedco LBI# 9259 Top [serial#]

Date: 7/29/80 Pouring Tech. MEH 7/29/80 12:45pm Colony Counter Biotran LBI No. M1112-000

Plates Removed from Incubator: Date: 8/1/80 Settings: Size: 0.2

Plates Counted By: Tech.: V.V.B Sensitivity: 8.5

Date: 8/1/80 Area: 4465

Calculations by: Tech: MEH

Calculations Reviewed by: V.B.

*C.E. based on average of solvent and untreated negative controls divided by 3.

N/A MEH 7/29/80

C-Contaminated ( )c = 1 contaminated plate, Ave. of 2 MEH 8/1/80

000050

LBI-0323 R 2/79 FORM 1685 R1/ 10/78 Page 5

ROHM AND HAAS COMPANY PHILADELPHIA

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Initiation Date: 8-25-80

Client's Compound Code: TD 8076 LBI Safety# 6035 Trial# III

Description: Clear yellow liquid Technician: JH

Solvent: DMSO Solubility: Excellent Protocol#: 431

#Tube	[Dose] ml/ml	Day 0 Viable Cell Count*	Day 0 Calculated Density**	Day 1 Viable Cell Count*	Day 1 Calculated Density**	Day 2 Tubes Cloned (✓)
501 +	50.0	85	8.5	69	6.8	✓
502 +	100	59	5.9	50	5.0	T
503 +	150	60	6.0	58	5.8	✓
504 +	175	32	3.2 x	61	6.1	✓
505 +	200	11	1.1 x	24	2.4 x	✓
506 +	225	8	0.8 x	33	3.3 x	✓
507 +	250	1	0.1 x	13	1.3 x	T
508 +	300	0	0 x	0	T	
+						
+						
511 +	Negative Control	99	9.9	76	7.6	✓
512 +	Solvent Control	54	5.4	71	7.1 11.20	✓
513 +	Solvent Control	72	7.2	74	7.4 5.92	✓
514 +	Positive Control	65	6.5	53	5.3	T
515 +	Positive Control	68	6.8	44	4.4	✓
Activation: Aroclor-Induced Fisher 344 Rat Liver S-9	Tech:	JH		JH		JH
	Date:	8-26-80		8-27-80		8-27-80
	Time:	9am	Split 9:30am	9am	Split 9:30am	

*Viable Cell Count = actual no. of viable cells counted in 5 squares of hemacytometer

**Calculated cell density = calculated number of cells x 10^5 cells/ml; diluted to 3 x 10^5 cells/ml after count.

x Not diluted [split] to 3 x 10^5 cells/ml

Positive Controls: +LI = DMN 0.3 µl/ml [Dimethylnitrosamine]

+LI = Activation mixture added to tubes: S-9 liver homogenate and core.
C = Contaminated
T = Terminated [due to too much or too little observed toxicity]
L = Lost [due to technical error]

000051

LBI-0320 R 2/79

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 8-25-80

Tech.: Jf

Stock Cells Used: LBI-2 date cleansed of mutants 8-20-80 THMG batch# 5

Stock Cells Viable Density 0.38 x10^6 cells/ml; 7.9 ml/tube = 3.0 x 10^6 cells/tube

Compound: Description clear yellow liquid

Stock Solution: 300 µl ~~mg~~ of #5145 + 9.7 ml of DMSO =

Description: clear colorless liquid formed quickly. Final Concentration of 30 µl/ml ~~mg~~ = A.
[solubility, appearance, etc.] 1:2 dilution made of A.

B= 0.4ml A+ 0.2ml DMSO = 20ul/ml. Serial 1:2 dil. was made from B.
C= 0.5ml A + 0.1ml DMSO = 25ul/ml.
D= 0.3ml A + 0.1ml DMSO = 22.5 ul/ml. E= 0.3ml A + 0.5ml DMSO = 17.5ul/ml.

Appearance of dosed tubes:
#501-508 had normal color. No precipitate was detectable due to cloudy appearance of activation mixture.

Note: All tubes received 0.1 ml of compound + solvent ~~except:~~

All dilutions made 1:2 in same solvent except:

Stocks B, C, D & E.

Negative Controls received F$_0$ medium with ~~and without~~ S-9, core.

Solvent Controls received 0.1 ml of DMSO w/~~wo~~ S-9 and core.

Positive Controls received:

~~EMS: 0.1ml dissolved in 9.9ml of _____, filter-sterilized, dosed with _____ ml/tube without activation.~~

DMN: 0.1ml dissolved in 9.9ml of H2O, ~~filter-sterilized~~, dosed with 0.3 ml/tube with activation.

