



**Report Title:** CHO/HGPRT Mutation Assay

**Test Type:** Genotoxicity Study

**Conducting Laboratory and Location:** Microbiological Associates, Bethesda, MD

**Test Substance(s):** G0539.02 – OP in ethanol

**Species:** Chinese Hamster Ovary cell culture

**Test Conditions:** CHO/HGPRT mutation tested in the absence and presence of an Aroclor-induced rat liver S-9 activation system. This was conducted at dose levels of 20,15,10,5 without S-9 and at 150,80,40,10 and 1 ug/ml in the presence of S-9.

**Results:** Test material considered negative in the absence of activation and suspect in the presence of a S-9 activated system in the CHO/HGPRT mutation assay.

**Study #:** T4920.332

**Report Date:** 7/31/86

**QA report/GLP compliance:** Yes

**Accession #:** 32637

CHO/HGPRT MUTATION ASSAY

TEST ARTICLE  
G0539.02

FINAL REPORT

FOR

The Procter & Gamble Company  
Beauty Care Division  
Sharon Woods Technical Center  
11511 Reed Hartman Hwy.  
Cincinnati, Ohio 45241

BY

MICROBIOLOGICAL ASSOCIATES, INC.  
5221 RIVER ROAD  
BETHESDA, MARYLAND 20816

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OPERATIONS SECTION

CHO/HGPRT MUTATION ASSAY

FINAL REPORT

Test Article I.D.: G0539.02

Test Article Lot No.: .02

MA Study No.: T4920.332

Test Article Description: White powder

Storage Conditions: Room Temperature

Date Sample Received: May 2, 1986

Initiation Date: May 7, 1986

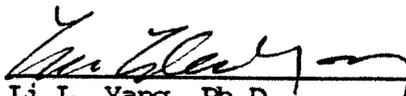
Completion Date: July 31, 1986

Sponsor: The Procter & Gamble Company  
Sharon Woods Technical Center  
11511 Reed Hartman Hwy. - Room HB2D29  
Cincinnati, Ohio 45241

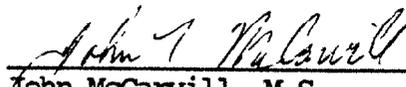
Authorized Representative: Dr. James E. Weaver/Dr. Edward D. Thompson

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.  
5221 River Road  
Bethesda, Maryland 20816

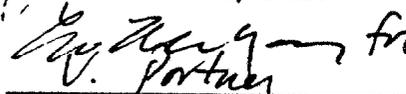
Study Director:

 7/31/86  
Li L. Yang, Ph.D. Date

Lead Technician:

 7/31/86  
John McCarvill, M.S. Date

Laboratory Technician:

 7/31/86  
Virginia Portner, B.S. Date

QUALITY ASSURANCE STATEMENT

Study Title: CHO/HGPRT MUTATION ASSAY

Study Number: T4920.332

Study Director: Li L. Yang, Ph.D.

Initiation Date: 86/05/07

Review Completed Date: 86/07/31

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice regulations (21CFR58), the U.S. EPA GLPs (40CFR792 and 40CFR160), and the OECD guidelines and to assure that the study is conducted according to the protocol.

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

INSPECT ON 86/05/06 - 86/05/06, TO STUDY DIR 86/05/06, TO MGMT 86/05/06  
PHASES: PROTOCOL REVIEW

INSPECT ON 86/06/30 - 86/06/30, TO STUDY DIR 86/06/30, TO MGMT 86/07/02  
PHASES: MUTATION ASSAY: SCORING AND RECORDING PLATES

INSPECT ON 86/07/30 - 86/07/30, TO STUDY DIR 86/07/30, TO MGMT 86/07/31  
PHASES: FINAL REPORT

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

Dana Mambh 7/31/86  
Quality Assurance Date  
RA/QA Department

SUMMARY

The test article, G0539.02, was tested in the CHO/HGPRT mutation assay in the absence and presence of an Aroclor-induced rat liver S-9 activation system. The assay was conducted at dose levels of 20, 15, 10, 5 and 1 ug/ml in the non-activated study and at 150, 80, 40, 10 and 1 ug/ml in the presence of S-9. Under the conditions of the assay described in this report, G0539.02 was considered negative in the absence of activation and suspect in the presence of a S-9 activated system in the CHO/HGPRT mutation assay.

## INTRODUCTION

Mammalian cell culture systems provide a valuable tool for assessing the genetic hazards of a variety of potentially mutagenic agents. The CHO/HGPRT mutation assay was designed to select for mutant cells which have become resistant to the purine analogue 6-thioguanine as a result of mutation at the X-chromosome-linked hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of Chinese hamster ovary (CHO) cells. This system has been demonstrated to be sensitive to the mutagenic action of a variety of chemicals (1).

## PURPOSE

The purpose of this study is to assess the mutagenic potential of the test article based upon its ability to induce forward mutations at the HGPRT locus of CHO cells.

## CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, G0539.02 was received by Microbiological Associates, Inc. on May 2, 1986 and was assigned the code number T4920. The test article was characterized by the Sponsor as powder.

Upon receipt, the test article was described as white powder and was stored at room temperature. At the time of dose administration, the test article was dissolved in ethanol (EtOH), lot 1094, from Pharmco Publicker Industries, Inc., Dayton, NJ, and lot BIN81M24 from U.S. Industrial Chemical Co. The Sponsor has assumed responsibility for the determination of the stability of the test article.

Ethyl methanesulfonate (EMS), lot 95F-0226, and Benzo(a)pyrene (BaP), lot 13F-9006, were obtained from Sigma Chemical Company, St. Louis, MO.

## MATERIALS AND METHODS

### Materials

Mammalian Cells: CHO-K<sub>1</sub>-BH<sub>4</sub> cells (Dr. Abraham Hsie, Oak Ridge National Laboratories, Oak Ridge, TN)

### Biological

#### Reagents:

Ham's F-12 medium supplemented with 5% dialyzed fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% L-glutamine (F12FBS5)  
Ham's F-12 medium without hypoxanthine supplemented with 5% dialyzed FBS, 1% penicillin-streptomycin and 1% L-glutamine (F12FBS5-Hx)  
Hank's balanced salt solution (HBSS)  
HBSS, Ca<sup>++</sup> and Mg<sup>++</sup>-free (CMF-HBSS)  
Trypsin, 0.05% in CMF-HBSS  
6-Thioguanine (TG, 2-amino-6-mercaptopurine), 20 mM in 0.1 N NaOH  
Cofactor pool: 4 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 mM glucose-6-phosphate, 30 mM potassium chloride (KCl), 10 mM calcium chloride (CaCl<sub>2</sub>), 10 mM magnesium chloride (MgCl<sub>2</sub>) and 50 mM sodium phosphate buffer, pH 8.0  
S-9, 9000 x g supernatant of an Aroclor-1254 induced Fischer 344 rat liver homogenate

#### Supplies:

Glass tubes with screw caps  
Pipettes, assorted sizes  
Plastic tissue culture flasks and dishes

#### Chemicals:

Solvent for test article (EtOH)  
Benzo(a)pyrene (BaP)  
Ethyl methanesulfonate (EMS)

### Methods

The S-9 was prepared according to established procedures. Adult male Fischer rats, 200-250 gm, were induced by a single intraperitoneal injection of Aroclor-1254 at a dosage of 500 mg/kg body weight two days prior to sacrifice. The animals were sacrificed and the livers aseptically removed. The excised tissue was rinsed three times in cold sterile 0.15 M KCl and then homogenized in a Polytron Tissuemizer at a concentration of 1:3 (w/v) in 0.15 M KCl. The supernatant fraction (S-9) was collected following centrifugation at 9000 x g for 10 minutes at 4±2°C, portioned into aliquots for daily use, and stored frozen at -70°C until used. Each bulk preparation of S-9 is assayed for its ability to metabolize 2-aminoanthracene and 7,12 dimethyl-

benz(a)anthracene to forms mutagenic to Salmonella typhimurium TA100.

Immediately prior to use, the S-9 was mixed with the cofactor pool to contain 100 ul S-9/ml cofactor pool, 4 mM NADP, 5 mM glucose-6-phosphate, 30 mM KCl, 10 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, and 50 mM sodium phosphate buffer, pH 8.0. The S-9 reaction mixture was stored on ice until used.

The optimal dose levels for the mutation assay were selected following a preliminary toxicity test based on colony-forming efficiency. CHO cells were exposed to solvent alone and to nine concentrations of test article ranging from 1000 ug/ml to 0.1 ug/ml for 5 hours at 37 ± 1°C in the presence and absence of an exogenous source of metabolic activation. The following day, the treated cells were trypsinized and reseeded at a density of 100 cells/60 mm dish. The cloning efficiency was determined 7-10 days later. The cell survival of the test article treated groups are expressed relative to the solvent control (relative cloning efficiency).

The mutation assay was performed according to a protocol developed from published methodologies (2,3). Exponentially growing CHO-K<sub>1</sub>-BH<sub>4</sub> cells were plated in F12FBS5 at a density of 5 x 10<sup>5</sup> cells/25 cm<sup>2</sup> flask and were incubated at 37±1°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 18-24 hours.

The time of initiation of chemical treatment was designated as day 0. Cells were exposed in duplicate to five concentrations of the test article for 5 hours at 37±1°C. The treatment medium consisted of 4 ml F12FBS5 containing various concentrations of test article, and 1 ml S-9 reaction mixture for the activated study, and 5 ml F12FBS5 containing various concentrations of test article for the non-activated study. After the treatment period, all media were aspirated, the cells washed with HBSS and cultured in F12FBS5 for an additional 18-24 hours at 37±1°C. At this time, the cells were subcultured to assess cytotoxicity and to continue the phenotypic expression period.

For evaluation of cytotoxicity, the replicates from each treatment condition were pooled and subcultured in F12FBS5-Hx<sup>1</sup>, in triplicate, at a density of 100 cells/60 mm dish. After 7-10 days incubation, the colonies were fixed with methanol, stained with 10% aqueous Giemsa, and counted.

For expression of the mutant phenotype, the replicates from each treatment condition were pooled and subcultured in F12FBS5 in duplicate, at a density of approximately 10<sup>6</sup> cells/100 mm dish. Subculture as above at 2-3 day intervals was employed for the 7-9 day expression period. At this time, selection for the mutant phenotype was performed.

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<sup>1</sup>The concurrent cytotoxicity was performed using F12FBS5-Hx as medium. Hypoxanthine is not an essential component for CHO cell growth or cloning. This is documented in the raw data book with a deviation report.

For selection of the TG-resistant phenotype, the replicates from each treatment condition were pooled and replated, in quintuplicate, at a density of  $2 \times 10^5$  cells/100 mm dish in F12FBS5-Hx containing 10 uM TG. For cloning efficiency determinations, at the time of selection, 100 cells/60 mm dish were plated in triplicate. After 7-10 days of incubation, the colonies were fixed, stained and counted for both cloning efficiency and mutant selection.

#### Controls

EMS was used as the positive control in the non-activated study at a concentration of 0.2 ul/ml. BaP was used as the positive control in the activated study at a concentration of 4 ug/ml. The solvent vehicle for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Growth medium was used in the untreated (negative) control.

#### Evaluation of Test Results

The cytotoxicity effects of each treatment condition are expressed relative to the solvent treated-control (relative cloning efficiency). The mutant frequency (MF) for each treatment condition is calculated by dividing the total number of mutant colonies by the number of cells selected (usually  $10^6$  cells: 5 plates at  $2 \times 10^5$  cells/plate), corrected for the cloning efficiency of cells prior to mutant selection, and is expressed as TG-resistant mutants per  $10^6$  clonable cells. For experimental conditions in which no mutant colonies are observed, mutant frequencies will be expressed as less than the frequency obtained with one mutant colony. Mutant frequencies generated from doses giving  $\leq 10\%$  relative survival are not considered as valid data points.

The calculation of mutagenic response in terms of fold increase in mutant frequency above the background rate does not provide a reliable indication of the significance of the observed response, since for some loci with spontaneous mutant frequencies which are very low and for which only a few colonies may be observed in the control experiments, a 2- to 3-fold increase in mutant frequency may not be significant. For assays characterized by a wide degree of variation in the frequency of spontaneous mutants found in the negative or solvent controls, a confidence interval can be calculated by the application of a one-sided Student's t test ( $p \leq 0.05$ ) from the historic background mutant frequency (4). In this laboratory, the confidence interval for the CHO/HGPRT assay is  $8.7/10^6$  clonable cells. Therefore, the mutagenic response after treatment will be considered significant only when the treatment mutant frequency is increased above that of the solvent control and the untreated control by at least 8.7 mutants/ $10^6$  clonable cells and also is at least twice that of the solvent control and the untreated control.

The following criteria will be used as guidelines for interpretation of the data; however, the conclusion of the study will be based upon the Study

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Director's evaluation and interpretation of the data. The assay will be considered positive in the event a dose-dependent increase in mutant frequency is observed with one or more of the five concentrations tested inducing a mutant frequency which is at least twice that of the solvent control and untreated control, and also is increased above that of the solvent control and the untreated control by at least 8.7 mutants/10<sup>6</sup> clonable cells. The assay will be considered suspect if there is no dose response but one or more doses induce a mutant frequency which is considered significant. The assay will be considered negative if none of the doses tested induce a mutant frequency which is considered significant.

