6) OECD 471-OPPTS 870.5100,
Bacterial reverse mutation test
(Ames test), 0623-2140

FINAL REPORT

Primedica Redfield

Test Article: Potassium Perfluorobutane Sulfonate

SITEK Study No. 0623-2140

July 12, 2001

SITEK RESEARCH LABORATORIES
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FINAL REPORT

Study Title

Evaluation of a Test Article in the *Salmonella typhimurium/Escherichia coli* Plate Incorporation/Preincubation Mutation Assay in the Presence and Absence of Induced Rat Liver S-9

Test Article I.D.

Potassium Perfluorobutane Sulfonate

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Laboratory Project ID

SITEK Study No. 0623-2140
Sponsor's Study No. 132-008

Study Initiation Date

August 1, 2000

Study Completion Date

July 13, 2001

Sponsor

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100 E. Boone Street
Redfield, AR 72132

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Study No. 0623-2140

Sponsor's Test Article ID: Potassium Perfluorobutane Sulfonate

The study described in this report was conducted in compliance with the following Good Laboratory Practice standard:

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 1998.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45, Paris 1992,

except for the strength and stability of the test article dosing solutions and controls under experimental conditions, which were not determined by SITEK Research Laboratories. However, the dosing solutions were analyzed by Southern Research Institute, Birmingham, Alabama. The analysis was not done under GLP conditions.

Signature Kamala Pant 7.13.01

Kamala J. Pant, M.S.
Study Director

Date

QUALITY ASSURANCE UNIT'S STATEMENT

Study No. 0623-2140

Sponsor's Test Article I.D.: Potassium Perfluorobutane Sulfonate

The performance of this study was audited for adherence to the Good Laboratory Practice regulations for nonclinical laboratory studies by the Quality Assurance Unit of SITEK Research Laboratories. In this context, the facilities, equipment, personnel, methods, practices, controls, original data and reports have been inspected as per SITEK's Quality Assurance Unit's Standard Operating Procedures. The information contained within this report accurately reflects the raw data generated from this study.

Protocol Review Date: 08-02-00

The following phases were inspected for this study:

Inspection Date	Phases Inspected	Date Findings Reported to Study Director	Date Findings Reported to Management
9-25-00	Confirmation of the Tester Strain Genotypes	09-25-00	09-25-00
10-04-00	Workbook Audit	10-04-00	10-04-00
10-04-00	Draft Report Audit	10-04-00	10-04-00
07-13-01	Final Report Audit	07-13-01	07-13-01

Signature _____ 7-13-01

Paul E. Kirby, Ph.D.
Quality Assurance Unit

Date

STUDY DIRECTOR'S SIGNATURE PAGE

This study was performed under the supervision of Kamala J. Pant, M.S., Study Director for *Salmonella typhimurium* and *Escherichia coli* Gene Mutation Assays, at SITEK Research Laboratories, 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

The Final Report for this study was written by the Study Director and released on July 13, 2001.

Signature

Kamala J. Pant, M.S. Kamala Pant Date 7.13.01
Study Director

ABSTRACT

The test article, Potassium Perfluorobutane Sulfonate, was tested for its potential to cause mutations at the histidine operon of *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and at the tryptophan operon of *Escherichia coli* strain WP2uvrA. The test article, dissolved in DMSO, was tested for toxicity to strains TA100 and WP2uvrA in a Range Finding Test (RFT) at test article concentrations ranging from 5.0-5000 μg/plate. The tester strains were exposed to the test article in the absence of exogenous activation and in the presence of induced rat liver S-9 plus cofactors. The toxicity was evaluated based on: 1) reversion frequency, 2) viability, and 3) integrity of the background lawn.

The first (definitive) Mutation Assay (B-1), using the plate incorporation method of treatment, was performed with the four *Salmonella typhimurium* tester strains and with *Escherichia coli* strain WP2uvrA. Based on the results of the RFT (A-1), the test article was tested at the following concentrations in the Mutation Assay:

50, 100, 500, 1000 and 5000 μg/plate.

The second (confirmatory) Mutation Assay (B-2), using the preincubation method of treatment, was performed to confirm the results of the definitive assay using the same test article concentrations with and without activation.

Both negative and positive controls fulfilled the requirements of the test.

The results of both Mutation Assays indicate that the test article did not induce any significant increase in the number of revertant colonies for any of the tester strains in the presence or absence of induced rat liver S-9.

Under the conditions of this study, Potassium Perfluorobutane Sulfonate was negative in the *Salmonella typhimurium/Escherichia coli* Plate Incorporation/Preincubation Mutation Assay with and without metabolic activation.

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INTRODUCTION

The purpose of this study was to evaluate the test article, Potassium Perfluorobutane Sulfonate, for its potential to cause mutations in the histidine operon of *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and the tryptophan operon of *Escherichia coli* strain WP2uvrA. The *Salmonella typhimurium/Escherichia coli* Plate Incorporation/Preincubation Mutation Assay has been used extensively and has been demonstrated to be effective in detecting mutations caused by compounds from a wide range of chemical classes.

The Ames Assay is the most widely used of all methods for determining the mutagenicity of chemicals. Because the bacterial strains used in this assay lack the enzymes necessary for metabolizing promutagens to ultimate mutagens, rat liver S-9 (induced with either Aroclor 1254 or phenobarbital) was added as a substitute for mammalian metabolism. This assay detects point mutations only and measures reverse mutation from acid auxotrophy to prototrophy. In this method, bacterial strains were used which carry base substitution or frame–shift mutations in operons coding for synthesis of specific amino acids. Therefore, these mutants (unlike their wild-type counterparts) cannot synthesize all their required amino acids from inorganic sources of nitrogen, being auxotrophic for the specific amino acid. This assay determines whether the test article can reverse the effect of the pre-existing mutation by introducing a second mutation, either at the structural gene or at another site on the bacterial chromosome. The following are the details of mutations in the different strains (4):

Strain	Mutation	rfa	uvrB	R Factor (pKM101)	Type of Mutation
TA98*	HISD 3052	Yes	Yes	Yes	Frame Shift
TA100**	HIS G46	Yes	Yes	Yes	Base Pair Sub. Frame Shift
TA1535**	HIS G46 B-P	Yes	Yes	No	Base Pair Sub.
TA1537	HISC 3076	Yes	Yes	No	Frame Shift
E. coli	trp-	Yes	No (uvrA)	No	Base Pair Sub.

* TA98 was derived from TA1538 (pKM101 plasmid added).

** TA100 was derived from TA1535 (pKM101 plasmid added).

rfa - Defective lipopolysaccharide coat. More permeable to chemicals. (Sensitive to crystal violet.)

uvrB - Reduced error-free repair of some types of DNA damage. (Sensitive to UV light.)

R Factor (pKM101) - Increases sensitivity by enhancing error-prone DNA repair. (Ampicillin resistant if plasmid present.)

uvrA - Less DNA repair.

This study was conducted by Kamala J. Pant, M.S., Sonia Srivastava, M.S., and Jieyu Chen, M.D., from August 4, 2000 to September 25, 2000, at SITEK Research Laboratories. The experimental procedures used to perform this study were essentially those of B. N. Ames, et al. (1), D. Maron and B. N. Ames (2), M. H. L. Green and W. J. Muriel (3), and S. Venitt and J. M. Parry (eds.) (4).