Selective Agent: BrdU [5-Bromo-2-deoxyuridine) 100 µg/ml final concentration.

S-9 liver homogenate, batch# 00027, dose 0.5 ml/tube final concentration.

Core, batch# 43, dose 1.0 ml/tube final concentration.

Activation added: Core and S-9 mixed immediately before addition to tubes.

Note: 10-ml volume includes the volume of S-9 and Core.

4 hour exposure: tubes dosed 8:30am, washed 12:30pm

controls dosed 8:30am, washed 12:30pm

Centrifuge LBI# 913

Shaker LBM #273

000052

LBI-0321 R 2/79 8-25-80.

FORM 4685 REV. 10/78

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 8-25-80

Tech.: JH

Lot Numbers:

1. Growth Medium, Batch Number N-216
   - F_0 Medium [HEM Research] # 19003
   - Horse Serum [KC Biologicals] # 200004
   - L-Glutamine [GIBCO] # A196601
   - Sodium Pyruvate Solution # N-21 6-27-80
   - Pluronic Solution # N-14 6-6-80
   - Pen/Strep [GIBCO] # 16N6100
2. Cloning Medium, Batch Number N-132
   - F_0 Medium [HEM Research] # 19003
   - Horse Serum [KC Biologicals] # 200004
   - L-Glutamine [GIBCO] # 13N5202
   - Sodium Pyruvate Solution # N-21 6-27-80
   - Pen/Strep [GIBCO] # 10P9105
   - Gentamycin [Schering] # 96RB2
3. DMSO [Eastman] # B8A
4. Agar Noble [Difco] # N-41 674167
5. BrdU [Sigma] # 89C-0159
6. Tissue culture plates used in cloning
   - Supplier: Costar ~~1005~~ 8-25-80 Lot# 011C261
7. EMS N.A.
8. DMN Aldrich CAS 62-75-9

Notes: Glass tubes & pipets were used to handle the stock concentrations of compound. JH 8-25-80.

000053

LBI-0922 R 2/79

FORM 4685 (Page 4)

MOUSE LYMPHOMA ASSAY

Client: Rohm + Haas Cloning Efficiency* [C.E.] of Assay 64.4 %

LBI Assay No. 5145 C.E.* Nonactivation — %C.E.* Activation 64.4 %

Agar Plate Colony Counts:

Dose	Plate#	B Plate Counts	B Total	VC Plate Counts	VC Total	B/VC
–						
–						
–						
–						
–						
500 +	501	7, 7, 8,	22	68, 76, 48	192	0.11
150 +	503	8, 7, 9,	24	60, 54, 40,	154	0.16
175 +	504	6, 3, 6,	15	40, 38, 42,	120	0.13
200 +	505	3, 8, 3	14	31, 30, 51	112	0.13
225 +	506	4, 4, 5,	13	50, 84, 60,	194	0.07
N –						
N +	511	6, 6, 5,	17	60, 74, 84,	218	0.08
S –						
S –						
S +	512	4, 5, 5	14	30, 62, 70,	162	0.09
S +	513	8, 6, 6,	20	60, 50, 90,	200	0.10
P –						
P +	515	10, 12, 6,	28	18, 14, 18	50	0.56

Plates Cloned: Dilutions Tech. Jf 10am Incubator Wedco LBI# 9259 Top [serial#]

Date: 8-27-80 Pouring Tech. Jf 11am Colony Counter Biotran LBI No. 8764

Plates Removed from Incubator: Date: 9/5/80 Settings: Size: 0-2

Plates Counted By: Tech.: S.D. Sensitivity: 2.5

Date: 9/5/80 Area: 4710

Calculations by: Tech: Jf

Calculations Reviewed by: NCH 8-9/5/80

*C.E. based on average of colvent and untreated negative controls divided by 3.

000054

LBI-0623 R 2/79

Colonies were counted too early. Jf 9-5-80. against supervisor's orders.

FORM 4685 REV. 10/79 Page 5

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Initiation Date: 9/10/80

Client's Compound Code: TD ~~2096~~ 20-16 wrong entry RCM 10/17/80 LBI Safety# 6035 Trial# 1

Description: clear yellow liquid Technician: S. Belyea

Solvent: DMSO Solubility: Excellent Protocol#: 431

	Day 0	Day 1		Day 2		
#Tube	[Dose] µl/ml	Viable Cell Count*	Calculated Density**	Viable Cell Count*	Calculated Density**	Tubes Closed (✓)
761 +	50.0 "	74	7.4	61	6.1	✓
762 +	100 "	43	4.3	83	8.3	T
763 +	150 "	64	6.4	107	10.7	✓
764 +	175 "	40	4.0	66	6.6	T
765 +	200 "	47	4.7	87	8.7	✓
766 +	225 "	21	2.1*	39	3.9*	✓
767 +	250 "	28	2.8*	60	6.0	✓
768 +	300 "	01	0.1*	0	0	T
+						
+						
781 +	Negative Control	63	6.3	98	9.8	✓
782 +	Solvent Control	67	6.7	63	6.3	✓
783 +	Solvent Control	64	6.4	56	5.6	✓
784 +	Positive Control	36	3.6*	42	4.2	✓
785 +	Positive Control	52	5.2	39	3.9*	✓