#### Criteria for Determination of a Valid Test

The cloning efficiency of the solvent and untreated controls must be no less than 50%. The spontaneous mutant frequency in the solvent and untreated controls must fall within the range of 0-20 mutants per 10<sup>6</sup> clonable cells.

The positive control must induce a mutant frequency at least three times that of the solvent control.

#### Records

All raw data, final report, and stained mutant plates are maintained in the archives of Microbiological Associates, Inc. located at 5221 River Road, Bethesda, Maryland 20816.

## RESULTS AND DISCUSSION

Dose levels for the CHO/HGPRT assay were selected following a preliminary toxicity test based upon cloning efficiency after treatment relative to the solvent control (Table 1). CHO cells were exposed to solvent alone and to nine concentrations of test article ranging from 1000 to 0.1 ug/ml in the toxicity test in the absence and presence of an S-9 reaction mixture. Dose levels of 20, 15, 10, 5 and 1 ug/ml in the absence of S-9 and 150, 80, 40, 10 and 1 ug/ml in the presence of S-9 were selected for further study.

The cytotoxic effects of a 5 hour treatment of CHO cells in the absence and presence of an exogenous metabolic activation system are presented in Table 2 (concurrent cytotoxicity with mutation assay reported in Tables 3 and 4). Relative to the solvent control, survival (relative cloning efficiency) was 3%, 11%, 68%, 86% and 97% at 20, 15, 10, 5 and 1 ug/ml respectively, in the absence of S-9 and was 5%, 26%, 46%, 58% and 91% at 150, 80, 40, 10 and 1 ug/ml in the presence of S-9.

The activity of T4920 in the CHO/HGPRT mutation assay after a 5 hour treatment in the absence of an exogenous metabolic activation system is presented in Table 3. Thirteen mutant colonies were observed in the solvent control group for a mutant frequency of 12.9 per  $10^6$  clonable cells. Nineteen mutant colonies were observed in the untreated control group for a mutant frequency of 18.8 per  $10^6$  clonable cells. At dose levels of 15, 10, 5 and 1 ug/ml, a total of 11, 15, 24 and 4 thioguanine-resistant mutants were observed. The mutant frequency of none of the test article treated group was increased significantly above the untreated control. The mutant frequency at 20 ug/ml was too toxic to evaluate. EMS induced a total of 221 mutants to yield a mutant frequency of 381.0 mutants/ $10^6$  clonable cells.

The activity of T4920 in the CHO/HGPRT mutation assay after a 5 hour treatment in the presence of an S-9 reaction mixture is presented in Table 4. Thirteen mutant colonies were observed in the solvent control group for a mutant frequency of 13.0 per  $10^6$  clonable cells. Fifteen mutant colonies were observed in the untreated control group for a mutant frequency of 14.9 per  $10^6$  clonable cells. At dose levels of 80, 40, 10 and 1 ug/ml, a total of 24, 29, 10 and 18 thioguanine-resistant mutants were observed. The mutant frequencies of two of the test article treated groups was increased significantly above the controls. The mutant frequency at 150 ug/ml was too toxic to consider as a valid data point. BaP induced a total of 409 mutants to yield a mutant frequency of 629.2 mutants/ $10^6$  clonable cells.

TABLE 1  
CHO/HGPRT MUTATION ASSAY  
Preliminary Toxicity Test

| -S-9                   |                    |                                    |   | +S-9                   |                    |                                    |   |
|------------------------|--------------------|------------------------------------|---|------------------------|--------------------|------------------------------------|---|
| Treatment <sup>1</sup> | Colonies/<br>Plate | Cloning<br>Efficiency <sup>2</sup> | Relative<br>Cloning<br>Efficiency <sup>3</sup><br>(%) | Treatment <sup>1</sup> | Colonies/<br>Plate | Cloning<br>Efficiency <sup>2</sup> | Relative<br>Cloning<br>Efficiency <sup>3</sup><br>(%) |
| Solvent<br>(EtOH)      | 82<br>92<br>99     | 0.91                               | 100   | Solvent<br>(EtOH)      | 92<br>99<br>92     | 0.94                               | 100   |
| T4920<br>1000 ug/ml    | 0<br>0<br>0        | -                                  | -   | T4920<br>1000 ug/ml    | 0<br>0<br>0        | -                                  | -   |
| 300 ug/ml              | 0<br>0<br>0        | -                                  | -   | 300 ug/ml              | 3<br>4<br>11       | 0.06                               | 6   |
| 100 ug/ml              | 0<br>0<br>0        | -                                  | -   | 100 ug/ml              | 29<br>24<br>39     | 0.31                               | 33  |
| 30 ug/ml               | 0<br>1<br>0        | 0                                  | 0   | 30 ug/ml               | 24<br>30<br>50     | 0.35                               | 37  |
| 10 ug/ml               | 50<br>32<br>35     | 0.39                               | 43  | 10 ug/ml               | 44<br>55<br>54     | 0.51                               | 54  |
| 3 ug/ml                | 55<br>74<br>51     | 0.60                               | 66  | 3 ug/ml                | 68<br>72<br>75     | 0.72                               | 77  |
| 1 ug/ml                | 83<br>78<br>70     | 0.77                               | 85  | 1 ug/ml                | 88<br>92<br>98     | 0.93                               | 99  |
| 0.3 ug/ml              | 80<br>99<br>85     | 0.88                               | 97  | 0.3 ug/ml              | 95<br>92<br>90     | 0.92                               | 98  |
| 0.1 ug/ml              | 86<br>92<br>96     | 0.91                               | 100   | 0.1 ug/ml              | 98<br>92<br>95     | 0.95                               | 101   |

<sup>1</sup> Cells were exposed to the test article in the absence (- S-9) or presence (+ S-9) of an exogenous metabolic activation for 5 hours at 37±1°C.

<sup>2</sup> Cloning efficiency =  $\frac{\text{total colonies counted}}{100 \text{ cells} \times \text{number replicates}}$

<sup>3</sup> Relative Cloning Efficiency =  $\frac{\text{cloning efficiency of treatment group}}{\text{cloning efficiency of solvent group}} \times 100$

TABLE 2  
CHO/HGPRT MUTATION ASSAY  
Concurrent Toxicity Test

| -S-9                   |                                 |                                    |   | +S-9                   |                                 |                                    |   |
|------------------------|---------------------------------|------------------------------------|---|------------------------|---------------------------------|------------------------------------|---|
| Treatment <sup>1</sup> | Colonies/<br>Plate <sup>4</sup> | Cloning<br>Efficiency <sup>2</sup> | Relative<br>Cloning<br>Efficiency <sup>3</sup><br>(%) | Treatment <sup>1</sup> | Colonies/<br>Plate <sup>4</sup> | Cloning<br>Efficiency <sup>2</sup> | Relative<br>Cloning<br>Efficiency <sup>3</sup><br>(%) |
| Untreated<br>control   | 74<br>76<br>*                   | 0.75                               | 95  | Untreated<br>control   | *<br>66<br>71                   | 0.69                               | 93  |
| Solvent (EtOH)         | *<br>83<br>75                   | 0.79                               | 100   | Solvent (EtOH)         | 73<br>*<br>75                   | 0.74                               | 100   |
| T4920<br>20 ug/ml      | 3<br>1<br>1                     | 0.02                               | 3   | T4920<br>150 ug/ml     | 3<br>4<br>6                     | 0.04                               | 5   |
| 15 ug/ml               | 10<br>7<br>10                   | 0.09                               | 11  | 80 ug/ml               | 17<br>22<br>18                  | 0.19                               | 26  |
| 10 ug/ml               | 55<br>47<br>59                  | 0.54                               | 68  | 40 ug/ml               | 34<br>33<br>36                  | 0.34                               | 46  |
| 5 ug/ml                | 68<br>68<br>*                   | 0.68                               | 86  | 10 ug/ml               | 45<br>40<br>44                  | 0.43                               | 58  |
| 1 ug/ml                | 80<br>*<br>73                   | 0.77                               | 97  | 1 ug/ml                | 64<br>69<br>*                   | 0.67                               | 91  |
| EMS                    | 41<br>44<br>*                   | 0.43                               | 54  | BaP                    | 10<br>11<br>9                   | 0.10                               | 14  |

<sup>1</sup> Cells were exposed to the test article in the absence (- S-9) or presence (+ S-9) of an exogenous metabolic activation for 5 hours at 37±1°C.

<sup>2</sup> Cloning efficiency =  $\frac{\text{total colonies counted}}{100 \text{ cells} \times \text{number replicates}}$

<sup>3</sup> Relative Cloning Efficiency =  $\frac{\text{cloning efficiency of treatment group}}{\text{cloning efficiency of solvent group}} \times 100$

<sup>4</sup> \*, plates lost due to contamination

TABLE 3  
 CHO/HGPRT MUTATION ASSAY  
 NON-ACTIVATED STUDY

| Treatment <sup>1</sup> | Cloning Efficiency at Selection |     |     |                |                                 | Mutant Colonies/Selection Dish    |    |    |    |    | Total Mutant Colonies | Mutants/10 <sup>6</sup> Clonable Cells <sup>3</sup> |
|------------------------|---------------------------------|-----|-----|----------------|---------------------------------|-----------------------------------|----|----|----|----|-----------------------|---|
|                        | Colonies per Dish               |     |     | Total Colonies | Cloning Efficiency <sup>2</sup> | 1                                 | 2  | 3  | 4  | 5  |                       |   |
|                        | 1                               | 2   | 3   |                |                                 |                                   |    |    |    |    |                       |   |
| Untreated control      | 100                             | 104 | 100 | 304            | 1.01                            | 4                                 | 8  | 0  | 2  | 5  | 19                    | 18.8  |
| Solvent (EtOH)         | 98                              | 105 | 100 | 303            | 1.01                            | 4                                 | 3  | 2  | 1  | 3  | 13                    | 12.9  |
| T4920                  |                                 |     |     |                |                                 |                                   |    |    |    |    |                       |   |
| 20 ug/ml               | 14                              | 23  | 22  | 59             | 0.20                            | ----- too toxic to evaluate ----- |    |    |    |    |                       |   |
| 15 ug/ml               | 69                              | 71  | 76  | 216            | 0.72                            | 1                                 | 4  | 2  | 1  | 3  | 11                    | 15.3  |
| 10 ug/ml               | 76                              | 80  | 82  | 238            | 0.79                            | 1                                 | 3  | 5  | 2  | 4  | 15                    | 19.0  |
| 5 ug/ml                | 76                              | 80  | 77  | 233            | 0.78                            | 4                                 | 2  | 8  | 6  | 4  | 24                    | 30.8  |
| 1 ug/ml                | 82                              | 81  | 81  | 244            | 0.81                            | 0                                 | 1  | 0  | 3  | 0  | 4                     | 4.9   |
| EMS                    | 55                              | 65  | 54  | 174            | 0.58                            | 40                                | 43 | 44 | 50 | 44 | 221                   | 381.0   |

<sup>1</sup> Cells were exposed to the test article for 5 hours at 37±1°C.

<sup>2</sup> Cloning efficiency =  $\frac{\text{Total Colonies Counted}}{\text{Dishes Counted} \times 100 \text{ Cells/dish}}$

<sup>3</sup> Mutants/10<sup>6</sup> cloning cells =  $\frac{\text{Total Mutant Colonies}}{\text{Number Selection dishes} \times \text{Cloning Efficiency} \times 2 \times 10^5 \text{ cells}} \times 10^6$

TABLE 4

## CHO/HGPRT MUTATION ASSAY

## ACTIVATED STUDY

| Treatment <sup>1</sup> | Cloning Efficiency at Selection |     |     |                |                                 | Mutant Colonies/Selection Dish |    |    |    |    | Total Mutant Colonies | Mutants/10 <sup>6</sup> Clonable Cells <sup>3</sup> |
|------------------------|---------------------------------|-----|-----|----------------|---------------------------------|--------------------------------|----|----|----|----|-----------------------|---|
|                        | Colonies per Dish               |     |     | Total Colonies | Cloning Efficiency <sup>2</sup> | 1                              | 2  | 3  | 4  | 5  |                       |   |
|                        | 1                               | 2   | 3   |                |                                 |                                |    |    |    |    |                       |   |
| Untreated control      | 96                              | 110 | 98  | 304            | 1.01                            | 3                              | 0  | 6  | 1  | 5  | 15                    | 14.9  |
| Solvent (EtOH)         | 100                             | 101 | 100 | 301            | 1.00                            | 2                              | 5  | 1  | 4  | 1  | 13                    | 13.0  |
| T4920                  |                                 |     |     |                |                                 |                                |    |    |    |    |                       |   |
| 150 ug/ml              | 59                              | 57  | 60  | 176            | 0.59                            | 6                              | 5  | 6  | 3  | 6  | 26                    | 44.1  |
| 80 ug/ml               | 60                              | 63  | 68  | 191            | 0.64                            | 6                              | 2  | 5  | 4  | 7  | 24                    | 37.5  |
| 40 ug/ml               | 70                              | 71  | 70  | 211            | 0.70                            | 8                              | 3  | 7  | 3  | 8  | 29                    | 41.4  |
| 10 ug/ml               | 74                              | 70  | 76  | 220            | 0.73                            | 0                              | 1  | 1  | 4  | 4  | 10                    | 13.7  |
| 1 ug/ml                | 89                              | 82  | 82  | 253            | 0.84                            | 3                              | 2  | 7  | 3  | 3  | 18                    | 21.4  |
| BaP                    | 66                              | 59  | 71  | 196            | 0.65                            | 75                             | 87 | 79 | 84 | 84 | 409                   | 629.2   |

<sup>1</sup> Cells were exposed to the test article in the presence of a S-9 reaction mixture for 5 hours at 37±1°C.