MATERIALS

TEST ARTICLE

1. Name:	Potassium Perfluorobutane Sulfonate
2. Batch/Lot No.:	C4F9S03-K/Lot 2
3. CAS No.:	29420-49-3
4. Physical Appearance:	White Powder
5. Date Received:	June 8, 2000 and September 7, 2000
6. Storage Conditions:	Room Temperature
7. Purity Information:	100% Pure
8. Expiration Date:	04-06-01

CONTROL SUBSTANCES

Positive Controls

The positive control chemicals used for the tester strains in the presence and absence of exogenous metabolic activation are presented below:

Strain	S-9	Chemical	Concentration (µg/plate)
TA98	-	2-NF (2-Nitrofluorene)	5.0
TA98	+	2-AA (2-Aminoanthracene)	1.25
TA100	-	NaAz (Sodium Azide)	1.0
TA100	+	2-AA (2-Aminoanthracene)	1.25
TA1535	-	NaAz (Sodium Azide)	1.0
TA1535	+	2-AA (2-Aminoanthracene)	1.25
TA1537	-	9-AA (9-Aminoacridine)	50
TA1537	+	2-AA (2-Aminoanthracene)	1.25
WP2uvrA	-	MMS (Methyl Methanesulfonate)	4000
WP2uvrA	+	2-AA (2-Aminoanthracene)	10

The following is the information for each of the positive controls used in this assay:

Chemical	Source	CAS No.	Lot No.	Storage Conditions	Expiration Date
2-AA	Sigma	613-13-8	39H0945	1-5°C	09-16-04
9-AA	Aldrich	52417-22-8	08326HN	1-5°C	07-15-01
2-NF	Aldrich	607-57-8	BY01703EV	1-5°C	04-06-02
NaAz	Sigma	26628-22-8	110H0269	1-5°C	04-06-02
MMS	Aldrich	66-27-3	08109BU	1-5°C	08-11-04

All of the positive control substances, except NaAz and MMS (dissolved in sterile, distilled, deionized water), were dissolved in dimethyl sulfoxide (DMSO). The source, lot number and expiration date of the DMSO are given below:

Source: Mallinckrodt — Lot No.: 4948N44H11

Storage Conditions: Room Temperature — Expiration Date: April 2003

CAS Number: 67-68-5

The source, batch number and expiration date of the sterile, distilled, deionized water are given below:

Source: SITEK Research Laboratories — Batch Nos.: 23, 24 and 25

Storage Conditions: Room Temperature — Expiration Dates: 12-20-00, 01-14-01 and 02-15-01 respectively.

Solvent Control

The stock solutions of the test article were prepared in DMSO. Therefore, DMSO was used as the solvent control. The source, lot number and expiration date of the DMSO are the same as stated above.

INDICATOR CELLS

Source

The *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 were originally obtained from Dr. Bruce N. Ames, University of California, Berkeley. The *Escherichia coli strain* WP2uvrA was obtained from Ms. Judy Mayo of Pharmacia and Upjohn Co., Kalamazoo, Michigan.

CULTURE CONDITIONS

The cells were grown in Oxoid Nutrient Broth No. 2 in a shaker incubator rotating at approximately 120 rpm and maintained at a temperature of 37 ± 1°C. Stock cultures of the tester strains were cryopreserved at SITEK Research Laboratories. Scrapes from the cryopreserved stock were used to initiate the overnight cultures for the test.

S-9 METABOLIC ACTIVATION SYSTEM

For the activated portion of the Mutation Assays, the cells were exposed to the test article in conjunction with an exogenous metabolic activation system consisting of Aroclor 1254 or phenobarbital-induced rat liver S-9 in 0.15M KCl plus cofactors (S-9 mix). The components of the standard S-9 mix were 8mM $MgCl_2$, 33mM KCl, 5mM glucose-6-phosphate, 4mM NADP, 100mM sodium phosphate buffer (pH 7.4), and 10% rat liver homogenate prepared from Aroclor 1254 or phenobarbital-induced, Sprague-Dawley rats. The S-9 batches used in this study were also evaluated for their ability to metabolically activate a promutagen in the *Salmonella typhimurium* Plate Incorporation Mutation Assay (Ames Test) in tester strain TA100 using a single dose of 2-AA. The following is the information pertaining to the S-9 batches used in this study:

Source:	Molecular Toxicology, Inc.	SITEK Research, Labs.
Inducing Agent:	Aroclor 1254	Phenobarbital
S-9 Batch No.:	1064, 1092 and 1120	101299
Protein Content:	39.9, 46.1, 45.8 mg/mL, respectively.	36.8 mg/mL
Storage Conditions:	≤ -70°C	≤ -70°C
Expiration Date:	03-01-02, 04-10-02, 06-27-02 and 10-12-02, respectively.	

EXPERIMENTAL PROCEDURES

DOCUMENTATION

The materials, experimental procedures used in the performance of the study, experimental results and methods used in the evaluation of the results were documented in the study workbook.

TEST SYSTEM IDENTIFICATION

Labeling Plates for the Mutation Assays

A sufficient number of Vogel-Bonner agar plates were removed from refrigerated storage and allowed to warm to room temperature. Each plate was then labeled with the following information: SITEK's test article number, experiment phase, presence or absence of rat liver S-9 mixture, dose level code, and strain code. The following strain and dose level codes were used:

Strain Codes:

1 = TA98 3 = TA1535 5 = WP2uvrA
2 = TA100 4 = TA1537

Dose Level Codes:

0 = Solvent for the Test Article
1 = 1st or highest Test Article dose level
2 = 2nd Test Article dose level
3 = 3rd Test Article dose level
4 = 4th Test Article dose level
5 = 5th Test Article dose level or lowest Test Article dose level for the Mutation Assays
6 = 6th Test Article dose level
7 = 7th Test Article dose level or lowest Test Article dose level for the Range Finding Test

In addition to the above, Mutation Assay viability plates that contained 10X histidine-biotin or 10X tryptophan were designated with the prefix "T".

Labeling Positive Control Plates

Vogel-Bonner agar plates were removed from refrigerated storage and allowed to warm to room temperature. Triplicate sets were labeled with the test article number, identity and dose of the particular positive control, experimental phase, strain code, and the presence or absence of rat exogenous metabolic activation.

Labeling Tester Strain Titer Plates

Each tester strain titer plate was labeled with the following information: SITEK test article number, tester strain identity, and experimental phase and the prefix T.

Labeling Tester Strain Characterization Plates

Histidine Requirement

A single histidine-biotin plate was divided into four zones by drawing horizontal lines on the bottom of the plate with a marking pen and labeling each zone with a different *Salmonella* tester strain. A biotin-only control plate was labeled in a similar manner.

rfa Mutation

Nutrient agar plates were labeled with the *Salmonella* tester strain identification and "CV" (crystal violet).

R-Factor

A single ampicillin agar plate was labeled in a similar manner as the histidine-biotin plate.

Tryptophan Requirement

A tryptophan plate and a Vogel-Bonner agar control plate were labeled with the code for strain WP2uvrA and used for confirmation of the tryptophan requirement.

SOLUBILITY TEST

Since Sponsor had not provided the information regarding a preferred solvent to be used, a solubility test was performed with water and DMSO. Two 30 mg samples were weighed and each solvent was added in 0.1 mL increments until the test article dissolved or until 5.0 mL had been added.

PREPARATION OF TEST CULTURES

The methods used for the cryopreservation and cultivation of the tester strains are modifications of the procedures used by B. N. Ames, et al. (1) and D. Maron and B. N. Ames (2).

Inoculation Procedures

Frozen ampules of strains TA98, TA100, TA1535, TA1537 and WP2uvrA for the Mutation Assays were removed from liquid nitrogen and placed into crushed dry ice to prevent thawing. Scrapes were made using the tip of a sterile pipet, and these scrapes were transferred to a shaker flask containing approximately 50 mL of sterile Oxoid Nutrient Broth No. 2. The flasks were placed in a shaker incubator, and a timer was set to start the unit at a time which allowed the strains to incubate at approximately 120 rpm and 37 $\pm$ 1°C for a period of 8-12 hours for the *Salmonella* strains and 4-6 hours for the *E. coli* strain before being harvested.