Activation: Aroclor-induced Fisher 344 Rat Liver S-9	Tech:	S. Belyea	S. Belyea
	Date:	9/11/80	9/12/80
	Time:	9:30 AM (Split back 10:00 AM)	9:30 A.M. (Split back 10:00 AM)

WHS 9/12/80

*Viable Cell Count = actual no. of viable cells counted in 5 squares of hemacytometer

**Calculated cell density = calculated number of cells x 10^5 cells/ml; diluted to 3 x 10^5 cells/ml after count.

* + Not diluted [split] to 3 x 10^5 cells/ml

Positive Controls: +LI = DMN 0.3 µl/ml [Dimethylnitrosamine]

+LI = Activation mixture added to tubes: S-9 liver homogenate and core.
C = Contaminated
T = Terminated [due to too much or too little observed toxicity]
L = Lost [due to technical error]

000055

FORM 4685 REV. 10/78 Page 2

LBI-0320 R 2/79

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 9/10/80

Tech.: H. Balmer

Stock Cells Used: L5178Y date cleansed of mutants 9/8/80 THMG batch# 5

Stock Cells Viable Density 0.45 x10^6 cells/ml; 6.7 ml/tube = 30 x 10^6 cells/tube

Compound: Description clear yellow liquid.

Stock Solution: 300 (µl) mg of #5145 + 9.7 ml of DMSO =

Description: Final Concentration of 30 (µl/ml) mg
[solubility, appearance, etc.]

Excellent solubility, clear colorless liquid.
Used glass tubes and glass pipettes to handle the compound.

Appearance of dosed tubes:

Tubes #761-#768 had normal color, no ppt.

Note: All tubes received 0.1 ml of compound + solvent ~~except~~*:

All dilutions made 1:2 in same solvent except: 1:2 → 150; 2:1 → 200; 1:2 → 100; 1:2 → 50; 5:6 → 250; 5:4 → 225; 7:12 → 175; → 800

Negative Controls received F_0 medium with and ~~without~~* S-9, core.

Solvent Controls received 0.1 ml of DMSO w/~~wo~~* S-9 and core.

Positive Controls received:

~~EMS: 0.1ml dissolved in 0.9ml of _____, filter-sterilized, dosed with _____ ml/tube without activation.~~

DMN: 0.1ml dissolved in 9.9ml of H2O, ~~filter-sterilized,~~* dosed with 0.3 ml/tube with activation.

Selective Agent: BrdU [5-Bromo-2-deoxyuridine] 100 µg/ml final concentration.

S-9 liver homogenate, batch# RH088, dose 0.5 ml/tube final concentration.

Core, batch# 40, dose 1.0 ml/tube final concentration.

Activation added: Core and S-9 mixed immediately before addition to tubes.

Note: 10-ml volume includes the volume of S-9 and Core.

4 hour exposure: tubes dosed 10:30 AM washed 2:30 PM

controls dosed 10:30 AM washed 2:30 PM

Centrifuge LBI# 9113

Shaker LBI# 9573

Was W.S. Hole

000056

LBI-0321 R 2/79

FORM 4585 REV. 10/78

COMPANY PHILADELPHIA

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 9/10/80

Tech.: N. Balmer

Lot Numbers:

1. Growth Medium, Batch Number N-220
   - F_0 Medium [HEM Research] # 19003
   - Horse Serum [KC Biologicals] # 200004
   - L-Glutamine [GIBCO] # A196601
   - Sodium Pyruvate Solution # N-21 6/27/80
   - Pluronic Solution # N-14 6/6/80
   - Pen/Strep [GIBCO] # 10P9105
2. Cloning Medium, Batch Number N-13H
   - F_0 Medium [HEM Research] # 19003
   - Horse Serum [KC Biologicals] # 200008
   - L-Glutamine [GIBCO] # 13N15202
   - Sodium Pyruvate Solution # N-21 6/27/80
   - Pen/Strep [GIBCO] # 16N6100
   - Gentamycin [Schering] # 9HRB2
3. DMSO [Eastman] # BBA
4. Agar Noble [Difco] # N-H3 674167
5. BrdU [Sigma] # 88C-0159
6. Tissue culture plates used in cloning
   - Supplier: Costar Lot# 016B974
7. EMS ~~Kodak NPA~~ VVR bd ester alcohol N.A.
8. DMN Aldrich #42-859