<sup>2</sup> Cloning efficiency =  $\frac{\text{Total Colonies Counted}}{\text{Dishes Counted} \times 100 \text{ cells/dish}}$

<sup>3</sup> Mutants/10<sup>6</sup> clonable cells =  $\frac{\text{Total Mutant Colonies}}{\text{Number Selection dishes} \times \text{Cloning Efficiency} \times 2 \times 10^5 \text{ cells}} \times 10^6$

CONCLUSION

The positive and negative controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, G0539.02 should be considered negative in the absence of activation and suspect in the presence of S-9 activation in the CHO/HGPRT mutation assay.

REFERENCES

1. Hsie, A.W., Casciano, D.A., Couch, D.B., Krahn, B.F., O'Neill, J.P., and Whitfield, B.L. 1981. The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. A report of the Gen-Tox Program. *Mutation Research* 86:193-214.
2. Machanoff, R., O'Neill, J.P., and Hsie, A.W. 1981. Quantitative analysis of cytotoxicity and mutagenicity of benzo(a)pyrene in mammalian cells (CHO/HGPRT). *Chem. Biol. Interactions* 34:1-10.
3. O'Neill, J.P., Brimer, P.A., Machanoff, R., Hirsch, G.P. and Hsie, A.W. 1977. A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Development and definition of the system. *Mutation Research* 45:91-101.
4. Gupta, R.S. and Sing, B. 1982. Mutagenic responses of five independent genetic loci in CHO cells to a variety of mutagens: development and characteristics of a mutagen screening system based on selection for multiple drug resistant markers. *Mutation Res.* 94:449-466.

PROTOCOL AMENDMENT

DATE: July 30, 1986

SPONSOR: Procter & Gamble Company

SPONSOR'S TEST ARTICLE DESIGNATION: G0539.02

MA STUDY NO: T4920.332 PROTOCOL NO.: SPGT332

PROTOCOL TITLE: CHO/HGPRT Mutation Assay

AMENDMENT(S): (INCLUDE LOCATION IN PROTOCOL, AMENDMENT, AND REASON)

Page 1 of 7, Section 2.1, Test Article Identification should read "G0539.02" instead of "G0539.01".

Reason for Amendment: To correct an entry error.

APPROVAL:

*Janet E. Wood*  
SPONSOR REPRESENTATIVE/  
INVESTIGATOR

*Jan Mary*  
STUDY DIRECTOR

*8/1/86*  
DATE

7/30/86  
DATE



**TEST SUBSTANCE CHARACTERIZATION REPORT  
(TSCR)**

For Tox Office  
Use Only:  
DPD # 210392  
TSIN # 60539.4

*Jew*  
Testing  
Lab or  
Data Source

11. Characterization, Microbial and Properties Information:

|    | Date Submitted | Submitter Code (if exists) or Lab Notebook # | Component or Property | ( <input checked="" type="checkbox"/> )* | Measured Value | Limits    | Testing Lab or Data Source     |
|----|----------------|--|-----------------------|--|----------------|-----------|--------------------------------|
| 1  | 4/16/86        | PEC-079                                      | MCT                   | <input checked="" type="checkbox"/>      | Pass           | Must Pass | Micro                          |
| 2  | 7/10/85        | 8514-9005                                    | Assay                 | <input type="checkbox"/>                 | 99.5           | 98-102    | Mfg. GMP <sup>PL</sup> 5-23-85 |
| 3  |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 4  |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 5  |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 6  |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 7  |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 8  |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 9  |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 10 |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 11 |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 12 |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 13 |                |  |                       | <input type="checkbox"/>                 |                |           |                                |
| 14 |                |  |                       | <input type="checkbox"/>                 |                |           |                                |

\* Analysis required by Toxicologist or Microbiologist.

12. Approvals:

The test substance as made and characterized is a representative example of the intended formulation. Making records for plant-made product are being/have been obtained and evaluated by Products Research.

a. Process Development: *BD Barberol* (Signature) BD Barberol (Name) 4/23/86 (Date)

b. Products Research: N/A (Signature) \_\_\_\_\_ (Name) \_\_\_\_\_ (Date)

\_\_\_\_\_ finished product samples will be retained by Quality Assurance.  
# samples

c. GMP-Quality Assur.: *Rodger F Stanfield* (Signature) \_\_\_\_\_ (Name) 4/23/86 (Date)

(Attach any Batch Records for Quality Assurance/GMP Review.)

13. The characterization tests requested are appropriate and the test substance is acceptable for: [] acute animal test; [] subchronic animal test; [] chronic animal test; [] human safety test; [] in vitro test; [] environmental safety test.

*James E. Cleaver* (Toxicologist's Signature) \_\_\_\_\_ (Name) 4/24/86 (Date)

7. Distribution: Original - Tox Office; Copies - Toxicologist, GMP/QA, Products Research and Process Dev.



# THE PROCTER & GAMBLE COMPANY

MIAMI VALLEY LABORATORIES

P. O. BOX 39175  
CINCINNATI, OHIO 45247

April 30, 1986

Dr. Steve Haworth  
Microbiological Associates, Inc.  
5221 River Road  
Bethesda, MD 20816

Dear Dr. Haworth:

This is to authorize you to carry out the following study according to the attached protocol, and in conformance with the stipulations of our current Laboratory Services Agreement.

Notice: 1) This study is expected to be submitted to the following regulatory agency: FDA. The study should be listed on the Test Facility's Master list of regulated studies. The stipulations of the protocol are to be implemented in complete conformance with the appropriate regulations [FDA Good Laboratory Practice Regulations (21 CFR, Part 58) or EPA GLP Regulations (40 CFR, Part 792)] with the following exception:

If two or more test substances appear on the protocol, it may be conducted as a single study, resulting in a single final report.

2) Documentation of the derivation, characterization, and stability testing of the test substance(s) will be the responsibility of the Sponsor.

Test: CHO/HGPRT Mutation Assay  
Protocol No.: Special Protocol  
Test Substance No.: G0539.01                      Doc. Req. No.: BY0392S  
Physical Form: Powder

This is the sample that we were given a start date of 5/5/86, verbals on 7/7/86 and a final report date of 7/25/86.

Four copies of the final report are needed as soon as possible, and are to be sent to my attention at the above address.

Dr. Steve Haworth  
MBA  
April 30, 1986  
Page 2

Matters involving the scientific aspects of the work can be handled directly with the Sponsor's Divisional Toxicologist or with Dr. E. D. Thompson. All unused samples are to be returned to the Divisional Toxicologist at the following address (the cost of shipment should be included in the study cost):

Mr. J. E. Weaver Telephone No. (513) 530-2430  
The Procter & Gamble Company  
Sharon Woods Technical Center  
11511 Reed Hartman Hwy. - Room HB-2D29  
Cincinnati, OH 45241

Complete both copies of the attached protocol by adding your study number, proposed start and completion dates, and have the Study Director sign and date them. The Study Director should define the start and completion dates on the protocol. Retain one copy and return one copy (which includes the study cost) to me along with a letter stating that you agree to do the work specified in the attached protocol. In addition, if you cannot meet the report dates, please let me know.

The invoice for this study should be sent to:

Mr. R. T. Lyons  
The Procter & Gamble Company  
11511 Reed Hartman Highway - Room No. HB-2D31  
Cincinnati, OH 45241

Sincerely,

THE PROCTER & GAMBLE COMPANY  
Research & Development Department



H. A. Derner  
Human & Environmental Safety Division

Approved:



R. E. Winters, Ph.D  
Director, Human & Environmental Safety Division

bg  
Attachments  
cc: Study File  
J. E. Weaver  
E. D. Thompson

## CHO/HGPRT MUTATION ASSAY

### 1.0 PURPOSE

The purpose of this study is to assess the mutagenic potential of a test article or its metabolites based on its ability to induce forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of Chinese hamster ovary (CHO) cells.

### 2.0 TEST ARTICLE

2.1 Identification: *.G0539,01*

2.2 Analysis:

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

2.3 Study No: *BY0392S (Sponsor's DRD No.)*

### 3.0 SPONSOR

3.1 Name:

*The Procter + Gamble Co*

3.2 Address:

*Beauty Care Division  
Sharon Woods Technical Ctr  
11511 Reed Hartman Hwy  
Cincinnati, OH 45241*

3.3 Authorized Representatives:

*James E. Weaver, Ph.D.*

### 4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology  
Microbiological Associates, Inc.

4.2 Address: 5221 River Road  
Bethesda, Maryland 20816

4.3 Study Director: Li L. Yang, Ph.D.

## 5.0 TEST SYSTEM

The CHO-K<sub>1</sub>-BH<sub>4</sub> cell line is a proline auxotroph with a modal chromosome number of 20, a population doubling time of 12-14 hours, and a cloning efficiency of usually greater than 80% (2). This subclone (D1) was derived by Dr. Abraham Hsie, Oak Ridge National Laboratories, Oak Ridge, TN.

## 6.0 EXPERIMENTAL DESIGN

The CHO/HGPRT assay was designed to select for mutant cells which have become resistant to such purine analogues as 6-thioguanine (TG) and 8-azaguanine as a result of mutation at the X-chromosome-linked HGPRT locus (3,4). This system has been demonstrated to be sensitive to the mutagenic action of a variety of chemicals (2).

The assays will be performed by exposing CHO cells for 5 hours to five concentrations of test article as well as positive, negative, and solvent controls in the presence and absence of an exogenous source of metabolic activation, after which the cells will be cultured for a 7-9 day expression period. The treated cells will be grown in the presence of 10  $\mu$ M TG for selection of the mutant colonies. The mutagenic potential of a test article will be determined by its ability to induce a dose-related increase in the number of TG-resistant mutant colonies when compared to the solvent control.

### 6.1 Dose Levels

The optimal dose levels for the mutation assay will be selected following a preliminary toxicity test based upon colony-forming efficiency. Approximately  $5 \times 10^5$  CHO cells will be seeded in each flask and incubated at  $37 \pm 1^\circ\text{C}$ . Eighteen to 24 hours later, cells will be exposed to solvent alone and to nine concentrations of test article ranging from 0.1 to 1000  $\mu\text{g}/\text{ml}$  for solids and 0.001 to 10  $\mu\text{l}/\text{ml}$  for liquids for 5 hours at  $37 \pm 1^\circ\text{C}$  in the presence and absence of an exogenous source of metabolic activation. The following day, the treated cells will be trypsinized and reseeded at a density of 100 cells/60 mm dish. The cloning efficiency will be determined 7-10 days later. The cell survival of the test article-treated groups will be expressed relative to the solvent control (relative cloning efficiency).

Whenever possible, the high dose will be selected to give a cell survival of 10-30%. Three lower doses will be selected, one of which will be non-toxic. In the event the test article cannot be dissolved at a high enough concentration in an appropriate solvent to be cytotoxic or if excessive precipitation of the test article-solvent solution occurs upon addition to the treatment medium, the study will be conducted with the maximum concentration obtainable.

## 6.2 Frequency and Route of Administration

Target cells will be treated for 5 hours by incorporation of the test article-solvent mixture into the growth medium. This technique has been demonstrated to be an effective method for exposing mammalian cells in culture to concentrations of chemicals.

## 6.3 Controls

### 6.3.1 Negative control

Untreated cells will be used as the negative control.

### 6.3.2 Solvent control

The solvent vehicle for the test article will be used as the solvent control. The solvents compatible with this test system, in order of preference, include distilled water or F12 medium, dimethylsulfoxide, acetone and ethanol.

### 6.3.3 Positive control

Ethyl methanesulfonate (EMS) will be used as the positive control for the non-activated study. Benzo(a)pyrene (BaP) will be used as the positive control for the activated study.

## 6.4 S-9

Adult male Fischer-344 rats, 200-250 gm, will be induced by a single intraperitoneal injection of Aroclor 1254 at a dosage of 500 mg/kg body weight two days prior to sacrifice. The animals will be sacrificed and the livers aseptically removed. The excised tissue will be rinsed three times in cold sterile 0.15 M KCl and then homogenized in a Polytron Tissuemizer at a concentration of 1:3 w/v in 0.15 M KCl. The supernatant fraction (S-9) will be collected following centrifugation at 9000 x g for 10 min at 4 + 2°C, portioned into aliquots for daily use, and stored frozen at -70°C until used.