Harvesting Overnight Cultures

Before starting the experiment, the cultures were sampled and their Percent Transmittance (%T) was determined using a spectrophotometer set to a wavelength of 650 nm.

When the desired cell density of $5x10^8$ to $1x10^9$ cells/mL (represented by a %T of between 25% and 10%, Optical Density of 0.6-1.0) was achieved, the cultures were placed on wet ice or kept at 1-5°C until needed.

PREPARATION OF S-9 METABOLIC ACTIVATION SYSTEM

The S-9 cofactor mix was prepared as follows: For each mL of S-9 cofactor mix required, 0.335 mL of sterile, deionized, distilled water was combined with 0.5 mL of 0.2M sodium phosphate buffer (pH 7.4), 0.04 mL of a 0.1M NADP solution, 5.0 µL of 1M glucose-6-phosphate, and 0.02 mL of a 0.4M $MgCl_2$/1.65M KCl salt solution. This mixture was maintained on ice until just prior to use, whereupon 0.10 mL of S-9 in 0.15N KCl was added to the mixture.

PREPARATION OF TEST ARTICLE DOSING SOLUTIONS

The test article was weighed in glass tubes and solubilized in DMSO prior to its use in the experiment. Further serial dilutions were also prepared in DMSO.

All of the test article and control substance treatments were done under UV-filtered lights to avoid possible problems of photoinactivation. Stability of the test article dosing solutions under experimental conditions was not determined by SITEK Research Laboratories. However, 2.0 mL samples from the Mutation Assays were saved frozen and shipped on dry ice to the Sponsor for analysis.

RANGE FINDING TEST

In order to determine the toxicity and to select the appropriate test article concentrations for the Mutation Assays, a Range Finding Test (A-1) was performed using strains TA100 and WP2uvrA. Seven doses of the test article, ranging from 5.0-5000 μg/plate, were evaluated with and without induced rat liver S-9, using one plate per dose.

Spontaneous Reversion Frequency

Treatment was performed by adding either 500 μL of sterile, deionized, distilled water or 500 μL of S-9 cofactor mix to tubes containing 2.0 mL of top agar supplemented with 1X histidine-biotin or 1X tryptophan solution. Immediately thereafter, 100 μL of TA100 or WP2uvrA was added, followed by 50 μL of the appropriate test article dose or solvent. The highest concentration of 5000 μg/plate had a slight precipitate in the treatment tube. Each tube was vortexed for 2-3 seconds, and the contents were evenly distributed over a Vogel-Bonner bottom agar plate. Each plate was placed on a level surface until the top agar solidified. The plates were inverted and incubated at 37 ± 1°C for approximately 70 hours.

Viable Count Determination

Treatment and incubation were performed as described in the preceding paragraphs, except that approximately 250-500 cells of TA100 or WP2uvrA were added to top agar supplemented with 10X histidine-biotin or 10X tryptophan solution.

After the incubation period was completed, the plates, starting with the highest test article concentration, were observed for the presence of precipitate. Plates having no interfering precipitate were counted using an automatic colony counter (ARTEK Counter, Model 880). Plates with precipitate that interfered with automatic counting were counted by hand. Three counts were taken by rotating the plate on the counter stage and the median count was entered into a validated, Lotus 123 (version 3.4) spreadsheet program (2140A.WK3 for the Range Finding Test).

The background lawn was also evaluated. The following notations were used for the precipitate and background lawn evaluation:

Chemical Precipitate:

NP = No precipitate present.

SP = Slight precipitate - Noticeable compound on the plate; however, no influence on automated plate counting.

MP = Moderate precipitate - Marked precipitate requiring hand counting for colony enumeration.

HP = Heavy precipitate - Large amount of compound on the plate rendering hand counting difficult.

Background Lawn Evaluation:

NL = Normal, healthy microcolony lawn.

SR = A noticeable thinning of the microcolony lawn compared to that of the solvent control plates.

MR = Marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plates.

ER = Extreme thinning of the microcolony lawn and a large increase in the size of the microcolonies compared to the solvent control plates.

AB = Absence of any microcolony bacterial lawn.

OP = Obscured by precipitate.

Determination of Relative Cloning Efficiency

The corrected viability counts from each dose with and without activation in *Salmonella* strain TA100 and in *Escherichia coli* strain WP2uvrA were compared with the respective solvent control viability counts. The ratio was converted into a percentage, and the data were included in the Range Finding Test results.

MUTATION ASSAYS

Definitive Mutation Assay (B-1)

Doses for the definitive Mutation Assay (B-1) were selected based on the results of the Range Finding Test (A-1). The definitive Mutation Assay was performed with the four *Salmonella typhimurium* tester strains (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* strain WP2uvrA using the plate incorporation method of treatment. The test article was tested with the following concentrations in the Mutation Assay:

50, 100, 500, 1000 and 5000 μg/plate.

Treatment was performed by adding either 500 μL of deionized, distilled water or 500 μL of rat S-9 cofactor mix to tubes containing 2.0 mL of top agar supplemented with 1X histidine-biotin or 1X tryptophan solution. Immediately thereafter, 100 μL of strains TA98, TA100, TA1535, TA1537 or WP2uvrA were added, followed by 100 μL of the appropriate test article dose or solvent. The positive controls were treated with 100 μL of the appropriate stock solutions. Each tube was vortexed for 2-3 seconds, and the contents were evenly distributed over a Vogel-Bonner bottom agar plate. Each plate was placed on a level surface until the top agar solidified. The plates then were inverted and incubated at 37 ± 1°C for approximately 70.5 hours.

Tester Strain Titer Determination

Each tester strain was diluted to determine the approximate number of viable cells delivered to the assay plates. Therefore, approximately 250-500 cells were added to top agar supplemented with 10X histidine-biotin or 10X tryptophan solution. Each tube was vortexed for 2-3 seconds, and the contents were evenly distributed on bottom agar plates. The plates were incubated at 37 ± 1°C for approximately 68 hours.

Tester Strain Characterization

All of the *Salmonella typhimurium* strains used in the assay were confirmed for the histidine requirement and the rfa mutation. In addition, strains TA98 and TA100 were tested for the presence of the pKM101 plasmid. *Escherichia coli* strain WP2uvrA was confirmed for the tryptophan requirement.

Histidine or Tryptophan Requirement

A streak of each tester strain was made by dipping a flamed wire loop into the appropriate undiluted tester strain suspension and drawing it across the surface in the appropriate region of a labeled histidine-biotin or tryptophan plate, as well as control plates. The plates were incubated at 37 ± 1°C for approximately 20 hours.

rfa Mutation

For each of the *Salmonella* tester strains, a 100 µL aliquot of the undiluted culture was added to a tube containing 2.0 mL of 1X histidine-biotin solution top agar. Each tube was vortexed for 2-3 seconds, and the contents were poured onto an appropriately labeled nutrient agar plate. After allowing the plate to solidify, a sterile disc was aseptically placed in the center of the agar overlay. Ten µL of a 1.0 mg/mL crystal violet solution was then added to the disc. The plates were incubated at 37 ± 1°C for approximately 20 hours.

R-Factor Plasmid

A streak of each of the *Salmonella* tester strains was made by dipping a flamed wire loop into the appropriate suspension and drawing it across the surface in the appropriate region of an ampicillin plate. The plates were incubated at 37 ± 1°C for approximately 20 hours.