000057

LBI-0322 R 2/79

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas Cloning Efficiency* [C.E.] of Assay 76.6 %

LBI Assay No. 5145 C.E.* Nonactivation _____ %C.E.* Activation 76.6 %

Agar Plate Colony Counts:

Dose ml/ml	Plate#	B Plate Counts	B Total	VC Plate Counts	VC Total	B/VC
–						
–						
–						
–						
–						
50.0 +	761	9, 14, 21	44	59, 46, 72	177	0.25
150 +	763	22, 32, 55	109	46, 54, 79	179	0.61
200 +	765	24, 20, 37	81	30, 28, 81	139	0.58
225 +	766	32, 19, 39	90	50, 49, 92	191	0.47
250 +	767	25, 28, 46	99	51, 44, 80	175	0.57
N –						
N +	781	18, 13, 18	49	87, 54, 77	218	0.22
S –						
S –						
S +	782	14, 38, 14	66	85, 84, 76	245	0.27
S +	783	11, 10, 23	44	82, 84, 60	226	0.19
P +	784	16, 13, 39	68	8, 17, 10	35	1.94
P +	785	19, 27, 55	101	21, 13, 26	60	1.68

Plates Cloned: Dilutions Tech. V. Rolino 11:45 AM Incubator Wedco LBI# 9259 Top. [serial#]

Date: 9/18/80 Pouring Tech. V. Rolino 12:15 PM Colony Counter Biotran LBI No. 8764

Plates Removed from Incubator: Date: 9-22-80 Settings: Size: 8.2

Plates Counted By: Tech.: JF Sensitivity: 8.75

Date: 9-22-80 Area: 4800

Calculations by: Tech: V.B.

Calculations Reviewed by: DJ

*C.E. based on average of solvent and untreated negative controls divided by 3.

000058

MOUSE LYMPHOMA ASSAY

Client: Rohm + Haas LBI Assay# 5145 Initiation Date: 9-30-80

Client's Compound Code: TD ~~8076~~ 80-16 wrong entry BCM 10/13/80 LBI Safety# 6035 Trial# IV

Description: Clear pale yellow liquid Technician: JH

Solvent: DMSO Solubility: Excellent Protocol#: 431

Dark I confused with T BCM 10/17/80

#Tube	[Dose] µl/ml	Day 0 Viable Cell Count*	Day 0 Calculated Density**	Day 1 Viable Cell Count*	Day 1 Calculated Density**	Tubes Cloned (✓)
521 +	50.0	49	4.9	125	12.5	✓
522 +	100	46	4.6	81	8.1	✓
523 +	150	30	3.0x	129	12.9	✓
524 +	175	32	3.2x	85	8.5	✓
525 +	200	18	1.8x	79	7.9	✓
526 +	225	0	0x	0	T	
527 +	250	0	0x	T		
528 +	300	0	0x	T		
+						
+						
531 +	Negative Control	66	6.6	85	8.5	✓
532 +	Solvent Control	65	6.5	126	12.6	✓
533 +	Solvent Control	74	7.4	110	11.0	✓
534 +	Positive Control	48	4.8	80	8.0	T
535 +	Positive Control	48	4.8	67	6.7	✓
Activation: Aroclor-Induced Fisher 344 Rat Liver S-9	Tech:	JH		JH		JH
	Date:	10-1-80		10-2-80		10-2-80
	Time:	9am	Split 10am	9am	Split 9:30am	

*Viable Cell Count = actual no. of viable cells counted in 5 squares of hemacytometer

**Calculated cell density = calculated number of cells x 10^5 cells/ml; diluted to 3 x 10^5 cells/ml after count.

x Not diluted (split) to 3 x 10^5 cells/ml

Positive Controls: +Li = DMN 0.3 µl/ml [Dimethylnitrosamine]

+Li = Activation mixture added to tubes: S-9 liver homogenate and core.
C = Contaminated
T = Terminated [due to too much or too little observed toxicity]
L = Lost [due to technical error]

000059

LBI-0320 R 2/79

FORM 4685 REV 10/78 Page 2

MOUSE LYMPHOMA ASSAY

Client: Rohm + Haas LBI Assay# 5145 Date: 9-30-80

Tech.: Jf

Stock Cells Used: LBI-2 date cleansed of mutants 9-24-80 THMG patch# 5

Stock Cells Viable Density 0.45 x10^6 cells/ml; 6.7 ml/tube = 3.0 x 10^6 cells/tube

Compound: Description Clear pale yellow liquid #5145 Jf 9-30-80 Jf 9-30-80

Stock Solution: 150 µl ~~mg~~* of 4.85 + 4.85 ml of DMSO =

Description: [solubility, appearance, etc.] Clear colorless liquid formed quickly. Final Concentration of 30 µl/ml ~~mg~~* = A.