Immediately prior to use, the S-9 will be mixed with the cofactor pool to contain 100 ul S-9/ml reaction mixture of approximately 4mM NADP, 5mM glucose-6-phosphate, 10mM MgCl<sub>2</sub>, 30mM KCl, 10mM CaCl<sub>2</sub>, and 50mM sodium phosphate buffer, pH<sup>2</sup> 8.0 (3). The S-9 reaction mixture will be stored on ice until used.

## 7.0 METHODS

### 7.1 Preparation of Target Cells

Exponentially growing CHO-K<sub>1</sub>-BH<sub>4</sub> cells will be plated in F12 medium supplemented with 5% dialyzed serum (F12FBS5) at a density of 5 x 10<sup>5</sup> cells/25 cm<sup>2</sup> flask and will be incubated at 37 + 1°C in a humidified atmosphere of 5 + 1% CO<sub>2</sub> in air for 18-24 hours.

## 7.2 Treatment of Target Cells

The time of initiation of chemical treatment will be designated as day 0. Cells will be exposed, in duplicate cultures, to five concentrations of the test article for 5 hours at  $37 \pm 1^\circ\text{C}$ . The treatment medium will consist of 4 ml F12FBS5, 1 ml S-9 reaction mixture, and 50 ul of control or test article diluted to the appropriate concentration in solvent for the activated study or 5 ml F12FBS5 and 50 ul of control or test article diluted to the appropriate concentration in solvent for the non-activated study. After the treatment period, all media will be aspirated, the cells washed with HBSS and cultured in F12FBS5 at  $37 \pm 1^\circ\text{C}$ . After 18-24 hours incubation, the cells will be subcultured to assess cytotoxicity and to continue the phenotypic expression period.

## 7.3 Identification of Test System

Using a permanent marking pen, or computer generated labels, the cytotoxicity and mutation plates will be identified by the study number and a code system to designate the treatment condition, test phase and replicate plate number.

## 7.4 Estimation of Cytotoxicity

For evaluation of cytotoxicity, the replicate from each treatment condition will be pooled and subcultured in F12FBS5, in triplicate, at a density of 100 cells/60 mm dish. After 7-10 days incubation at  $37 \pm 1^\circ\text{C}$  in  $5 \pm 1\%$   $\text{CO}_2$  in air, colonies will be fixed with 95% methanol, stained with 10% aqueous Giemsa, and counted. Cytotoxicity will be expressed relative to the solvent-treated control cultures.

## 7.5 Expression of the Mutant Phenotype

For expression of the mutant phenotype, the replicates from each treatment condition will be pooled and subcultured in F12FBS5 in duplicate, at a density of no greater than  $10^6$  cells/100 mm dish. Subculture as above at 2-3 day intervals will be performed for the 7-9 day expression period. At this time, selection for the mutant phenotype will be performed.

## 7.6 Selection of the Mutant Phenotype

For selection of the TG-resistant phenotype, the duplicate flasks from each treatment condition will be pooled and five replicate dishes plated, at a density of  $2 \times 10^5$  cells/100 mm dish in F12FBS5-Hx containing 10 uM TG. For cloning efficiency at the time of selection, 100 cells/60 mm dish will be plated in triplicate in medium free of TG. After 7-10 days of incubation, the colonies will be fixed, stained and counted for both cloning efficiency at selection and mutant selection.

## 8.0 EVALUATION OF TEST RESULTS

The cytotoxicity effects of each treatment condition are expressed relative to the solvent-treated control (relative cloning efficiency). The mutation frequency (MF) for each treatment condition is calculated by dividing the total number of mutant colonies by the number of cells selected (usually  $10^6$  cells: 5 plates at  $2 \times 10^5$  cells/plate), corrected for the cloning efficiency of cells prior to mutant selection, and is expressed as TG-resistant mutants per  $10^6$  clonable cells. For experimental conditions in which no mutant colonies are observed, mutation frequencies will be expressed as less than the frequency obtained with one mutant colony.

The calculation of mutagenic response in terms of fold increase in mutation frequency above the background rate does not provide a reliable indication of the significance of the observed response, since for some loci with spontaneous mutation frequencies which are very low and for which only a few colonies may be observed in the control experiments, a 2- to 3-fold increase in mutation frequency may not be significant. For assays characterized by a wide degree of variation in the frequency of spontaneous mutants found in the negative or solvent controls, a confidence interval can be calculated by the application of a one-sided Student's t test ( $p < 0.05$ ) from the historic background mutation frequency (1). In this laboratory, the confidence interval for the CHO/HGPRT assay is  $8.7/10^6$  clonable cells. Therefore, the mutagenic response after treatment will be considered significant only when the treatment mutation frequency is increased above that of the solvent control by at least 8.7 mutants/ $10^6$  clonable cells and also is at least twice that of the solvent control.

The following criteria will be used as guidelines for interpretation of the data; however, the conclusion of the study will be based upon the Study Director's evaluation and interpretation of the data. The assay will be considered positive in the event a dose-dependent increase in mutation frequency is observed with one or more of the five concentrations tested inducing a mutation frequency which is at least twice that of the solvent control and also is increased above that of the solvent control by at least 8.7 mutants/ $10^6$  clonable cells. The assay will be considered suspect if there is no dose response but one or more doses induce a mutation frequency which is considered significant. The assay will be considered negative if none of the doses tested induce a mutation frequency which is considered significant.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The cloning efficiency of the solvent and untreated controls must be no less than 50%. The spontaneous mutation frequency in the solvent and untreated controls must fall within the range of 0-20 mutants per  $10^6$  clonable cells.

The positive control must induce a mutation frequency at least three times that of the solvent control.

10.0 FINAL REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of data. Data will be presented in tabular form for the relative cytotoxic effects of each condition, the total number of mutants observed, and the mutation frequency for each treatment group.

11.0 RECORD AND SPECIMEN ARCHIVES

11.1 Records

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

11.2 Specimens

All specimens, such as stained plates, will be held in storage as long as the quality of the preparation affords evaluation and in accordance with the Terms and Conditions.

12.0 -GOOD LABORATORY PRACTICES

This study will be performed in compliance with the provisions of the Good Laboratory Practices for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? Likely  
If so, to which agency or agencies? U.S. FDA

Does the Sponsor request that samples of the Test Article dosing solutions be returned? Yes. 100 ml each

Does the Sponsor request that the test article be returned? Yes

13.0 SPECIAL INSTRUCTIONS

Dose in aqueous solution if solubility (<1%) permits.  
Ethanol (2<sup>nd</sup> choice; >10% solubility) or acetone (3<sup>rd</sup> choice; >10% solubility) may be used only if distilled water cannot be used.



TO OPERATIONS SECTION  
SUBJECT NONCLINICAL STUDY - REGULATORY STATUS

ATTENTION

ATTENTION

Notifications pertaining to:

DRD # BYCRO392S  
TSIN G0539.01

*JFW*  
*5/2/80*

1. Studies requested on the above document:

- are expected to be submitted to the following regulatory agencies as a GLP regulated study: FDA

- are expected to be submitted to the following regulatory agencies but is not a GLP regulated study: \_\_\_\_\_

Metabolism,  Pharmacological Screen,  Other: \_\_\_\_\_

- are not expected to be submitted to a regulatory agency. (Boxes #3 and #4 below need not be checked).

2.  - The test substance has been characterized and results are shown on the test substance characterization report which accompanies the DRD.

3.  - The method of synthesis fabrication or derivation of the test related substances has been documented. (Required for regulated studies).

4.  - Stability testing has been done or will be done on the test substance. (Required for regulated studies).

Sponsor's Divisional Toxicologist: *James E. Weaver*

Date: 3/7/85

lg:MEQANZ



# SITEK RESEARCH LABORATORIES

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

## FINAL REPORT

Test Substance Identification: G0539.02

Divisional Request Document Number: BY0440

Test Substance Lot Number: 02

SITEK's Study Number: 0024-2400

Test Substance Description: White Powder

Storage Conditions: Room Temperature

Date Test Substance Received: September 13, 1985

Test Initiation Date: October 31, 1985

Final Report Date: December 5, 1985

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

Sponsor's Representative: E. D. Thompson, Ph.D.

Testing Facility: SITEK Research Laboratories  
12111 Parklawn Drive  
Rockville, Maryland 20852

RECEIVED BY

DEC 10 1985

OPERATIONS SECTION

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

12/5/85  
Date

Roger M. Brauning  
Roger M. Brauning, B.S., Sr. Laboratory Technician

12/5/85  
Date

Kamala Pant  
Kamala Pant, M.S., Research Associate

12.5.85  
Date

Karen Schweitzer  
Karen Schweitzer, B.S., Laboratory Technician

12/5/85  
Date

Tracey Vandermark  
Tracey Vandermark, B.S., Laboratory Technician

12/5/85  
Date



**SITEK RESEARCH LABORATORIES**

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

STUDY NO. 0024-2400

SPONSOR'S TEST ARTICLE I. D. G0539.02

To the best of my knowledge this study was performed in compliance with the GLP regulations for nonclinical laboratory studies and the GLP standards for health effects as described in 21 CFR Part 58 and 40 CFR Part 772, respectively. In this context the facilities, equipment, personnel, methods, practices, records, controls and reports have been inspected per the GAU standard operating procedures and found to be in compliance with the above regulatory requirements.

The following phases were inspected for this study:

| <u>Inspection Date</u> | <u>Critical Phase</u>   |
|------------------------|---|
| <u>11/4/85</u>         | <u>Preparation of cloning medium for the</u><br><u>Mutation Assay</u> |
| <u>11/4/85</u>         | <u>Addition of cells to the cloning flasks</u>                        |
| _____                  | _____   |
| _____                  | _____   |
| _____                  | _____   |
| _____                  | _____   |
| _____                  | _____   |

Findings Reported to Study Director 11/5/85  
Date

Findings Reported to Management 11/5/85  
Date

Signature *Patricia Postal* Date 12/5/85

SUMMARY

The Procter and Gamble Company's test article G0539.02 was tested for its potential to induce mutations in the L5178Y TK+/- Mouse Lymphoma Mutation Assay at the thymidine kinase locus. The test article was also tested to determine if it was differentially toxic to L5178Y TK+/- cells versus L5178Y TK-/- cells. Cultures were treated at concentrations of 1.0, 0.56, 0.31, 0.18, 0.10, 0.056, 0.031 and 0.017 ug/ml in the absence of exogenous activation and at concentrations of 20.0, 15.0, 11.25, 8.44, 6.33, 4.75, 3.56 and 2.67 ug/ml in the presence of Aroclor-induced rat liver S-9 plus cofactors (S-9 mix).

The results indicated that in the absence of exogenous activation, the test article induced a significant increase in the mutant frequency of treated cultures as compared to the mutant frequency of the corresponding solvent control cultures. The response was also dose dependent. In the presence of S-9, all of the treated cultures that were cloned had mutant frequencies that were not significantly greater than the mean mutant frequency of the corresponding solvent control cultures. The relative total growth for these cultures ranged from 9% to 23 %. In regard to differential toxicity to TK+/- cells versus TK-/- cells, no significant difference in toxicity was observed. The suspension growth, relative cloning efficiencies and total growth for the TK+/- cells and TK-/- cells were similar throughout the concentration range tested.

INTRODUCTION

This study was conducted by P. Kirby, Ph.D., K. Pant, M.S., R. Brauningner, B.S., K. Schweitzer, B.S., and T. Vandermark, B.S., from November 2, 1985, to November 22, 1985, at SITEK Research Laboratories. The experimental procedure used to perform this study is described in the study protocol.

The purpose of this study was to evaluate the test article for its ability to induce mutations at the thymidine kinase locus of L5178Y TK+/- Mouse Lymphoma cells and to determine if the test article was differentially toxic to L5178Y TK+/- cells as compared to L5178Y TK-/- cells.

MATERIALS AND METHODS

INDICATOR CELLS

Source

The L5178Y TK+/- Mouse Lymphoma cells, clone 3.7.2C were originally obtained from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina, on October 17, 1984. The cells were subcultured, cleansed of TK-/- cells, and cryopreserved in a large number of ampules for L5178Y TK+/- assays. A sample of these cells was reconstituted, tested for mycoplasma contamination, and found to be free of mycoplasma.

The L5178Y TK-/- Mouse Lymphoma cells were derived from the L5178Y TK+/- line described above by selection in soft agar cloning medium containing trifluorothymidine (3 ug/ml). After 10 to 12 days' incubation in the soft agar cloning medium, this colony of TK-/- cells was isolated and cultured to a large population. These cells were cryopreserved and were designated by SITEK as lot number 032885A.

CONTROL SUBSTANCES

Positive Controls

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

Source: Kodak

Lot No.: A11E

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

Source: Kodak

Lot No.: C13A

Solvent Controls

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

Source: Fisher Scientific Co.