Evaluation of Assay Results

After the incubation period was completed, the plates, starting with the highest test article concentration, were observed for the presence of precipitate. Plates having no interfering precipitate were counted using an automatic colony counter (ARTEK Counter, Model 880). Plates with precipitate that interfered with automatic counting were counted by hand. Three counts were taken by rotating the plate on the counter stage and the median count was entered into a validated, Lotus 123 (version 3.4) spreadsheet program (2140B.WK3 or 2150B.WK3 for the definitive and the confirmatory Mutation Assays, respectively).

The background lawn was also evaluated. Same notations as in the Range Finding Test were used to evaluate the precipitate and background lawn.

Evaluation of Tester Strain Characterization

The requirement for histidine or tryptophan was demonstrated by the growth of the tester strains on plates supplemented with histidine or tryptophan and the lack of growth on the control plates.

The presence of the rfa mutation was evaluated by measuring the zone of inhibition around the crystal violet disc. A zone about 14 to 15 mm in diameter was evidence of appropriate inhibition.

The presence of the pKM101 plasmid was demonstrated by the growth of strains TA98 and TA100 and the lack of growth of strains TA1535 and TA1537 streaked on ampicillin plates.

Tabulation of Colony Counts

The colony counts provided by the automatic colony counter were raw counts and were not corrected to reflect actual counts. Correction of the counts was performed by computer. The correction factor was determined by comparing a wide range of manual and automatic counts, as described in SITEK's SOP No. 21.0. The relationship was linear, and the counts were corrected by using the following formula:

Corrected Count = Raw Counts (1.0571607) + 3.09496

Confirmatory Mutation Assay (B-2)

To confirm the results of the definitive assay (B-1), a second (confirmatory) Mutation Assay (B-2) was performed with and without activation using the same test article concentrations. All test article concentrations, including the controls, were tested in triplicate. The preincubation method of treatment was used.

For the confirmatory assay, 500 µL of deionized, distilled water or S-9 mix was dispensed into a series of labeled culture tubes, and treatment was performed by adding 100 µL of tester strain and 100 µL of test article or the solvent. The positive control cultures were treated with 100 µL of the appropriate stock solutions. The tubes were vortexed gently and preincubated at 37 ± 1°C for 20 minutes. The tubes were shaken at a moderate speed during the preincubation. 2.0 mL of top agar containing 1X histidine-biotin or 1X tryptophan was added to each culture tube after the preincubation period. The contents of each tube were mixed by vortexing, poured onto a bottom agar plate, and evenly distributed by gently tilting and rotating the plates. All of the plates were placed on a level surface until solidified. After all treatments had been performed, the plates were inverted and incubated at 37 ± 1°C for approximately 68 hours. Remainder of the experiment was performed in the similar manner as the definitive Mutation Assay.

CRITERIA FOR A VALID ASSAY

The following criteria were used as guidelines in evaluating the acceptability of the Mutation Assay. Since it is impossible to formulate criteria that would apply to every configuration of data generated by the assay, the Study Director was responsible for the ultimate decision regarding the acceptability of the results.

Solvent Control Cultures

The mean reversion frequency of the test article solvent control plates for each tester strain should have fallen within the following ranges:

TA98	20 ± 15	WP2uvrA	15 ± 10
TA100	100 ± 70		
TA1535	20 ± 15		
TA1537	15 ± 12		

Positive Controls

The results for the positive control cultures were considered acceptable if the treated strains had a mean reversion frequency that was three times or more greater than the mean reversion frequency of the solvent control plates.

Tester Strain Characterization

All of the *Salmonella typhimurium* strains were confirmed positive for histidine dependence. *Escherichia coli* strain WP2uvrA was confirmed positive for tryptophan dependence.

All of the *Salmonella typhimurium* strains were confirmed positive for the rfa mutation as evidenced by sensitivity to crystal violet.

The R-factor strains, TA98 and TA100, were confirmed positive for the pKM101 plasmid as evidenced by ampicillin resistance.

The titer of the stock cultures for each strain indicated that the stock cultures contained approximately between 5.0×10^8 and 1.0×10^9 bacteria per mL.

EVALUATION OF TEST RESULTS

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

Criteria for a Negative Response

A response was considered to be negative if all of the strains treated with the test article had mean reversion frequencies that were less than twice that of the mean reversion frequencies of the corresponding solvent control plates in TA98 and TA100 and less than three times in TA1535, TA1537 and WP2uvrA, and there was no evidence of a dose-dependent response.

Criteria for a Positive Response

A response was considered to be positive if either strain TA98 or TA100 exhibited a mean reversion frequency that was at least double the mean reversion frequency of the corresponding solvent control in at least one dose, or if either strain TA1535, TA1537 or WP2uvrA exhibited a three-fold increase in the mean reversion frequency compared to the solvent control in at least one dose. In addition, the response must have been dose dependent or increasing concentrations of the test article must have showed increasing mean reversion frequencies. In evaluating the results, consideration was given to the degree of toxicity exhibited by the dose causing the two-fold/three-fold or greater increase in reversion frequency and the magnitude of the increase in reversion frequency.

Criteria for an Equivocal Response

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

ARCHIVES

All of the raw data, documentation, protocol, protocol amendments/deviations, and final report along with an electronic file containing the data tables and final report of the study, will be maintained at SITEK Research Laboratories' Archives at 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

RESULTS

SOLUBILITY TEST

The test article was tested for solubility in water and DMSO. Two 30 mg samples were weighed and tested for their solubility in water and DMSO. The test article was not soluble in up to 0.5 mL of water, however, 0.1 mL of DMSO was enough to solubilize a 30 mg sample. Thus, the maximum solubility of test article in DMSO was 300 mg/mL. Based on the results of the solubility test, DMSO was used as the solvent for the test article.

RANGE FINDING TEST (A-1)

Summaries of the results of the Range Finding Test are presented in Tables 1 and 2 and included in Appendix I. The individual plate counts and background lawn evaluation are presented in Appendix II.

TA100 With and Without Activation:

There were no signs of toxicity related with the background lawn or the number of revertants. The Relative Cloning Efficiencies (RCEs) among the test article concentrations of 5.0 to 5000 μg/plate ranged from 94% to 78% in the non-activated portion and all concentrations were greater than 100% in the activated portion.

WP2uvrA With and Without Activation:

There were essentially no signs of toxicity related to the test article treatment. The RCEs for the test article concentrations of 5.0 to 5000 μg/plate ranged from 108% to 94% without activation and from 106% to 94% with activation.

MUTATION ASSAYS

Definitive Mutation Assay (B-1)

Summaries of the results of the definitive Mutation Assay are presented in Tables 3-4 included in Appendix I. The individual plate counts and background lawn evaluation are presented in Appendix II.

The definitive Mutation Assay, using the plate incorporation method of treatment, was performed with the four *Salmonella* tester strains (TA98, TA100, TA1535, TA1537) and with *E. coli* strain WP2uvrA. Based on the results of the Range Finding Test, the following concentrations were tested in the Mutation Assay:

50, 100, 500, 1000 and 5000 μg/plate.

All the test article concentrations were nontoxic.

In the definitive Mutation Assay (B-1) with and without activation, all the tester stains treated with test article exhibited a mean reversion frequency that was similar to or less than double the corresponding solvent control, and there was no dose-related response.

Both the negative and positive controls fulfilled the requirements of the test.

Confirmatory Mutation Assay (B-2)

Summaries of the results of the confirmatory Mutation (B-2) Assay are presented in Tables 5-6 included in Appendix I. The individual plate counts and background lawn evaluation are presented in Appendix II.

The confirmatory Mutation Assay (B-2) was performed with the same test article concentrations as the definitive Mutation Assay (B-1) with and without activation, but using the preincubation method of treatment.