1:2 dilution made from A. Dosed tube nos 523 & 529.

B = 0.4 ml A + 0.2 ml DMSO = 20 ul/ml. Serial 1:2 dilutions made from B. Dosed tube nos. 521, 522, 525.

C = 0.5 ml A + 0.1 ml DMSO = 25 ul/ml. Dosed tube no. 527.

D = 0.3 ml A + 0.1 ml DMSO = 22.5 ul/ml. Dosed tube no. 526

E = 0.7 ml A + 0.5 ml DMSO = 17.5 ul/ml. Dosed tube no. 524.

Appearance of dosed tubes: #521-528 had normal color. No precip tate was detectable due to cloudiness of activation mixture.

Note: All tubes received 0.1 ml of compound + solvent ~~except~~*:

Clarification of tube nos. dosed from each stock series made by DCM 7-15-82.

All dilutions made 1:2 in same solvent except:

Stocks B, C, D + E

Negative Controls received F_0 medium with ~~and without~~* S-9, core.

Solvent Controls received 0.1 ml of DMSO w/~~wo~~* S-9 and core.

Positive Controls received:

~~EMS: 0.1ml dissolved in 9.9ml of _____, filter-sterilized, dosed with _____ ml/tube without activation.~~

DMN: 0.1ml dissolved in 9.9ml of H_2O, ~~filter-sterilized~~*, dosed with 0.3 ml/tube with activation.

Selective Agent: BrdU [5-Bromo-2'-deoxyuridine] 100 µg/ml final concentration.

S-9 liver homogenate, batch# RDH088, dose 0.5 ml/tube final concentration.

Core, batch# 43, dose 1.0 ml/tube final concentration.

Activation added: Core and S-9 mixed immediately before addition to tubes

Note: 10-ml volume includes the volume of S-9 and Core.

4 hour exposure: tubes dosed 9:25am, washed 1:25pm

controls dosed 9:25am, washed 1:25pm

Centrifuge LBI# 9163

Shaker LBI# 10066

000060

* N.A. Jf 9-30-80

LBI-0321 R 2/79

FORM 4 Page 3 0/78

PROPERTY OF ROHM AND HAAS COMPANY PHILADELPHIA

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 9-30-80

Tech.: Jf

Lot Numbers:

1. Growth Medium, Batch Number N-224
   - F_0 Medium [HEM Research] # 19038
   - Horse Serum [KC Biologicals] # 200008
   - L-Glutamine [GIBCO] # A196601
   - Sodium Pyruvate Solution # N-21 6-27-80
   - Pluronic Solution # N-14 6-6-80
   - Pen/Strep [GIBCO] # 10P9105
2. Cloning Medium, Batch Number N-137
   - F_0 Medium [HEM Research] # 19038
   - Horse Serum [KC Biologicals] # 200008
   - L-Glutamine [GIBCO] # A196601
   - Sodium Pyruvate Solution # N-21 6-27-80
   - Pen/Strep [GIBCO] # 10P9105
   - Gentamycin [Schering] # 96R82
3. DMSO [Eastman] # JF 9-30-80 ~~Wong 10-8-8A~~ B8A
4. Agar Noble [Difco] # N-44 674167
5. BrdU [Sigma] # 89C-0159
6. Tissue culture plates used in cloning
   - Supplier: Costar Lot# D11B261
7. EMS N.A.
8. DMN Aldrich CAS 62-75-9

Notes: Glass tubes and pipets were used to handle the test material and solutions in DMSO.
Note added by BCM 7-15-82

000061

7-26-80

Rohm & Haas # 5145 Trial IV + DMSO

Doses:

21	50.0	nl/ml
22	100	
23	150	
24	175	
25	200	
26	225	
27	250	
28	300	

Stock: 30,000 nl/ml = 30 ul/ml = 150 ul/5 ml = 0.15 ml # 5145 + 4.85 ml DMSO

Dilutions: 300 —1:2 (0.1 ml + 0.1 ml DMSO)→ 150

—2:3 (0.4 ml + 0.2 ml DMSO)→ 200 —1:2 (0.2 ml + 0.2 ml DMSO)→ 100 —1:2 etc.→ 100

—5:6 (0.5 ml + 0.1 ml DMSO)→ 250

—3:4 (0.3 ml + 0.1 ml DMSO)→ 225

—7:12 (0.7 ml + 0.5 ml DMSO)→ 175

Dose: 0.1 ml / tube

Notes: 1. Carefully prepare DMN + S-9 mix to insure good positive control response.