Lot No.: 744197

CAS Registry Number: 67-68-5

Certificate of Actual Lot Analysis:

|                                      |        |
|--------------------------------------|--------|
| Appearance (clear, colorless liquid) | P.T.   |
| Density (grams/ml) at 25°C           | 1.095  |
| Freezing point                       | 18.2°C |
| Residue after evaporation            | 0.002% |
| Color (APHA)                         | 5      |
| Water                                | 0.08%  |

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

Source: Fisher Scientific Co.      Lot No.: 735031

CAS Registry Number: 67-64-1

Certificate of Actual Lot Analysis:

|                                      |         |
|--------------------------------------|---------|
| Appearance (clear, colorless liquid) |         |
| Density (grams/ml) at 25°            | 0.7857  |
| Residue after evaporation            | 0.0002% |
| Color (APHA)                         | 5       |
| Water                                | 0.5%    |

## EXPERIMENTAL PROCEDURES

The experimental procedures used for this study are described in the study protocol.

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

## DESCRIPTION OF DATA CALCULATIONS

Suspension Growth (SG)

The SG for each culture was determined by the following formula:

$$SG = \frac{\text{Day 1 Cell Conc.}}{\text{Day 1 Cell Conc. After Adjustment}} \times \frac{\text{Day 2 Cell Conc.}}{\text{Day 2 Cell Conc. After Adjustment}}$$

Note: If the concentration of cells per ml in a culture never exceeded the starting concentration of  $0.3 \times 10^6$  cells/ml, the SG was rated at 0.

Relative Suspension Growth (RSG)

The RSG for each culture was determined as follows:

$$\text{RSG} = \frac{\text{SG}}{\text{Solvent Ave. SG}} \times 100$$

Cloning Efficiency (CE)

The CE for each culture was determined as follows:

$$\text{CE} = \frac{\text{Ave. Colonies/VC Plate}}{200 \text{ Cells Seeded}} \times 100$$

Mutant Frequency (MF)

The MF for each culture was determined by the following formula:

$$\text{MF} = \frac{\text{Ave. RM Colonies/Plate}}{\text{Ave. VC Colonies/Plate}} \times 200$$

Induced Mutant Frequency (IMF)

The IMF for each culture was determined by subtracting the average MF of the solvent control cultures from the culture's MF.

Relative Cloning Efficiency (RCE)

The RCE for each culture was determined as follows:

$$\text{RCE} = \frac{\text{Culture's Ave. Colonies/VC Plate}}{\text{Solvent Controls' Ave. Colonies/VC Plate}} \times 100$$

Relative Total Growth (RTG)

The RTG for each culture was determined as follows:

$$\text{RTG} = \frac{\text{RSG} \times \text{RCE}}{100}$$

ARCHIVES

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

RESULTS

## RANGE FINDING TEST

A Range Finding Test was not performed on test article G0539.02. Dr. Ed Thompson of the Procter & Gamble Company had specified the concentration range for testing the test article in the assay (see Protocol, page 2, Special Instructions).

## MUTATION ASSAY

In the absence of exogenous activation, the cultures were treated at concentrations of 1.0, 0.56, 0.31, 0.18, 0.10, 0.056, 0.031 and 0.017 ug/ml; and in the presence of S-9 plus cofactors, cultures were treated with concentrations of 20.0, 15.0, 11.25, 8.44, 6.33, 4.75, 3.56 and 2.67 ug/ml. The results of the assay are presented in Tables 1-9.

Key to Tables 1-9

| <u>Table No.</u> | <u>Substance</u>  | <u>Type of Data</u>   | <u>Cell Type</u> |
|------------------|-------------------|-----------------------|------------------|
| 1                | G0539.02 NA       | Cloning Data          | TK+/-            |
| 2                | G0539.02 S-9      | Cloning Data          | TK+/-            |
| 3                | Pos. Controls     | Cloning Data          | TK+/-            |
| 4                | G0539.02 NA & S-9 | Total Growth          | TK+/-            |
| 5                | Pos. Controls     | Total Growth          | TK+/-            |
| 6                | G0539.02 NA       | Cloning Data          | TK-/-            |
| 7                | G0539.02 S-9      | Cloning Data          | TK-/-            |
| 8                | G0539.02 NA & S-9 | Total Growth          | TK-/-            |
| 9                | G0539.02 NA & S-9 | Differential Toxicity | TK+/-&TK-/-      |

These results indicate that in the absence of exogenous activation the test article induced significant dose-dependent increases in mutant frequency in cultures treated with 0.31 ug/ml and above. These cultures had total growth of 33% or less. The next lower concentration of 0.18 ug/ml had a mutant frequency that was approximately 1.70 times greater than the mutant frequency of the solvent control cultures. This culture had 128% relative total growth.

Two cultures treated in the presence of S-9 had mutant frequencies that were elevated, but not twofold greater than the mean mutant frequency of the solvent controls. The cultures treated with 11.25 ug/ml and 8.44 ug/ml had 1.78 and 1.97 fold increases, respectively. These cultures had 16% and 15% relative total growth. The remaining cultures had mutant frequencies that were very near the background level.

As expected, the positive controls caused a significant increase in the number of mutants per  $10^6$  surviving cells.

An additional set of controls was also run in this assay to check the resistance of TK-/- cells and the susceptibility of TK+/- cells to TFT exposure. As expected, the growth of TK+/- cells was effectively stopped while the TK-/- cells grew essentially at the same rate as cells not exposed to TFT. This data is not presented in this report, but is maintained in the study notebook.

#### Differential Toxicity Test

The results of the total growth data for the TK+/- cells and TK-/- cells have been summarized in Table 9. These results clearly indicate that the test article caused equivalent responses in suspension growth, clonal growth and total growth for the TK+/- and TK-/- cells.

TABLE 1 (TK+/- cells)

L517BY TK+/- ASSAY - CLONING DATA

WITHOUT ACTIVATION

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

STUDY NUMBER: 0024-2400

EXPERIMENT NO: B-1

TEST DOSES IN: ug/ml

TEST ARTICLE I.D.: SC-0024

SOLVENT: ETOH

| TEST ARTICLE CONCENTRATION | COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM) |     |     | AVERAGE/ COLONIES RM PLATE | COLONIES PER VIABLE COUNT (VC) PLATE |     |     | AVERAGE/ COLONIES VC PLATE | CLONING EFFI- CIENCY | MUTANT FREQUENCY (MF)/1 <sup>6</sup> | INDUCED MF/ 10 <sup>6</sup> | RELATIVE TOTAL GROWTH |
|----------------------------|---|-----|-----|----------------------------|--------------------------------------|-----|-----|----------------------------|----------------------|--------------------------------------|-----------------------------|-----------------------|
|                            | 1   | 2   | 3   |                            | 1                                    | 2   | 3   |                            |                      |                                      |                             |                       |
| 1.0                        | 123   | 132 | 155 | 137                        | 126                                  | 148 | 173 | 149                        | 75%                  | 184                                  | 130                         | 27%                   |
| 0.56                       | 184   | 188 | 187 | 186                        | 193                                  | 205 | 188 | 195                        | 98%                  | 191                                  | 177                         | 33%                   |
| 0.31                       | 224   | 228 | 272 | 241                        | 157                                  | 156 | 158 | 157                        | 79%                  | 307                                  | 253                         | 28%                   |
| 0.18                       | 101   | 119 | 122 | 114                        | 234                                  | 247 | 260 | 247                        | 124%                 | 92                                   | 38                          | 128%                  |
| 0.10                       | 73  | 60  | 29  | 54                         | 208                                  | 257 | 177 | 214                        | 107%                 | 50                                   | -4                          | 189%                  |
| 0.056                      | -2  | -2  | -2  | NA                         | -2                                   | -2  | -2  | NA                         | NA                   | NA                                   | NA                          | NA                    |
| 0.031                      | -2  | -2  | -2  | NA                         | -2                                   | -2  | -2  | NA                         | NA                   | NA                                   | NA                          | NA                    |
| 0.017                      | -2  | -2  | -2  | NA                         | -2                                   | -2  | -2  | NA                         | NA                   | NA                                   | NA                          | NA                    |

|           | 1  | 2  | 3  | Average | 1   | 2   | 3   | Average | Efficiency | Mutant Freq | Ave. Solvent MF |
|-----------|----|----|----|---------|-----|-----|-----|---------|------------|-------------|-----------------|
| SOLVENT 1 | 28 | 27 | 44 | 33      | 144 | 120 | 157 | 140     | 70%        | 47          | 54              |
| SOLVENT 2 | 44 | 22 | 41 | 36      | 105 | 114 | 132 | 117     | 59%        | 62          | 54              |

-1=CULTURE LOST  
-2=NOT CLONED

TABLE PREPARED BY: Paul E. Kirby DATE: 11/22/85

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TABLE 2 (TK+/- cells)

L5178Y TK+/- ASSAY - CLONING DATA

WITH S9 ACTIVATION

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

STUDY NUMBER: 0024-2400

EXPERIMENT NO: E-1

TEST DOSES IN: ug/ml

TEST ARTICLE I.D.: SC-0024

SOLVENT: ETOH

| TEST ARTICLE CONCENTRATION | COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM) |     |    | AVERAGE/ COLONIES/ RM PLATE | COLONIES PER VIABLE COUNT (VC) PLATE |     |     | AVERAGE/ COLONIES VC PLATE | CLONING EFFICIENCY | MUTANT FREQUENCY (MF)/1 <sup>6</sup> | INDUCED MF/ 10 <sup>6</sup> | RELATIVE TOTAL GROWTH |
|----------------------------|---|-----|----|-----------------------------|--------------------------------------|-----|-----|----------------------------|--------------------|--------------------------------------|-----------------------------|-----------------------|
|                            | 1   | 2   | 3  |                             | 1                                    | 2   | 3   |                            |                    |                                      |                             |                       |
| 20.0                       | -2  | -2  | -2 | NA                          | -2                                   | -2  | -2  | NA                         | NA                 | NA                                   | NA                          | NA                    |
| 15.0                       | 37  | 32  | 48 | 39                          | 116                                  | 113 | 137 | 122                        | 61%                | 64                                   | -5                          | 9%                    |
| 11.25                      | 70  | 94  | 88 | 84                          | 117                                  | 150 | 145 | 137                        | 69%                | 123                                  | 54                          | 16%                   |
| 8.44                       | 116   | 106 | 75 | 99                          | 152                                  | 148 | 138 | 146                        | 73%                | 136                                  | 67                          | 15%                   |
| 6.33                       | 55  | 46  | 70 | 57                          | 152                                  | 125 | 122 | 133                        | 67%                | 86                                   | 17                          | 22%                   |
| 4.75                       | 47  | 42  | 46 | 45                          | 123                                  | 139 | 122 | 128                        | 64%                | 70                                   | 1                           | 23%                   |
| 3.56                       | -2  | -2  | -2 | NA                          | -2                                   | -2  | -2  | NA                         | NA                 | NA                                   | NA                          | NA                    |
| 2.67                       | -2  | -2  | -2 | NA                          | -2                                   | -2  | -2  | NA                         | NA                 | NA                                   | NA                          | NA                    |
| SOLVENT 1                  | 67  | 72  | 61 | 67                          | 176                                  | 199 | 222 | 199                        | 100%               | 67                                   | AVE. SOL-<br>VENT MF        |                       |
| SOLVENT 2                  | 69  | 65  | 50 | 61                          | 181                                  | 175 | 163 | 173                        | 87%                | 71                                   | 69                          |                       |

-1=CULTURE LOST  
-2=NOT CLONED

TABLE PREPARED BY: Paul E. Kirby DATE: 11/22/85

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TABLE 3 (TK+/- cells)

L5178Y TK+/- ASSAY - CLONING DATA

POSITIVE CONTROLS

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

STUDY NUMBER: 0024-2400

EXPERIMENT NO: B-1

EMS DOSES IN: ul/ml  
DMBA DOSES IN: ug/ml

TEST ARTICLE I.D.: SC-0024

SOLVENT: EMS=DMSO  
DMBA=ACETONE

| TEST ARTICLE CONCENTRATION | COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM) |     |     | AVERAGE/ COLONIES RM PLATE | COLONIES PER VIABLE COUNT (VC) PLATE |     |     | AVERAGE/ COLONIES VC PLATE | CLONING EFFICIENCY | MUTANT FREQUENCY (MF) / 10 <sup>6</sup> | INDUCED MF. 10 <sup>6</sup> | RELATIVE TOTAL GROWTH |
|----------------------------|---|-----|-----|----------------------------|--------------------------------------|-----|-----|----------------------------|--------------------|---|-----------------------------|-----------------------|
|                            | 1   | 2   | 3   |                            | 1                                    | 2   | 3   |                            |                    |   |                             |                       |
| <b>EMS</b>                 |   |     |     |                            |                                      |     |     |                            |                    |   |                             |                       |
| 1.0                        | 177   | 171 | 181 | 176                        | 13                                   | 13  | 5   | 10                         | 5%                 | 3520                                    | 3464                        | 2%                    |
| 0.5                        | 336   | 373 | 354 | 354                        | 65                                   | 73  | 67  | 68                         | 34%                | 1041                                    | 985                         | 31%                   |
|                            |   |     |     |                            |                                      |     |     |                            |                    |   | AVE. SOL-VENT MF            |                       |
| SOLVENT 1                  | 36  | 50  | -1  | 43                         | 106                                  | 127 | 129 | 121                        | 60%                | 71                                      | 56                          |                       |
| SOLVENT 2                  | 25  | 25  | 18  | 23                         | 106                                  | 130 | 110 | 115                        | 58%                | 40                                      | 36                          |                       |
| =====                      |   |     |     |                            |                                      |     |     |                            |                    |   |                             |                       |
| <b>DMBA</b>                |   |     |     |                            |                                      |     |     |                            |                    |   |                             |                       |
| 7.5                        | 207   | 180 | 186 | 191                        | 92                                   | 88  | 88  | 89                         | 45%                | 424                                     | 350                         | 30%                   |
| 5.0                        | 188   | 181 | 164 | 178                        | 116                                  | 136 | 120 | 124                        | 62%                | 287                                     | 213                         | 67%                   |
|                            |   |     |     |                            |                                      |     |     |                            |                    |   | AVE. SOL-VENT MF            |                       |
| SOLVENT 1                  | 35  | 44  | 34  | 38                         | 138                                  | 135 | 161 | 145                        | 72%                | 50                                      | 74                          |                       |
| SOLVENT 2                  | 50  | 53  | 49  | 51                         | 145                                  | 175 | -1  | 106                        | 53%                | 96                                      | 74                          |                       |