In the confirmatory Mutation Assay (B-2), as in the definitive Mutation Assay with and without activation, all of the tester strains treated with the test article concentrations exhibited a mean reversion frequency that was similar to or less than double the corresponding solvent control. There was no dose-related response.

The test article in both Mutation Assays produced a negative response in the presence and absence of S-9 metabolic activation.

Both the positive and negative controls fulfilled the requirements of the test. All of the criteria for a valid assay were met.

DOSING SOLUTION ANALYSIS RESULTS

The analysis results are presented in Appendix V. All of the test article concentrations from the definitive (B-1) and confirmatory (B-2) Mutation Assays were within ± 5% of the targeted values.

CONCLUSIONS

The test article, Potassium Perfluorobutane Sulfonate, was tested in the *Salmonella typhimurium/ Escherichia coli* Plate Incorporation/Preincubation Mutation Assay in the presence and absence of induced rat liver S-9.

The definitive Mutation Assay (B-1), using the plate incorporation method, was performed with four *Salmonella typhimurium* tester strains, TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2uvrA. Since the results of the Mutation Assay were negative, a second (confirmatory) Mutation Assay (B-2) was performed to confirm the results using the preincubation method of treatment.

The results of the Mutation Assays indicated that the test article did not induce any significant increase in the number of revertant colonies for any of the tester strains in the presence or absence of induced rat liver S-9. The positive and negative controls fulfilled the requirements of the test.

Under the conditions of this study, Potassium Perfluorobutane Sulfonate was negative in the *Salmonella typhimurium/Escherichia coli* Plate Incorporation/Preincubation Mutation Assay.

REFERENCES

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APPENDIX I

DATA TABLES

TABLE 1

SALMONELLA TYPHIMURIUM PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST RESULTS

SPONSOR: Primedica Redifield
EXPERIMENT NO.: A-1
TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SITEK STUDY NO.: 0623-2140
SOLVENT: DMSO
STRAIN: TA100

WITHOUT ACTIVATION						WITH S-9 ACTIVATION					
Test Article Conc. µg/plate	No. of Revertants/ Plate	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/ Plate	Relative Cloning Efficiency (RCE)	Test Article Conc. µg/plate	No. of Revertants/ Plate	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/ Plate	Relative Cloning Efficiency (RCE)
5.0	59	NP	NL	212	78%	5.0	69	NP	NL	540	115%
10	62	NP	NL	244	90%	10	70	NP	NL	574	122%
50	52	NP	NL	240	89%	50	84	NP	NL	539	115%
100	72	NP	NL	211	78%	100	67	NP	NL	545	116%
500	55	NP	NL	254	94%	500	59	NP	NL	508	108%
1000	71	NP	NL	256	94%	1000	63	NP	NL	523	112%
5000	58	NP	NL	249	92%	5000	62	NP	NL	541	115%
SOLV. CONT.	59	NP	NL	271	100%	SOLV. CONT.	73	NP	NL	469	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

TABLE 2

ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST RESULTS

SPONSOR: Primedica Redifield
EXPERIMENT NO.: A-1
TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SITEK STUDY NO.: 0623-2140
SOLVENT: DMSO
STRAIN: WP2uvrA

WITHOUT ACTIVATION						WITH S-9 ACTIVATION					
Test Article Conc. µg/plate	No. of Revertants/ Plate	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/ Plate	Relative Cloning Efficiency (RCE)	Test Article Conc. µg/plate	No. of Revertants/ Plate	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/ Plate	Relative Cloning Efficiency (RCE)
5.0	13	NP	NL	1016	107%	5.0	18	NP	NL	1100	96%
10	13	NP	NL	998	105%	10	16	NP	NL	1144	100%
50	10	NP	NL	1000	106%	50	13	NP	NL	1196	104%
100	10	NP	NL	887	94%	100	12	NP	NL	1080	94%
500	8	NP	NL	1018	108%	500	12	NP	NL	1122	98%
1000	15	NP	NL	1006	106%	1000	17	NP	NL	1181	103%
5000	10	NP	NL	1000	106%	5000	22	NP	NL	1218	106%
SOLV. CONT.	17	NP	NL	946	100%	SOLV. CONT.	20	NP	NL	1149	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

TABLE 3
SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
MUTATION ASSAY RESULTS – WITHOUT ACTIVATION

SPONSOR: Primedica Redfield
EXPERIMENT NO.: B-1
TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SITEK STUDY NO.: 0623-2140
SOLVENT: DMSO
CONC. IN: µg/plate

S. typhimurium		Average No. of Revertants Per Plate						
		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	740	30	38	37	37	34	30
DATE PLATED: 08/15/00	STD. DEV.	28	4	6	6	4	11	12
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.692×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	484	67	83	78	80	78	74
DATE PLATED: 08/15/00	STD. DEV.	33	6	8	14	11	11	7
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 0.964×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	453	20	17	23	16	16	13
DATE PLATED: 08/15/00	STD. DEV.	16	7	3	3	3	2	1
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.798×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	165	9	6	5	9	7	7
DATE PLATED: 08/15/00	STD. DEV.	56	4	2	1	4	2	2
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.128×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	466	16	14	14	15	16	16
DATE PLATED: 08/15/00	STD. DEV.	7	2	4	5	3	5	4
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.334×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

TABLE 4
SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
MUTATION ASSAY RESULTS – WITH S-9 ACTIVATION

SPONSOR: Primedica Redfield
EXPERIMENT NO.: B-1
TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SITEK STUDY NO.: 0623-2140
SOLVENT: DMSO
CONC. IN: µg/plate

S. typhimurium		Average No. of Revertants Per Plate						
		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	1507	42	43	50	56	42	45
DATE PLATED:	STD. DEV.	47	10	4	2	7	3	8
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.692×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	1258	82	74	87	73	75	75
DATE PLATED:	STD. DEV.	94	2	16	6	4	6	11
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
0.964×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	199	16	15	14	10	15	11
DATE PLATED:	STD. DEV.	10	9	8	4	3	2	1
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.798×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	288	9	9	11	9	5	7
DATE PLATED:	STD. DEV.	3	5	3	4	1	2	1
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.128×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	197	21	23	20	25	17	17
DATE PLATED:	STD. DEV.	24	3	3	7	2	2	4
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.334×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

TABLE 5
SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PRINCUBATION MUTATION ASSAY
MUTATION ASSAY RESULTS – WITHOUT ACTIVATION

SPONSOR: Primedica Redfield Labs
EXPERIMENT NO.: B-2
TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SITEK STUDY NO.: 0623-2140
SOLVENT: DMSO
CONC. IN: µg/plate

S. typhimurium		Average No. of Revertants Per Plate						
		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	544	24	24	24	27	27	33
DATE PLATED: 09/20/00	STD. DEV.	23	7	9	6	6	2	3
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.160×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	495	69	75	76	82	74	83
DATE PLATED: 09/20/00	STD. DEV.	30	8	8	2	6	9	4
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.026×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	405	16	19	16	15	17	17
DATE PLATED: 09/20/00	STD. DEV.	39	2	4	7	3	4	4
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.002×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	290	13	7	6	7	6	6
DATE PLATED: 09/20/00	STD. DEV.	48	3	3	2	3	2	2
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 0.814×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	552	18	18	22	21	21	17
DATE PLATED: 09/20/00	STD. DEV.	17	6	1	7	3	4	8
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.360×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

TABLE 6
SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PRINCUBATION MUTATION ASSAY
MUTATION ASSAY RESULTS – WITH S-9 ACTIVATION

SPONSOR: Primedica Redfield Labs
EXPERIMENT NO.: B-2
TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SITEK STUDY NO.: 0623-2140
SOLVENT: DMSO
CONC. IN: µg/plate