2. Use glass tubes + pipets for the stocks.

3. Clone a range of doses including most toxic.

PROPERTY OF ROHM AND HAAS COMPANY PHILADELPHIA

FORM 4685 REV. 10/78

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas Cloning Efficiency* [C.E.] of Assay 87.0 %

LBI Assay No. 5145 C.E.* Nonactivation — %C.E.* Activation 87.0 %

Agar Plate Colony Counts:

µl/ml Dose	Plate#	B Plate Counts	B Total	VC Plate Counts	VC Total	B/VC
–						
–						
–						
–						
–						
50.0 +	521	28, 29, 13	70	100, 65, 81	246	0.28
100 +	522	17, 31, 37	85	90, 103, 102	295	0.29
150 +	523	12, 29, 40	81	65, 84, 86	235	0.34
175 +	524	19, 27, 31	77	49, 75, 95	219	0.35
200 +	525	15, 30, 23	68	76, 76, 101	253	0.27
N –						
N +	531	27, 19, 16	62	91, 101, 99	291	0.21
S –						
S –						
S +	532	24, 27, 24	75	64, 95, 103	262	0.29
S +	533	13, 7, 23	43	84, 68, 78	230	0.19
P –						
P +	535	51, 85, 82	218	9, 16, 27	52	4.19

Plates Cloned: Dilutions Tech. JH 10am Incubator Wedco LBI# 8616 T90 [serial#]

Date: 10-2-80 Pouring Tech. JH 10:30am Colony Counter Biotran LBI No. 10778

Plates Removed from Incubator: Date: 10-3-80 Settings: Size: 1.3

Plates Counted By: Tech.: JH Sensitivity: 7.1

Date: 10-3-80 Area: 4800

Calculations by: Tech: JH

Calculations Reviewed by: V.B.

*C.E. based on average of solvent and untreated negative controls divided by 3.

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LBI-0323 R 2/79

FORM 4685 REV. 10/7

PROJECT CHANGE SHEET

LBI Project No.: 20989	Change No.: 1
Assay No.: 5145	Date: 1/22/81
LBI Safety No.: 6035	Compound: TD 80-16

Sponsor:	Study Director:	Departments Involved:
Rohm & Haas Company	Brian C. Myhr Sign. Brian C. Myhr	Genetics

1. Title and Description of Study: MLA Protocol No. 431

2. Description of Change with Effective Date: 1/22/81

According to discussion with client on January 2, 1981, by telephone, this study will be supplementary to an earlier study. No solubility or toxicity tests will be performed - refer to earlier study for compound handling.

Perform with S9 activation only for following conditions

4 solvent controls

2 positive controls (DMN)

Doses: 100, 150, 175 (dupl.), 200, 225, 250, 275 (triplicate), nl/ml, 300 nl/ml (dupl.)

Plan to done approx. 10-12 test doses - see ne for selection

3. Justification for Change:

Attempt to define the response to highly toxic treatments for a compound that has a sharp survival curve. Protocol change meets Sponsor's approval.

DISTRIBUTION: White, Client File; Yellow, Principal Investigator; Pink, Report Folder.

FORM 4685 REV. 10/78

LBI-0417 R5/79

PROPERTY OF MERIDIAN COMPANY PHILADELPHIA

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Initiation Date: 1-26-81

Client's Compound Code: TD 80-16 LBI Safety# C^35 Trial# VI

Description: Clear yellow liquid Technician: JH

Solvent: DMSO Solubility: Excellent Protocol#: 434

#Tube	[Dose] ml/ml	Day 0 Viable Cell Count*	Day 0 Calculated Density**	Day 1 Viable Cell Count*	Day 1 Calculated Density**	Tubes Cloned ✓
501 +	100	10.1	10.1	120	12.0	✓
502 +	150	85	8.5	140	14.0	✓
503 +	175	69	6.9	105	10.5	T
504 +	175	103	10.3	115	11.5	T
505 +	200	37	3.7 x	98	9.8	✓
506 +	200	46	4.6	78	7.8	✓
507 +	200	55	5.5	74	7.4	✓
508 +	225	20	2.0 x	67	6.7	✓
509 +	225	27	2.7 x	98	9.8	✓
510 +	225	63	6.3	59	5.9	✓
+	Negative Control					
521 +	Solvent Control	164	16.4	166	16.6	✓
522 +	Solvent Control	161	16.1	184	18.4	✓
+	Positive Control					
525 +	Positive Control	91	9.1	75	7.5	✓
Activation: Aroclor-Induced Fisher 344 Rat Liver S-9	Tech:	JH		JH		JH
	Date:	1-27-81		1-28-81		1-28-81
	Time:	10:30am	split 11:30am	11:30am	split 1:00pm	