EMS=Ethyl methanesulfonate  
DMBA=7,12-dimethylbenz(a)anthracene

-1=CULTURE LOST  
-2=NOT CLONED

TABLE PREPARED BY: Paul E. Kirby DATE: 11/22/85

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TABLE 4 (TK+/- cells)

LS178r Tr +/- AEC40

TOTAL GROWTH DATA

STUDY DIRECTOR: Paul E. Kirby, Ph.D. STUDY NUMBER: 0024-2400 EXPERIMENT NUMBER:

TEST DOSES IN: µg/ml

TEST ARTICLE I.D.: 90-0024

SOLVENT: CTG-

| CONCENTRATION             | CELL CONCENTRATION |       | SUSPENSION GROWTH | RELATIVE SUSPENSION GROWTH | AVERAGE COLONIES/ VC PLATE | RELATIVE CLONING EFFICIENCY | RELATIVE TOTAL GROWTH |
|---------------------------|--------------------|-------|-------------------|----------------------------|----------------------------|-----------------------------|-----------------------|
|                           | DAY 1              | DAY 2 |                   |                            |                            |                             |                       |
| <b>WITHOUT ACTIVATION</b> |                    |       |                   |                            |                            |                             |                       |
| 1.0                       | 0.516              | 0.632 | 5.6               | 23%                        | 149                        | 116%                        | 27%                   |
| 0.56                      | 0.518              | 0.601 | 3.5               | 22%                        | 195                        | 151%                        | 33%                   |
| 0.31                      | 0.522              | 0.643 | 3.7               | 23%                        | 157                        | 122%                        | 28%                   |
| 0.18                      | 0.814              | 1.184 | 10.7              | 67%                        | 247                        | 191%                        | 128%                  |
| 0.10                      | 1.086              | 1.503 | 18.1              | 114%                       | 214                        | 166%                        | 189%                  |
| 0.056                     | 1.022              | 1.291 | 14.7              | 92%                        | NA                         | NA                          | NA                    |
| 0.031                     | 1.134              | 1.388 | 17.5              | 110%                       | NA                         | NA                          | NA                    |
| 0.017                     | 0.966              | 1.400 | 15.0              | 95%                        | NA                         | NA                          | NA                    |

| SOLVENT   | DAY 1 | DAY 2 | SUSPENSION GROWTH | SOLVENT AVERAGE | AVE. NO. COLONIES |
|-----------|-------|-------|-------------------|-----------------|-------------------|
| SOLVENT 1 | 0.952 | 1.477 | 15.6              | 15.9            | 140<br>129        |
| SOLVENT 2 | 0.911 | 1.593 | 16.1              |                 | 117               |

| <b>WITH S-9 ACTIVATION</b> |       |       |                   |                            |                            |                             |                       |
|----------------------------|-------|-------|-------------------|----------------------------|----------------------------|-----------------------------|-----------------------|
| CONCENTRATION              | DAY 1 | DAY 2 | SUSPENSION GROWTH | RELATIVE SUSPENSION GROWTH | AVERAGE COLONIES/ VC PLATE | RELATIVE CLONING EFFICIENCY | RELATIVE TOTAL GROWTH |
| 20.0                       | 0.187 | 0.206 | 0.0               | 0%                         | NA                         | NA                          | NA                    |
| 15.0                       | 0.365 | 0.569 | 2.3               | 13%                        | 122                        | 66%                         | 9%                    |
| 11.25                      | 0.506 | 0.685 | 3.9               | 22%                        | 137                        | 74%                         | 16%                   |
| 8.44                       | 0.407 | 0.756 | 3.4               | 19%                        | 146                        | 78%                         | 15%                   |
| 6.33                       | 0.528 | 0.908 | 5.3               | 30%                        | 133                        | 72%                         | 22%                   |
| 4.75                       | 0.521 | 1.011 | 5.9               | 33%                        | 128                        | 69%                         | 23%                   |
| 3.56                       | 0.702 | 1.199 | 9.4               | 53%                        | NA                         | NA                          | NA                    |
| 2.67                       | 0.839 | 1.417 | 13.2              | 74%                        | NA                         | NA                          | NA                    |

| SOLVENT   | DAY 1 | DAY 2 | SUSPENSION GROWTH | SOLVENT AVERAGE | AVE. NO. COLONIES |
|-----------|-------|-------|-------------------|-----------------|-------------------|
| SOLVENT 1 | 0.983 | 1.655 | 18.1              | 17.8            | 199<br>186        |
| SOLVENT 2 | 1.011 | 1.553 | 17.5              |                 | 173               |

-1=CULTURE LOST  
-2=NOT CLONED

TABLE PREPARED BY: Paul E. Kirby 11/22/85  
(Signature) (Date)



TABLE 6 (TK-/- cells)

L5178Y TK+/- ASSAY - CLONING DATA

WITHOUT ACTIVATION

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

STUDY NUMBER: 0024-2400

EXPERIMENT NO: B-1

TEST DOSES IN: ug/ml

TEST ARTICLE I.D.: SC-0024

SOLVENT: ETOH

| TEST ARTICLE CONCENTRATION | COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM) |    |    | AVERAGE/ COLONIES RM PLATE | COLONIES PER VIABLE COUNT (VC) PLATE |     |     | AVERAGE/ COLONIES VC PLATE | CLONING EFFICIENCY | MUTANT FREQUENCY (MF)/1 <sup>6</sup> | INDUCED MF/ 10 <sup>6</sup> | RELATIVE TOTAL GROWTH |
|----------------------------|---|----|----|----------------------------|--------------------------------------|-----|-----|----------------------------|--------------------|--------------------------------------|-----------------------------|-----------------------|
|                            | 1   | 2  | 3  |                            | 1                                    | 2   | 3   |                            |                    |                                      |                             |                       |
| 1.0                        | -2  | -2 | -2 | NA                         | 124                                  | 131 | 127 | 127                        | 64%                | NA                                   | NA                          | 21%                   |
| 0.56                       | -2  | -2 | -2 | NA                         | 114                                  | 84  | 139 | 112                        | 56%                | NA                                   | NA                          | 18%                   |
| 0.31                       | -2  | -2 | -2 | NA                         | 117                                  | 112 | 116 | 115                        | 58%                | NA                                   | NA                          | 25%                   |
| 0.18                       | -2  | -2 | -2 | NA                         | 120                                  | 116 | 99  | 112                        | 56%                | NA                                   | NA                          | 29%                   |
| 0.10                       | -2  | -2 | -2 | NA                         | 88                                   | 111 | 136 | 112                        | 56%                | NA                                   | NA                          | 68%                   |
| 0.056                      | -2  | -2 | -2 | NA                         | -2                                   | -2  | -2  | NA                         | NA                 | NA                                   | NA                          | NA                    |
| 0.031                      | -2  | -2 | -2 | NA                         | -2                                   | -2  | -2  | NA                         | NA                 | NA                                   | NA                          | NA                    |
| 0.017                      | -2  | -2 | -2 | NA                         | -2                                   | -2  | -2  | NA                         | NA                 | NA                                   | NA                          | NA                    |
| SOLVENT 1                  | -2  | -2 | -2 | NA                         | 163                                  | 132 | 139 | 145                        | 72%                | NA                                   | AVE. SOL-<br>VENT MF        |                       |
| SOLVENT 2                  | -2  | -2 | -2 | NA                         | 91                                   | 95  | 104 | 97                         | 48%                | NA                                   | NA                          |                       |

-1=CULTURE LOST  
-2=NOT CLONED

TABLE PREPARED BY: Paul E. Kirby DATE: 11/22/85

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TABLE 7 (TK-/- cells)

L5178Y TK+/- ASSAY - CLONING DATA

WITH S9 ACTIVATION

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

STUDY NUMBER: 0024-2400

EXPERIMENT NO: B-1

TEST DOSES IN: ug/ml

TEST ARTICLE I.D.: SC-0024

SOLVENT: ETOH

| TEST ARTICLE CONCENTRATION | COLONIES PER PLATE IN RESTRICTIVE MEDIUM (RM) |    |    | AVERAGE/ COLONIES RM PLATE | COLONIES PER VIABLE COUNT (VC) PLATE |     |     | AVERAGE/ COLONIES VC PLATE | CLONING EFFICIENCY | MUTANT FREQUENCY (MF)/1 <sup>6</sup> | INDUCED MF/ 10 <sup>6</sup> | RELATIVE TOTAL GROWTH |
|----------------------------|---|----|----|----------------------------|--------------------------------------|-----|-----|----------------------------|--------------------|--------------------------------------|-----------------------------|-----------------------|
|                            | 1   | 2  | 3  |                            | 1                                    | 2   | 3   |                            |                    |                                      |                             |                       |
| 20.0                       | -2  | -2 | -2 | NA                         | -2                                   | -2  | -2  | NA                         | NA                 | NA                                   | NA                          | NA                    |
| 15.0                       | -2  | -2 | -2 | NA                         | 88                                   | 132 | 139 | 120                        | 60%                | NA                                   | NA                          | 12%                   |
| 11.25                      | -2  | -2 | -2 | NA                         | 98                                   | 117 | 98  | 104                        | 52%                | NA                                   | NA                          | 13%                   |
| 8.44                       | -2  | -2 | -2 | NA                         | 168                                  | 173 | 179 | 173                        | 87%                | NA                                   | NA                          | 24%                   |
| 6.33                       | -2  | -2 | -2 | NA                         | 116                                  | 107 | 110 | 111                        | 56%                | NA                                   | NA                          | 19%                   |
| 4.75                       | -2  | -2 | -2 | NA                         | 116                                  | 88  | 103 | 102                        | 51%                | NA                                   | NA                          | 17%                   |
| 3.56                       | -2  | -2 | -2 | NA                         | -2                                   | -2  | -2  | NA                         | NA                 | NA                                   | NA                          | NA                    |
| 2.67                       | -2  | -2 | -2 | NA                         | -2                                   | -2  | -2  | NA                         | NA                 | NA                                   | NA                          | NA                    |

|           |    |    |    |    |     |     |     |     |     |    |                      |
|-----------|----|----|----|----|-----|-----|-----|-----|-----|----|----------------------|
| SOLVENT 1 | -2 | -2 | -2 | NA | 170 | 171 | 180 | 174 | 87% | NA | AVE. SOL-<br>VENT MF |
| SOLVENT 2 | -2 | -2 | -2 | NA | 151 | 170 | 186 | 169 | 85% | NA | NA                   |

-1=CULTURE LOST  
-2=NOT CLONED

TABLE PREPARED BY: Paul E. Kirby DATE: 11/22/85

TABLE 8 (TK-/- cells)