S. typhimurium		Average No. of Revertants Per Plate						
		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	623	35	38	36	40	39	38
DATE PLATED:	STD. DEV.	19	1	4	6	5	4	5
09/20/00	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 1.160 x 10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	495	82	97	88	98	95	88
DATE PLATED:	STD. DEV.	38	5	14	10	13	4	8
09/20/00	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.026 x 10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	123	17	18	19	16	16	13
DATE PLATED:	STD. DEV.	10	2	3	3	2	1	6
09/20/00	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.002 x 10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	58	11	6	9	9	10	9
DATE PLATED:	STD. DEV.	2	3	1	2	1	4	1
09/20/00	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 0.814 x 10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	229	19	23	25	24	20	19
DATE PLATED:	STD. DEV.	17	4	2	6	2	4	4
09/20/00	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED: 2.360 x 10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

APPENDIX II

DETAILED PLATE COUNTS AND

BACKGROUND LAWN EVALUATION

SALMONELLA TYPHIMURIUM PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: A-1 SITEK STUDY NO.: 0623-2140
TEST ARTICLE: Potassium Perfluorobutane Sulfonate SOLVENT: DMSO
STRAIN: TA100

WITHOUT ACTIVATION

Test Article Conc. µg/plate	No. of Revertants Per Plate (raw)	(corrected)	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/Plate (raw)	(corrected)	Relative Cloning Efficiency (RCE)
5.0	53	59	NP	NL	198	212	78%
10	56	62	NP	NL	228	244	90%
50	46	52	NP	NL	224	240	89%
100	65	72	NP	NL	197	211	78%
500	49	55	NP	NL	237	254	94%
1000	64	71	NP	NL	239	256	94%
5000	52	58	NP	NL	233	249	92%
SOLVENT CONTROL	53	59	NP	NL	253	271	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

SITEK RESEARCH LABORATORIES

SALMONELLA TYPHIMURIUM PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: A-1 SITEK STUDY NO.: 0623-2140
TEST ARTICLE: Potassium Perfluorobutane Sulfonate SOLVENT: DMSO
STRAIN: TA100

WITH S-9 ACTIVATION

Test Article Conc. µg/plate	No. of Revertants Per Plate (raw)	(corrected)	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/Plate (raw)	(corrected)	Relative Cloning Efficiency (RCE)
5.0	62	69	NP	NL	508	540	115%
10	63	70	NP	NL	540	574	122%
50	77	84	NP	NL	507	539	115%
100	60	67	NP	NL	513	545	116%
500	53	59	NP	NL	478	508	108%
1000	57	63	NP	NL	492	523	112%
5000	56	62	NP	NL	509	541	115%
SOLVENT CONTROL	66	73	NP	NL	441	469	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: A-1 SITEK STUDY NO.: 0623-2140
TEST ARTICLE: Potassium Perfluorobutane Sulfonate SOLVENT: DMSO
STRAIN: WP2uvrA

WITHOUT ACTIVATION

Test Article Conc. µg/plate	No. of Revertants Per Plate (raw)	(corrected)	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/Plate (raw)	(corrected)	Relative Cloning Efficiency (RCE)
5.0	9	13	NP	NL	958	1016	107%
10	9	13	NP	NL	941	998	105%
50	7	10	NP	NL	943	1000	106%
100	7	10	NP	NL	836	887	94%
500	5	8	NP	NL	960	1018	108%
1000	11	15	NP	NL	949	1006	106%
5000	7	10	NP	NL	943	1000	106%
SOLVENT CONTROL	13	17	NP	NL	892	946	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
RANGE FINDING TEST COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: A-1 SITEK STUDY NO.: 0623-2140
TEST ARTICLE: Potassium Perfluorobutane Sulfonate SOLVENT: DMSO
STRAIN: WP2uvrA

WITH S-9 ACTIVATION

Test Article Conc. µg/plate	No. of Revertants Per Plate (raw)	(corrected)	Chem. PPT. Eval.*	Background Lawn Evaluation**	No. of Viable Colonies/Plate (raw)	(corrected)	Relative Cloning Efficiency (RCE)
5.0	14	18	NP	NL	1038	1100	96%
10	12	16	NP	NL	1079	1144	100%
50	9	13	NP	NL	1128	1196	104%
100	8	12	NP	NL	1019	1080	94%
500	8	12	NP	NL	1058	1122	98%
1000	13	17	NP	NL	1114	1181	103%
5000	18	22	NP	NL	1149	1218	106%
SOLVENT CONTROL	16	20	NP	NL	1084	1149	100%

$$RCE = \frac{\text{No. of Colonies in Test Plates}}{\text{No. of Colonies in Solvent Control Plates}} \times 100$$

* Chemical Precipitate Evaluation
NP = No precipitate
SP = Slight precipitate; noticeable precipitate on the plate, but no interference with automated plate counting
MP = Moderate precipitate; marked precipitate necessitating hand counting for colony enumeration
HP = Heavy precipitate; large amount of precipitate rendering hand counting difficult or impossible

** Background Lawn Evaluation
NL = Normal, healthy microcolony lawn
SR = Noticeable thinning of the microcolony lawn compared to control
MR = Marked thinning of the microcolony lawn and increase in size of microcolonies compared to control
ER = Extreme thinning of the microcolony lawn and large increase in size of microcolonies compared to control
AB = Absence of microcolonies
OP = Obscured by precipitate

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
MUTATION ASSAY RAW COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-1 SITEK STUDY NO.: 0623-2140
TEST ARTICLE: Potassium Perfluorobutane Sulfonate SOLVENT: DMSO
CONC. IN: µg/plate

WITHOUT ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	683	21	34	37	31	17	20
	PER	727	27	26	25	28	32	39
DATE PLATED:	PLATE	680	27	38	35	36	38	19
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.692×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	423	55	80	63	77	70	75
	PER	485	61	67	63	61	82	65
DATE PLATED:	PLATE	457	66	78	87	81	61	62
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
0.964×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	408	8	16	22	16	13	10
	PER	436	21	12	17	10	10	8
DATE PLATED:	PLATE	432	18	11	18	11	12	10
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.798×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	108	4	1	2	3	4	2
	PER	141	9	3	3	10	6	6
DATE PLATED:	PLATE	211	3	5	2	4	2	4
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.128×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	444	11	14	9	13	14	16
	PER	438	14	7	15	8	7	12
DATE PLATED:	PLATE	431	11	9	7	12	16	8
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.334×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
MUTATION ASSAY CORRECTED COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-1
TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SITEK STUDY NO.: 0623-2140
SOLVENT: DMSO
CONC. IN: µg/plate

WITHOUT ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	725	25	39	42	36	21	24
	PER	772	32	31	30	33	37	44
DATE PLATED:	PLATE	722	32	43	40	41	43	23
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.692 x 10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	450	61	88	70	84	77	82
	PER	516	68	74	70	68	90	72
DATE PLATED:	PLATE	486	73	86	95	89	68	69
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
0.964 x 10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	434	12	20	26	20	17	14
	PER	464	25	16	21	14	14	12
DATE PLATED:	PLATE	460	22	15	22	15	16	14
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.798 x 10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	117	7	4	5	6	7	5
	PER	152	13	6	6	14	9	9
DATE PLATED:	PLATE	226	6	8	5	7	5	7
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.128 x 10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	472	15	18	13	17	18	20
	PER	466	18	10	19	12	10	16
DATE PLATED:	PLATE	459	15	13	10	16	20	12
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.334 x 10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
MUTATION ASSAY RAW COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-1 SITEK STUDY NO.: 0623-2140
TEST ARTICLE: Potassium Perfluorobutane Sulfonate SOLVENT: DMSO
CONC. IN: µg/plate