*Viable Cell Count = actual no. of viable cells counted in 5 squares of hemacytometer

**Calculated cell density = calculated number of cells x 10^5 cells/ml; diluted to 3 x 10^5 cells/ml after count.

x Not diluted [split] to 3 x 10^5 cells/ml

Positive Controls: +Li = DMN 0.3 µl/ml [Dimethylnitrosamine]

+Li = Activation mixture added to tubes: S-9 liver homogenate and core.
C = Contaminated
T = Terminated [due to too much or too little observed toxicity]
L = Lost [due to technical error]

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Page number change because no -S9 portion performed BCR 3/15/81

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Initiation Date: 1-26-81

Client's Compound Code: TD 80-16 LBI Safety# 6035 Trial# VI

Description: Clear yellow liquid Technician: JH

Solvent: DMSO Solubility: Excellent Protocol#: 431

	Day 0	Day 1		Day 2		
#Tube	[Dose] µl/ml	Viable Cell Count*	Calculated Density**	Viable Cell Count*	Calculated Density**	Tubes Cloned (✓)
511 +	250	4	0.4 x	13	1.3 x	T
512 +	250	14	1.4 x	36	3.6 x	✓
513 +	250	2.2	2.2 x	43	4.3	✓
514 +	275	0	0 x	0	0 x	T
515 +	275	2	0.2 x	0	0 x	T
516 +	275	1	0.1 x	7	0.7 x	T
517 +	300	0	0 x	0	0 x	T
518 +	300	0	0 x	0	0 x	T
+						
+						
+	Negative Control					
523 +	Solvent Control	139	13.9	158	15.8	✓
524 +	Solvent Control	109	10.9	193	19.3	✓
+	Positive Control					
526 +	Positive Control	66	6.6	72	7.2	✓
Activation: Aroclor-Induced Fisher 344 Rat Liver S-9	Tech:	JH		JH		JH 1-28-81
	Date:	1-27-81		1-28-81		
	Time:	10:30am	Split 11:30am	11:30am	Split noon	

*Viable Cell Count = actual no. of viable cells counted in 5 squares of hemacytometer

**Calculated cell density = calculated number of cells x 10^5 cells/ml; diluted to 3 x 10^5 cells/ml after count.

x Not diluted [split] to 3 x 10^5 cells/ml

Positive Controls: +Li = DMN 0.3 µl/ml [Dimethylnitrosamine]

+Li = Activation mixture added to tubes: S-9 liver homogenate and core.
C = Contaminated
T = Terminated [due to too much or too little observed toxicity]
L = Lost [due to technical error]

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page 2 is appropriate. BCM 3/15/81

FORM 4685 REV. 10/78

LBI-0320 R 2/79

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 1-26-81

Tech.: JJ

Stock Cells Used: LBI-2 date cleansed of mutants 1-21-81 THMG batch# 6

Stock Cells Viable Density 0.708 x10^6 cells/ml; 4.2 ml/tube = 3.0 x 10^6 cells/tube

Compound: Description Clear yellow liquid

Stock A Solution: 150 µl ~~mg~~ of #5145 + 485 ml of DMSO =

Description: formed rapidly clear colorless liquid
[solubility, appearance, etc.]

Final Concentration of 30 µl/ml /~~mg~~

B= 0.4ml A+ 0.2 ml DMSO = 20 ul/ml.
C= 1.1 ml A+ 0.1 ml DMSO = 27.5 ul/ml.
D= 0.5 ml A + 0.1 ml DMSO = 25 ul/ml.
E= 0.3 ml A+ 0.1 ml DMSO = 22.5 ul/ml.
1:2 dilutions made from A, B, ~~C~~ JJ 1-26-81 wrong stock.
F= 0.7 ml C+ 0.4 ml DMSO = 17.5 ul/ml.

omission corrected by BCM 7-15-82

Appearance of dosed tubes:
#501-518 were milky in color. Presence of precipitate was difficult to see due to activation mixture

Stock Series	Dosed tubes*
A	517, 518, 502
B	505-507, 501
C	514-516
D	511-513
E	508-510
F	503, 504

above clarification added by BCM 7-15-82

Note: All tubes received 0.1 ml of compound + solvent ~~except:~~

All dilutions made 1:2 in same solvent except:
stocks B, C, D & E & F.

Negative Controls received F/0 medium with ~~and without~~ S-9, core.