LS1751, T. +, - P. 5HJ

TOTAL GROWTH DATA

STUDY DIRECTOR: Paul E. Kirby, Ph.D.      STUDY NUMBER: 9024-2400      EXPERIMENT NO: 6-1

TEST DOSES IN: ug/ml

TEST ARTICLE I.D.: SC-0024

SOLVENT: ETOH

| CONCENTRATION             | CELL CONCENTRATION DAY 1 | CELL CONCENTRATION DAY 2 | SUSPENSION GROWTH | RELATIVE SUSPENSION GROWTH | AVERAGE COLONIES/ VC PLATE | RELATIVE CLONING EFFICIENCY | RELATIVE TOTAL GROWTH |
|---------------------------|--------------------------|--------------------------|-------------------|----------------------------|----------------------------|-----------------------------|-----------------------|
| <b>WITHOUT ACTIVATION</b> |                          |                          |                   |                            |                            |                             |                       |
| 1.0                       | 0.450                    | 0.576                    | 2.9               | 20%                        | 127                        | 105%                        | 21%                   |
| 0.56                      | 0.376                    | 0.665                    | 2.8               | 19%                        | 112                        | 93%                         | 18%                   |
| 0.31                      | 0.439                    | 0.763                    | 3.7               | 26%                        | 115                        | 95%                         | 25%                   |
| 0.18                      | 0.468                    | 0.897                    | 4.7               | 32%                        | 112                        | 92%                         | 29%                   |
| 0.10                      | 0.782                    | 1.235                    | 10.7              | 74%                        | 112                        | 92%                         | 68%                   |
| 0.056                     | 0.566                    | 1.325                    | 8.3               | 57%                        | NA                         | NA                          | NA                    |
| 0.031                     | 0.867                    | 1.233                    | 11.9              | 82%                        | NA                         | NA                          | NA                    |
| 0.017                     | 0.808                    | 1.412                    | 12.7              | 87%                        | NA                         | NA                          | NA                    |

|           |       |       |      |                      |     |                       |
|-----------|-------|-------|------|----------------------|-----|-----------------------|
| SOLVENT 1 | 0.979 | 1.291 | 14.0 | SOLVENT AVERAGE 14.5 | 145 | AVE. NO. COLONIES 121 |
| SOLVENT 2 | 0.993 | 1.364 | 15.1 |                      | 97  |                       |

| <b>WITH S-9 ACTIVATION</b> |       |       |      |     |     |      |     |
|----------------------------|-------|-------|------|-----|-----|------|-----|
| 20.0                       | 0.187 | 0.228 | 0.0  | 0%  | NA  | NA   | NA  |
| 15.0                       | 0.360 | 0.611 | 2.4  | 17% | 120 | 70%  | 12% |
| 11.25                      | 0.391 | 0.731 | 3.2  | 22% | 104 | 61%  | 13% |
| 8.44                       | 0.413 | 0.777 | 3.6  | 24% | 173 | 101% | 24% |
| 6.33                       | 0.428 | 0.881 | 4.2  | 29% | 111 | 65%  | 19% |
| 4.75                       | 0.461 | 0.816 | 4.2  | 29% | 102 | 60%  | 17% |
| 3.56                       | 0.613 | 1.177 | 8.0  | 55% | NA  | NA   | NA  |
| 2.67                       | 0.721 | 1.392 | 11.2 | 76% | NA  | NA   | NA  |

|           |       |       |      |                      |     |                       |
|-----------|-------|-------|------|----------------------|-----|-----------------------|
| SOLVENT 1 | 0.871 | 1.527 | 14.8 | SOLVENT AVERAGE 14.6 | 174 | AVE. NO. COLONIES 171 |
| SOLVENT 2 | 0.822 | 1.573 | 14.4 |                      | 169 |                       |

-1=CULTURE LOST

-2=NOT CLONED

TABLE PREPARED BY:

*Paul E. Kirby*      11/22/85  
(Signature)      (Date)

TABLE 9

Summary: Results of Differential Toxicity Test

| CONCENTRATION<br>ug/ml | RSG   |        | RCE   |        | RTG   |        |
|------------------------|-------|--------|-------|--------|-------|--------|
|                        | TK-/- | TK+/-  | TK-/- | TK+/-  | TK-/- | TK+/-  |
| WITHOUT ACTIVATION     |       |        |       |        |       |        |
| 1.0                    | 20%   | : 23%  | 105%  | : 116% | 21%   | : 27%  |
| 0.56                   | 19%   | : 22%  | 93%   | : 151% | 18%   | : 33%  |
| 0.31                   | 26%   | : 23%  | 95%   | : 122% | 25%   | : 28%  |
| 0.18                   | 32%   | : 67%  | 92%   | : 191% | 29%   | : 128% |
| 0.10                   | 74%   | : 114% | 92%   | : 166% | 68%   | : 189% |
| 0.056                  | 57%   | : 92%  | NA    | : NA   | NA    | : NA   |
| 0.031                  | 82%   | : 110% | NA    | : NA   | NA    | : NA   |
| 0.017                  | 87%   | : 95%  | NA    | : NA   | NA    | : NA   |
| WITH S-9 ACTIVATION    |       |        |       |        |       |        |
| 20.0                   | 0%    | : 0%   | NA    | : NA   | NA    | : NA   |
| 15.0                   | 17%   | : 13%  | 70%   | : 66%  | 12%   | : 9%   |
| 11.25                  | 22%   | : 22%  | 61%   | : 74%  | 13%   | : 16%  |
| 8.44                   | 24%   | : 19%  | 101%  | : 78%  | 24%   | : 15%  |
| 6.33                   | 29%   | : 30%  | 65%   | : 72%  | 19%   | : 22%  |
| 4.75                   | 29%   | : 33%  | 60%   | : 69%  | 17%   | : 23%  |
| 3.56                   | 55%   | : 53%  | NA    | : NA   | NA    | : NA   |
| 2.67                   | 76%   | : 74%  | NA    | : NA   | NA    | : NA   |

NA = Not Applicable

CONCLUSIONS

The results of the L5178Y TK+/- Mouse Lymphoma Assay conducted on test article G0539.02 indicated that under these test conditions the test article induced a positive, dose-dependent response in the absence of an exogenous activation system. In the presence of S-9, all of the cultures had mutant frequencies that were within the range of the mean mutant frequency of the corresponding solvent control cultures.

The results of the test for differential toxicity of the test article to TK+/- cells versus TK-/- cells indicated that no differential toxicity was evident. Equivalent responses for suspension growth, clonal growth and total growth were observed in cultures of the two cell types treated with the same doses of the test article.

Study Number 0024-2400

APPENDIX

STUDY PROTOCOL

PROTOCOL DEVIATION

Deviation No.: 1

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

Testing Facility: SITEK Research Laboratories  
12111 Parklawn Drive  
Rockville, Maryland 20852

Study No.: 0024-2400

Test Article I.D.: G0539.02

1. Reason for the Deviation

On page 2, Special Instructions, item 2, Dr. Thompson had specified that TFT (1 ug/ml) should also be used as a positive control to treat TK+/- and TK-/- cells. This procedure was mistakenly omitted when the assay was initiated.

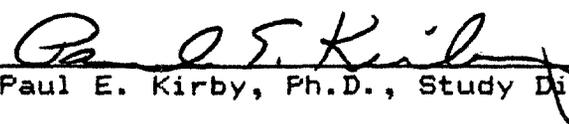
Corrective Action

The omission was realized on the second day of the expression period. Additional cultures were established from the solvent control cultures of the TK+/- and TK-/- cells. Half of these cultures were treated with TFT and half were left untreated as negative controls. Approximately 24 hours after addition of the TFT, the concentration of cells per ml was determined for each culture, and this parameter was used to determine if the TFT was differentially toxic to the TK+/- cells versus the TK-/- cells.

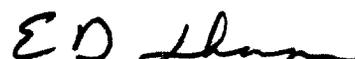
Effect on Study Integrity

This deviation did not have any compromising effect on the study's results; especially since the sensitivity of TK+/- cells and resistance of TK-/- cells to TFT was demonstrated as specified above.

APPROVAL

  
\_\_\_\_\_  
Paul E. Kirby, Ph.D., Study Director

11/13/85  
Date

  
\_\_\_\_\_  
Sponsor's Study Coordinator

11/12/85  
Date

PROTOCOL NO. C29B

Test for Chemical Induction of Mutation and Differential Toxicity in Mammalian Cells in Culture  
the L5178Y TK<sup>+</sup>/<sup>-</sup> Mouse Lymphoma Assay

Issue Date: September 12, 1985  
Supersedes Issue Dated: NEW

Test Substance Identification Number (TSIN) # G-0539.02

Divisional Request Document Number (DRD) # B40440

Sponsor: The Procter & Gamble Company  
Cincinnati, Ohio

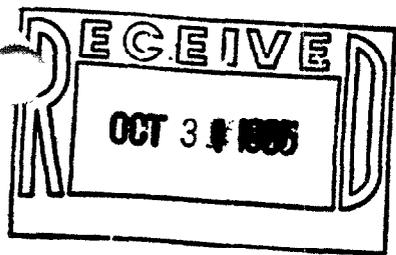
Testing Facility: SITEK Research Laboratories Study # 0024-2400  
(To be filled in by Operations Section) 12111 Parklawn Drive (To be filled in by Testing Facility)  
Rockville, MD 20852

Purpose: To determine the potential of a test substance to induce mutations at the thymidine kinase (TK) locus of cultured L5178Y TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells.<sup>1, 2, 3</sup> and to determine if the test substance is differentially toxic to TK<sup>-</sup>/<sup>-</sup> or TK<sup>+</sup>/<sup>-</sup> cells.

Justification for Selection of Test System: The L5178Y/TK<sup>+</sup>/<sup>-</sup> mouse lymphoma cells clone 3.7.2C is the system of choice due to the amount of background data available. The measurement of toxicity to TK<sup>-</sup>/<sup>-</sup> cells is appropriate because some classes of compounds produce false positive results due to differential toxicity to the two cell types (TK<sup>+</sup>/<sup>-</sup> vrs. TK<sup>-</sup>/<sup>-</sup>).

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

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



*Approved by*  
*QAD PP*  
*11-1-85*

Test for Chemical Induction of Mutation and Differential Toxicity in Mammalian Cells in Culture  
the L5178Y TK<sup>+</sup>/- Mouse Lymphoma Assay

Issue Date: September 12, 1985

| <u>Test Substance(s)</u> |                   | <u>Description</u> |                      | <u>Expiration Date</u> |
|--------------------------|-------------------|--------------------|----------------------|------------------------|
| <u>TSIN #</u>            | <u>DRD Number</u> | <u>Color</u>       | <u>Physical Form</u> |                        |
| G 0539.02                | B Y0440           | white              | Powder               | 7/29/86                |

Storage Conditions: (Check one)

- Room temperature                       Refrigerator                       Freezer  
 Other

Hazards: (Check one)

- None known. Take ordinary precautions in handling.  
 As follows: May be eye and skin irritant. For eye or skin contact, flush with water.

Special Instructions: (Check one)

- None                      ① It will not be necessary to perform the preliminary toxicity assay.  
 As follows: assay. Instead, use 60 µg/ml and 20 µg/ml as the high doses in the absence and presence of S-9 metabolic activation respectively.  
 ② In addition to the standard positive controls for the TK<sup>+</sup> mutation assay, include TFT at 1.0 µg/ml as a positive control for the TK<sup>+</sup> and TK<sup>-</sup>

Dose Preparation: Vehicles in order of preference cells for the differential toxicity assay.

- F<sub>0</sub>P<sup>+</sup>  
 DMSO  
 EtOH  
 Acetone  
 Other

LOT 10/29/85

Solubility Over 10%

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

\*See Appendix 1 for abbreviations and glossary of terms.

Test for Chemical Induction of Mutation and Differential Toxicity in Mammalian Cells in Culture  
the L5178Y TK<sup>+</sup> Mouse Lymphoma Assay

Issue Date: September 12, 1985

Dose Preparation

When possible dissolve the test substance in F<sub>0</sub>P. A 100X concentrated solution is preferable, but if the material is not soluble at that concentration, less concentrated solutions may be prepared. If the pH of the F<sub>0</sub>P changes, adjust to neutrality before proceeding. Do not test a suspension unless agreed to by the sponsor. Up to 3.0 ml of the F<sub>0</sub>P solution may be added to the final dosing solution. Since  $6 \times 10^6$  cells must be present in the final dosing solution, the cells will have to be concentrated such that the test substance solution (in F<sub>0</sub>P) and the cells make a total volume of 6.0 ml e.g. If 3.0 ml of the concentrated test substance (in F<sub>0</sub>P) were added to the final dosing solution, then 3.0 ml of cells with  $2 \times 10^6$  cells/ml would also be added. The remaining 4.0 ml will either be S-9 mix or F<sub>0</sub>P. Final volume of the dosing solution is 10 ml. The order of addition should be test substance, S-9 mix or F<sub>0</sub>P, then cells.

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

Chemicals:

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

Dosage Level:

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

Test for Chemical Induction of Mutation and Differential Toxicity in Mammalian Cells in Culture  
the L5178Y TK<sup>+</sup>/<sup>-</sup> Mouse Lymphoma Assay

Issue Date: September 12, 1985

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

[ ] Other, specify:

Note

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

If a concentration analysis is required:

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

Shipping Instructions

Send approximately 50 - 100 ml. Send [ ] frozen;  under ambient conditions; [ ] other \_\_\_\_\_

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

Test System Identification:

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

Test System:

L5178Y/TK<sup>+</sup>/<sup>-</sup>, clone 3.7.2C mouse lymphoma cells were obtained from D. Clive, Research Triangle Park, N.C., Burroughs-Wellcome Co. TK<sup>-</sup>/<sup>-</sup> cells are obtained from the above stock culture by plating in selective cloning medium. The clone is grown to saturation in F<sub>10</sub>P medium, then exposed to 1 µg/ml TFT in F<sub>10</sub>P overnight to remove TK<sup>+</sup>/<sup>-</sup> cells.

Test System Storage:

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

Test for Chemical Induction of Mutation and Differential Toxicity in Mammalian Cells in Culture  
the L5178Y TK<sup>+</sup>/<sup>-</sup> Mouse Lymphoma Assay

Issue Date: September 12, 1985

Methods:Cell Line

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

The TK<sup>+</sup>/<sup>-</sup> cultures are periodically cleansed free of spontaneous TK<sup>-</sup>/<sup>-</sup> mutants by treatment of stock cultures with THMG according to the Standard Operating Procedures of the Test Facility. Cultures used for the assay are cleansed within the two week period prior to initiation the study. TK<sup>-</sup>/<sup>-</sup> cultures are grown approximately 24 hrs in 1.0 µg TFT in F<sub>10</sub>P within a two week period before initiation of a study to remove TK<sup>+</sup>/<sup>-</sup> cells.