WITH S-9 ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	1386	29	37	43	46	37	40
	PER	1472	47	35	47	46	40	47
DATE PLATED:	PLATE	1409	35	42	43	58	34	32
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.692×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	1098	76	66	80	70	61	57
	PER	1276	73	82	84	67	71	72
DATE PLATED:	PLATE	1187	77	53	73	62	73	76
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
0.964×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	175	10	20	7	9	12	8
	PER	188	6	7	11	5	8	7
DATE PLATED:	PLATE	192	22	7	14	7	12	7
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.798×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	267	3	5	8	7	2	5
	PER	269	3	4	3	5	1	3
DATE PLATED:	PLATE	272	11	8	10	5	4	5
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.128×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	158	20	16	16	23	12	13
	PER	200	15	22	9	19	15	10
DATE PLATED:	PLATE	192	15	20	22	22	13	17
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.334×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
MUTATION ASSAY CORRECTED COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-1 SITEK STUDY NO.: 0623-2140
TEST ARTICLE: Potassium Perfluorobutane Sulfonate SOLVENT: DMSO
CONC. IN: µg/plate

WITH S-9 ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	1468	34	42	49	52	42	45
	PER	1559	53	40	53	52	45	53
DATE PLATED:	PLATE	1493	40	47	49	64	39	37
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.692×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	1164	83	73	88	77	68	63
	PER	1352	80	90	92	74	78	79
DATE PLATED:	PLATE	1258	84	59	80	69	80	83
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
0.964×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	188	14	24	10	13	16	12
	PER	202	9	10	15	8	12	10
DATE PLATED:	PLATE	206	26	10	18	10	16	10
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.798×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	285	6	8	12	10	5	8
	PER	287	6	7	6	8	4	6
DATE PLATED:	PLATE	291	15	12	14	8	7	8
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.128×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	170	24	20	20	27	16	17
	PER	215	19	26	13	23	19	14
DATE PLATED:	PLATE	206	19	24	26	26	17	21
08/15/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.334×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PRINCUBATION MUTATION ASSAY
MUTATION ASSAY RAW COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-2 SITEK STUDY NO.: 0623-2140
TEST ARTICLE: Potassium Perfluorobutane Sulfonate SOLVENT: DMSO
CONC. IN: µg/plate

WITHOUT ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	530	13	18	16	23	21	31
	PER	518	26	13	17	18	23	29
DATE PLATED:	PLATE	487	19	29	26	28	24	25
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.160×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	470	58	60	71	71	76	73
	PER	435	59	68	69	81	59	79
DATE PLATED:	PLATE	491	72	75	67	73	68	74
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.026×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	417	12	13	19	9	12	12
	PER	379	11	13	12	14	10	18
DATE PLATED:	PLATE	344	14	20	7	10	17	10
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.002×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	311	10	4	4	4	3	3
	PER	281	11	6	1	1	4	1
DATE PLATED:	PLATE	221	6	1	4	7	1	4
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
0.814×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	535	15	14	17	19	14	14
	PER	519	20	15	25	18	16	6
DATE PLATED:	PLATE	503	8	13	12	14	21	20
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.360×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PRINCUBATION MUTATION ASSAY
MUTATION ASSAY CORRECTED COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-2
TEST ARTICLE: Potassium Perfluorobutane Sulfonate
SITEK STUDY NO.: 0623-2140
SOLVENT: DMSO
CONC. IN: µg/plate

WITHOUT ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	563	17	22	20	27	25	36
	PER	551	31	17	21	22	27	34
DATE PLATED:	PLATE	518	23	34	31	33	28	30
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.160×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	500	64	67	78	78	83	80
	PER	463	65	75	76	89	65	87
DATE PLATED:	PLATE	522	79	82	74	80	75	81
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.026×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	444	16	17	23	13	16	16
	PER	404	15	17	16	18	14	22
DATE PLATED:	PLATE	367	18	24	10	14	21	14
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.002×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	332	14	7	7	7	6	6
	PER	300	15	9	4	4	7	4
DATE PLATED:	PLATE	237	9	4	7	10	4	7
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
0.814×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	569	19	18	21	23	18	18
	PER	552	24	19	30	22	20	9
DATE PLATED:	PLATE	535	12	17	16	18	25	24
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.360×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PRINCUBATION MUTATION ASSAY
MUTATION ASSAY RAW COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-2 SITEK STUDY NO.: 0623-2140
TEST ARTICLE: Potassium Perfluorobutane Sulfonate SOLVENT: DMSO
CONC. IN: µg/plate

WITH S-9 ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	567	30	37	29	30	37	37
	PER	603	30	30	27	38	36	33
DATE PLATED:	PLATE	588	31	32	38	38	29	28
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.160×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	445	73	96	78	76	85	76
	PER	507	79	74	91	97	85	77
DATE PLATED:	PLATE	443	71	96	72	97	92	90
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.026×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	104	11	11	15	11	13	11
	PER	113	14	16	17	15	12	4
DATE PLATED:	PLATE	122	15	14	12	11	12	14
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.002×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	52	8	2	7	6	7	5
	PER	53	4	4	4	7	3	6
DATE PLATED:	PLATE	50	9	3	7	5	9	7
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
0.814×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	226	19	17	15	20	13	15
	PER	196	12	21	21	22	21	19
DATE PLATED:	PLATE	218	15	18	26	19	14	11
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.360×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PRINCUBATION MUTATION ASSAY
MUTATION ASSAY CORRECTED COLONY COUNTS AND BACKGROUND LAWN EVALUATION

EXPERIMENT NO.: B-2 SITEK STUDY NO.: 0623-2140
TEST ARTICLE: Potassium Perfluorobutane Sulfonate SOLVENT: DMSO
CONC. IN: µg/plate

WITH S-9 ACTIVATION

S. typhimurium		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: TA98	REVERTANTS	603	35	42	34	35	42	42
	PER	641	35	35	32	43	41	38
DATE PLATED:	PLATE	625	36	37	43	43	34	33
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
1.160×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA100	REVERTANTS	474	80	105	86	83	93	83
	PER	539	87	81	99	106	93	84
DATE PLATED:	PLATE	471	78	105	79	106	100	98
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.026×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1535	REVERTANTS	113	15	15	19	15	17	15
	PER	123	18	20	21	19	16	7
DATE PLATED:	PLATE	132	19	18	16	15	16	18
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.002×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP
STRAIN: TA1537	REVERTANTS	58	12	5	10	9	10	8
	PER	59	7	7	7	10	6	9
DATE PLATED:	PLATE	56	13	6	10	8	13	10
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
0.814×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

E. coli		Positive Control	Solvent Control	Concentration per plate 50	100	500	1000	5000
STRAIN: WP2uvrA	REVERTANTS	242	23	21	19	24	17	19
	PER	210	16	25	25	26	25	23
DATE PLATED:	PLATE	234	19	22	31	23	18	15
09/20/00								
	LAWN	NL	NL	NL	NL	NL	NL	NL
CELLS SEEDED:								
2.360×10^8	PRECIPITATE	NP	NP	NP	NP	NP	NP	NP

NL = Normal, healthy microcolony lawn. NP = No precipitate.

APPENDIX III

STUDY PROTOCOL AND PROTOCOL AMENDMENTS

EVALUATION OF A TEST ARTICLE IN THE SALMONELLA TYPHIMURIUM/ESCHERICHIA COLI PLATE INCORPORATION/PREINCUBATION MUTATION ASSAY IN THE PRESENCE AND ABSENCE OF INDUCED RAT LIVER S-9

This protocol is presented in two parts. Part One is designed to collect specific information pertaining to the test article and study. Part Two describes the study design in detail. Please complete all sections in Part One and sign Section 8.0 to approve the protocol.