Solvent Controls received 0.1 ml of DMSO w/~~wo~~ S-9 and core.

Positive Controls received:

~~EMS: 0.1ml dissolved in 9.9ml of _____, filter-sterilized, dosed with _____ ml/tube without activation.~~

DMN: 0.1ml dissolved in 9.9ml of H_2O, ~~filter-sterilized~~, dosed with 0.3 ml/tube with activation.

Selective Agent: BrdU [5-Bromo-2-deoxyuridine] 100 µg/ml final concentration.

S-9 liver homogenate, batch# RDH088, dose 0.5 ml/tube final concentration.

Core, batch# 45, dose 1.0 ml/tube final concentration.

Activation added: Core and S-9 mixed immediately before addition to tubes.

Note: 10-ml volume includes the volume of S-9 and Core.

4 hour exposure: tubes dosed 8:45am; washed 12:45pm

controls dosed 8:45am, washed 12:45pm

Centrifuge LBI# 9163

Shaker LBI# 9273

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LBI-0321 R 2/79 JJ 1-26-81

FORM 466B REV. 10/78

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas LBI Assay# 5145 Date: 1-26-81

Tech.: Jf

Lot Numbers:

1. Growth Medium, Batch Number N-244
   - F_0 Medium [HEM Research] # 19128
   - Horse Serum [KC Biologicals] # 200011
   - L-Glutamine [GIBCO] # 10P3006 & R490402
   - Sodium Pyruvate Solution # N-24 10-31-80
   - Pluronic Solution # N-16 10-10-80
   - Pen/Strep [GIBCO] # 10P9105
2. Cloning Medium, Batch Number N-152
   - F_0 Medium [HEM Research] # 19128
   - Horse Serum [KC Biologicals] # 200011
   - L-Glutamine [GIBCO] # R490402
   - Sodium Pyruvate Solution # N-24 10-31-80
   - Pen/Strep [GIBCO] # 13N4004
   - Gentamycin [Schering] # 96RB2
3. DMSO [Eastman] # B8A
4. Agar Noble [Difco] # N-50 674167
5. BrdU [Sigma] # 840-0159
6. Tissue culture plates used in cloning
   - Supplier: Costar Lot# 1005-202
7. EMS N.A.
8. DMN Aldrich CAS 62-75-9

Notes: Glass tubes & pipets were used to handle the compound stocks. Jf 1-26-81.

COMPANY PHILADELPHIA

LBI-0322 R 2/79

FORM 4685 REV. 10/78

MOUSE LYMPHOMA ASSAY

Client: Rohm & Haas Cloning Efficiency* [C.E.] of Assay 70.5 %

LBI Assay No. 5145 C.E.* Nonactivation — %C.E.* Activation 70.5 %

Agar Plate Colony Counts:

Dose	Plate#	B Plate Counts	B Total	VC Plate Counts	VC Total	B/VC
100 +	501	23, 27, 25	75	65, 60, 83	208	0.30
150 +	502	16, 20, 27	63	25, 32, 81	138	0.46
200 +	505	10, 25, 23	58	45, 80, 70	195	0.30
200 +	506	20, 24, 34	78	63, 65, 109	237	0.33
200 +	507	32, 19, 34	85	59, 86, 99	244	0.35
225 +	508	8, 8, 19	35	56, 53, 51	160	0.22
225 +	509	15, 15, 12	42	29, 46, 50	125	0.34
225 +	510	6, 10, 27	43	59, 54, 80	193	0.22
250 +	512	7, 9, 17	33	41, 40, 72	153	0.22
250 +	513	20, 23, 29	72	69, 54, 66	189	0.38
+						
N −						
N +	5	JH 1-28-81 in one line				
S +	521	6, 16, 20	42	60, 62, 89	211	0.20
S +	522	16, 15, 19	50	61, 60, 94	215	0.23
S +	523	14, 20, 22	56	65, 82, 62	209	0.27
S +	524	13, 18, 22	53	74, 60, 77	211	0.25
P +	525	26, 36, 50	112	7, 10, 9	26	4.31
P +	526	31, 35, 52	118	9, 7, 11	27	4.37

Plates Cloned: Dilutions Tech. JH 1pm Incubator Wedco LBI# 9259/op [serial#]

Date: 1-28-81 Pouring Tech. JH 2pm Colony Counter Biotran LBI No. 8764

Plates Removed from Incubator: Date: 2-6-81 Settings: Size: 6.2

Plates Counted By: Tech.: JH Sensitivity: 8.75

Date: 2-6-81 Area: 5000

Calculations by: Tech: JH

Calculations Reviewed by: V.B.

*C.E. based on average of solvent and untreated negative controls divided by 3.

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