Preparation of the Microsomal Enzyme (S-9)  
Metabolic Activation System

Non-Induced S-9 Fraction

A liver microsomal enzyme (S-9) activation system is employed in this assay to detect promutagens.<sup>2,4</sup> S-9 is prepared by the homogenization of minced livers from commercially obtained male, Sprague-Dawley rats (200-275 gms). S-9 may be purchased or prepared according to the Standard Operating Procedures of the Test Facility.

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

Induced S-9 Fraction

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

Toxicity Test:

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

Test for Chemical Induction of Mutation and Differential Toxicity in Mammalian Cells in Culture  
the L5178Y TK<sup>+</sup>/<sup>-</sup> Mouse Lymphoma Assay

Issue Date: September 12, 1985

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

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

Mutagenicity Test:

S-9 Mix (Metabolic Activation System)

Prior to dosing the cells, S-9 mix will be prepared by combining S-9 fraction with a neutralized solution of NADP and sodium isocitrate. The final concentrations of each component in the cultures during treatment are 100  $\mu$ l/ml S-9, 2.4 mg/ml NADP, and 4.5 mg/ml sodium isocitrate in F<sub>0</sub>P. The S-9 mix will be prepared shortly before use from freshly thawed S-9 fraction. Unused portions should be discarded at the end of the day.

Dosing, Expression Growth and Cloning of Cells

Each sample will be prepared by combining the test substance, 4.0 ml of S-9 mix or 4.0 ml F<sub>0</sub>P and then adding  $6 \times 10^6$  L5178Y TK<sup>+</sup>/<sup>-</sup> or TK<sup>-</sup>/<sup>-</sup> cells to a labeled, sterile 50 ml centrifuge. The volume of cells will depend on the concentration of the test substance. Final volume of the test substance and cells should be 6.0 ml (See Dose Preparation Section).

Each sample vessel is then gassed with 5% CO<sub>2</sub>-in-air, sealed and incubated at  $37 \pm 2$  °C on a roller drum for four hours. The cell samples are then centrifuged (approximately 200 X g), the supernatant discarded, and the cells washed twice with fresh F<sub>10</sub>P. The cells are then resuspended in F<sub>10</sub>P at a concentration of approximately  $3 \times 10^5$  cells/ml, based on the original cell number of  $6 \times 10^6$  cells per culture prior to treatment with chemical, and all samples incubated as described above for a two or three day expression period according to the Standard Operating Procedures of the Test Facility. During the expression period,

Test for Chemical Induction of Mutation and Differential Toxicity in Mammalian Cells in Culture  
the L5178Y TK<sup>+</sup> Mouse Lymphoma Assay

Issue Date: September 12, 1985

Mutagenicity Test  
(Cont'd):

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

the cell concentration is determined daily and all cultures are diluted to  $3 \times 10^5$  cells/ml if necessary, in order to keep the cells in an active state of growth.

At the end of the expression period, doses are chosen for cloning based on the relative toxicity shown during the expression period. In general, dose levels which exhibit from 10 to 90% relative growth inhibition during the expression period are chosen for cloning. However, if that level of toxicity is not achieved within the solubility limits of the compound, then dose(s) showing less than 10% inhibition may be cloned. Dose levels showing greater than 90% growth inhibition will not be cloned. A portion of each culture is centrifuged and resuspended in F<sub>10</sub>P. The appropriate dilutions are then made and a portion of each sample is plated on Petri dishes in soft agar medium with and without the selective agent (TFT) according to the Standard Operating Procedures of the Test Facility. Three dishes for each sample at  $1 \times 10^6$  cells/plate are prepared in TFT medium. Three dishes for each sample at an estimated cell number from 100-200 cells/plate are prepared in cloning medium without selective agent. All petri dishes are then incubated at  $37 \pm 2$  °C for 10-14 days to allow colonies to form from individual cells. At the end of this time, the number of colonies on each plate is counted. The number of viable cells (survivors) originally placed on the plates containing the TFT medium is determined from the number of colonies in dishes containing the non-selective medium. The number of TK<sup>-/-</sup> mutants is determined from the number of colonies in dishes containing the TFT medium.

Test For Differential Toxicity

A culture of TK<sup>-/-</sup> cells is dosed by the same procedure outlined for TK<sup>+/+</sup> cells in the mutagenicity Test Section. Following the dosing, 200 cells per plate are added to the cloning medium without selective agent. The plates are incubated as outlined in the mutagenicity test.

A comparison between the number of TK<sup>+/+</sup> and TK<sup>-/-</sup> colonies in the non-selective growth medium is the measure of differential toxicity.

Test for Chemical Induction of Mutation and Differential Toxicity in Mammalian Cells in Culture  
the L5178Y TK<sup>+</sup> Mouse Lymphoma Assay

Issue Date: September 12, 1985

Protocol Changes:

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

Results:

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

Report:Final Report

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

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



9/12/85

## PROTOCOL - APPENDIX 1

## Abbreviations and Glossary of Terms:

1. 2-AAF - 2-acetylaminofluorene
2. TFT - Triflurothymidine
3. Cloning medium- Fischer's Medium for Leukemic Cells of Mice supplemented as described below for F<sub>0</sub>P and with approximately 20% (v/v) horse serum and 0.32-0.37% noble agar according to the Standard Operating Procedures of the Test Facility
4. DMN - dimethylnitrosamine
5. DMSO - dimethylsulfoxide
6. EMS - ethyl methanesulfonate
7. F<sub>0</sub>P - Fischer's Medium for Leukemic Cells of Mice supplemented with sodium pyruvate, Pluronic F68, and penicillin-streptomycin according to the Standard Operating Procedures of the Test Facility
8. F<sub>10</sub>P - F<sub>0</sub>P plus approximately 10% (v/v) horse serum
9. Gassing - Replacement of the air in a culture vessel with 5% CO<sub>2</sub>-in-air by purging with CO<sub>2</sub>-air mixture
10. NADP - β-nicotinamide adenine dinucleotide phosphate
11. Selective cloning medium - Cloning medium containing TFT according to the Standard Operating Procedures of the Test Facility
12. S-9 - The supernatant obtained by centrifugation of a homogenate of liver at 9000 X g.
13. TK - thymidine kinase

REFERENCES

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

PROTOCOL DEVIATION

Deviation No.: 1

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

Testing Facility: SITEK Research Laboratories  
12111 Parklawn Drive  
Rockville, Maryland 20852

Study No.: 0024-2400

Test Article I.D.: 60539.02

1. Reason for the Deviation

On page 2, Special Instructions, item 2, Dr. Thompson had specified that TFT (1 ug/ml) should also be used as a positive control to treat TK+/- and TK-/- cells. This procedure was mistakenly omitted when the assay was initiated.

Corrective Action

The omission was realized on the second day of the expression period. Additional cultures were established from the solvent control cultures of the TK+/- and TK-/- cells. Half of these cultures were treated with TFT and half were left untreated as negative controls. Approximately 24 hours after addition of the TFT, the concentration of cells per ml was determined for each culture, and this parameter was used to determine if the TFT was differentially toxic to the TK+/- cells versus the TK-/- cells.

Effect on Study Integrity

This deviation did not have any compromising effect on the study's results; especially since the sensitivity of TK+/- cells and resistance of TK-/- cells to TFT was demonstrated as specified above.

APPROVAL

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

11/13/85  
Date

ED Shyne  
Sponsor's Study Coordinator

11/12/85  
Date



# THE PROCTER & GAMBLE COMPANY

MIAMI VALLEY LABORATORIES

P. O. BOX 39175  
CINCINNATI, OHIO 45247

October 30, 1985

Dr. Paul E. Kirby  
Sitek Research Laboratories  
12111 Parklawn Dr.  
Rockville, Maryland 20852

Dear Dr. Kirby:

This is to authorize you to carry out the following study according to the attached protocol, and in conformance with the stipulations of our current Laboratory Services Agreement.

Notice: 1) This study is expected to be submitted to the following regulatory agency: FDA. The study should be listed on the Test Facility's Master list of regulated studies. The stipulations of the protocol are to be implemented in complete conformance with the appropriate regulations [FDA Good Laboratory Practice Regulations (21 CFR, Part 58) or EPA GLP Regulations (40 CFR, Part 792)] with the following exception:

If two or more test substances appear on the protocol, it may be conducted as a single study, resulting in a single final report.

2) Documentation of the derivation, characterization, and stability testing of the test substance(s) will be the responsibility of the Sponsor.

Test: Test for Chemical Induction of Mutation and Differential Toxicity in Mammalian Cells in Culture the L5178Y TK<sup>+</sup>/<sup>-</sup> Mouse Lymphoma Assay

Protocol No.: C29B  
Test Substance No.: G0539.02  
Physical Form: Powder

Issue Date: September 12, 1985  
Doc. Req. No.: BY0440

Four copies of the final report are needed as soon as possible, and are to be sent to my attention at the above address.

Dr. Paul E. Kirby  
Sitek  
October 30, 1985  
Page 2

Matters involving the scientific aspects of the work can be handled directly with the Sponsor's Divisional Toxicologist or with Dr. E. D. Thompson. All unused samples are to be returned to the Divisional Toxicologist at the following address (the cost of shipment should be included in the study cost):

Mr. J. E. Weaver Telephone No. (513) 530-2430  
The Procter & Gamble Company  
Sharon Woods Technical Center  
11511 Reed Hartman Hwy. - Room HB-2D29  
Cincinnati, OH 45241

Complete both copies of the attached protocol by adding your study number, proposed start and completion dates, and have the Study Director sign and date them. The Study Director should define the start and completion dates on the protocol. Retain one copy and return one copy (which includes the study cost) to me along with a letter stating that you agree to do the work specified in the attached protocol. In addition, if you cannot meet the report dates, please let me know.

The invoice for this study should be sent to:

Mr. R. T. Lyons  
The Procter & Gamble Company  
11511 Reed Hartman Highway - Room No. HB-2D31  
Cincinnati, OH 45241

Sincerely,

THE PROCTER & GAMBLE COMPANY  
Research & Development Department

*H. A. Derner /WRK*

H. A. Derner  
Human & Environmental Safety Division

Approved: 

R. E. Winters, Ph.D  
Director, Human & Environmental Safety Division

bg  
Attachments  
cc: Study File  
J. E. Weaver  
E. D. Thompson



**TEST SUBSTANCE CHARACTERIZATION REPORT  
(TSCR)**

For Tox Office <sup>12/12</sup>  
Use Only: B7C440  
DRD #BYCR0428  
TSIN #G0539.02

**Characterization, Microbial and Properties Information:**

|    | <u>Date Submitted</u> | <u>Submitter Code (if exists) or Lab Notebook #</u> | <u>Component or Property</u> | <u>(✓)</u> | <u>Measured Value</u> | <u>Limits</u> | <u>Testing Lab or Data Source</u> |
|----|-----------------------|---|------------------------------|------------|-----------------------|---------------|-----------------------------------|
| 1  | 7/29/85               | JDM-121   | MCT                          | ✓          | Pass                  | Must Pass     | Microbial                         |
| 2  | 7/10/85               | 85149005  | Assay                        |            | 99.5                  | 98-102        | 1B21                              |
| 3  |                       |   |                              |            |                       |               |                                   |
| 4  |                       |   |                              |            |                       |               |                                   |
| 5  |                       |   |                              |            |                       |               |                                   |
| 6  |                       |   |                              |            |                       |               |                                   |
| 7  |                       |   |                              |            |                       |               |                                   |
| 8  |                       |   |                              |            |                       |               |                                   |
| 9  |                       |   |                              |            |                       |               |                                   |
| 10 |                       |   |                              |            |                       |               |                                   |
| 11 |                       |   |                              |            |                       |               |                                   |
| 12 |                       |   |                              |            |                       |               |                                   |
| 13 |                       |   |                              |            |                       |               |                                   |
| 14 |                       |   |                              |            |                       |               |                                   |
| 15 |                       |   |                              |            |                       |               |                                   |

**12. Approvals:**

The test substance as made and characterized is a representative example of the intended formulation. Making records for plant-made product should be obtained and evaluated by Products Research.

a. Process Development: Jan (Signature) JOW MELANON (Name) 8/6/85 (Date)

b. Products Research: [Signature] (Signature) J. S. DOUGLASSON (Name) 8/6/85 (Date)

7 finished product samples will be retained by Quality Assurance.  
7 samples

c. GQP-Quality Assur.: \_\_\_\_\_ (Signature) \_\_\_\_\_ (Name) \_\_\_\_\_ (Date)

13. The characterization tests requested are appropriate and the test substance is acceptable for:  acute animal test;  subchronic animal test;  chronic animal test;  human safety test;  in vitro test;  environmental safety test.

James E. Weaver (Toxicologist's Signature) \_\_\_\_\_ (Name) 8-6-85 (Date)

TSCR Distribution: Original - Tox Office; Copies - Toxicologist, GQP/QA, Products REsearch and Process Dev.