PART ONE

1.0 SPONSOR

1.1 Name: Primedica Redfield

1.2 Address: 100 E. Boone Street
Redfield, AR 72132

1.3 Sponsor's Study Coordinator: John Seng, Ph.D.

2.0 TESTING FACILITY

2.1 Name: SITEK Research Laboratories

2.2 Address: 15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

2.3 Study Director: Kamala J. Pant, M.S.

3.0 STUDY NUMBERS

* 3.1 Testing Facility's Study No.: 0623-2140

3.2 Sponsor's Study No.: 132-008

4.0 TEST ARTICLE

4.1 Identification

Name: Potassium Perfluorobutane Sulfonate, CAS # 29420-49-3

Batch/Lot No.: Lot #2

* To be completed by the Testing Facility.

SITEK RESEARCH LABORATORIES

4.2 Description

Color: White

Physical Form: Solid, free flowing powder

4.3 Analysis

Purity Information: 100%

Does the Sponsor require the use of a correction factor to account for impurity?

___ Yes _X_ No

If yes, what is the correction factor? _____

Determination of the test article characteristics as defined by Good Laboratory Practices will be the responsibility of the Sponsor. The specific GLP references for U.S. agencies are: FDA = 21 CFR, 58.105; EPA TSCA = 40 CFR, 792.105 and EPA FIFRA = 40 CFR 160.105.

4.4 Stability

Storage Conditions (check one):

_X_ Room Temperature ___ Refrigerated (1-5°C)

___ Frozen (-10 to -20°C)

___ Other (please specify): _____

Expiration Date: 04-06-01

4.5 Preferred Solvent (check one):

___ H_2O ___ DMSO

___ Acetone ___ Ethanol

___ Other (please specify): _____

_X_ To be decided by the Testing Facility

4.6 Special Handling Instructions:

Standard precaution (MSDS attached)

SITEK RESEARCH LABORATORIES

5.0 REGULATORY AGENCY SUBMISSION

This study will be conducted in compliance with the following Good Laboratory Practice standards:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 1997.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 1998.

Japanese Ministry of Agriculture, Forestry and Fisheries, 59 Nohsan, Notification No. 3850, Agriculture Production Bureau, August 10, 1984.

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45, Paris 1992.

Will this study be submitted to a regulatory agency?

_X_ Yes ___ No

If so, which agency(ies)? OECD

6.0 TEST ARTICLE/DOSING SOLUTIONS CHARACTERIZATION

The U.S. requirements for analysis of dosing solutions are specified in: FDA = 21 CFR, 58.113; EPA TSCA = 40 CFR, 792.113; and EPA FIFRA = 40 CFR, 160.113.

Does the Sponsor want dosing solution analysis?

_X_ Yes** ___ No

If yes, please complete the rest of this section.

If requested by the Sponsor, SITEK Research Laboratories will determine the strength and stability of the dosing solutions. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

** Additional charges will apply. See Special Services price schedule.

Alternatively, the Sponsor will be responsible for determining the strength and stability of the dosing solutions.

Dosing solution analysis will be performed by:

___ SITEK Research Laboratories _X_ Sponsor

What dosing solutions will be analyzed?

From the Range Finding Test?

___ Yes _X_ No

From the Assay?

_X_ Yes ___ No

Which concentration(s)? All

What amount of each concentration? 2.0 mL

At what temperature should the dosing solutions be stored?

___ Room Temperature _X_ Frozen (-10 to -20°C)

___ Refrigerated (1-5°C)

7.0 STUDY DATES

* 7.1 Proposed Experimental Start Date: 8/7/00

Defined as the first date the test article is applied to the test system.

* 7.2 Anticipated Experimental Completion Date: 10/1/00

Defined as the last date on which data are collected directly from the study.

* To be completed by the Testing Facility.

SITEK RESEARCH LABORATORIES

8.0 PROTOCOL APPROVAL

* Kamala Pant — Study Director — Date 8/1/00

John Heng — Study Coordinator — Date 7/18/00

John L. Butenhoff — Sponsor's Authorized Representative — Date July 17, 2000

* M Bauernschub — Quality Assurance Manager — Date 8/2/00

* M Bauernschub — Safety Officer — Date 8/2/00

* To be completed by the Testing Facility.

SITEK RESEARCH LABORATORIES

STUDY DESIGN

PART TWO

9.0 PURPOSE

The purpose of this study is to evaluate the test article for its potential to cause mutations in the histidine operon of Salmonella typhimurium strains TA98, TA100, TA1535 and TA1537 and the tryptophan operon of Escherichia coli strain WP2uvrA.

10.0 JUSTIFICATION FOR SELECTION OF TEST SYSTEM

The Salmonella typhimurium and Escherichia coli strains have been used extensively in the Plate Incorporation Mutation Assay and have been demonstrated to be effective in detecting the mutagenic activity of chemicals from a wide range of classes (1-4).

11.0 ABBREVIATIONS

2-AA	-	2-Aminoanthracene
2-NF	-	2-Nitrofluorene
9-AA	-	9-Aminoacridine
DMSO	-	Dimethyl Sulfoxide
MMS	-	Methyl Methanesulfonate
NaAz	-	Sodium Azide
NADP	-	Nicotinamide-adenine Dinucleotide Phosphate
O.D.	-	Optical Density
%T	-	Percent Transmittance
S-9	-	Induced Rat Liver Homogenate

12.0 INDICATOR CELLS

12.1 Source

The Salmonella typhimurium strains TA98, TA100, TA1535 and TA1537 were obtained from Dr. Bruce N. Ames, University of California, Berkeley, California. The Escherichia coli strain WP2uvrA was obtained from Dr. Elena C. McCoy, Case Western University, Cleveland, Ohio.

SITEK RESEARCH LABORATORIES

12.2 Culture Conditions

The Salmonella typhimurium and Escherichia coli strains are routinely grown in Oxoid Nutrient Broth No. 2 in a shaker incubator rotating at approximately 120 rpm and maintaining a temperature of 37 ±1°C.

12.3 Stock Cultures

The Salmonella typhimurium and Escherichia coli strains were propagated to obtain a sufficient number of cells for freezing a large number of stock ampules. The cells were cryopreserved in Oxoid Nutrient Broth No. 2 supplemented with 8-9% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen vapor phase. Scrapes from stock ampules are used to initiate the stock cultures for the test.

13.0 METABOLIC ACTIVATION

The standard rat liver S-9 will be prepared by inducing male Sprague-Dawley rats with Aroclor-1254 or phenobarbital and/or ß-naphthoflavone. The livers will be aseptically removed, washed, minced, homogenized and centrifuged at 9000xg. The supernatant fraction will be pooled, dispensed in appropriate aliquots, and stored below -70°C for up to 3 years. Prior to its use in this study, the S-9 will be evaluated for acceptable metabolic activity in a standard Salmonella typhimurium mutation assay using strain TA100 and a single dose of 2-AA.

14.0 ROUTE OF ADMINISTRATION OF TEST ARTICLE

The test article will be administered in vitro directly or through a solvent compatible with the test cultures. This is the only route of administration available in this test system.

15.0 TEST SYSTEM IDENTIFICATION

All test plates will be labeled using an indelible pen with a code system which clearly identifies the experiment number, the SITEK test article number, controls, doses, and whether or not the plate was treated in conjunction with an exogenous activation system.

The test article will be designated by the unique four-digit number assigned by SITEK when the test article is received (e.g., 0074). The experiment phase will be designated by the letter A (Range Finding Test) or B (Mutation Assay) followed by a number designating the trial number. This will be followed by the letter N (No Activation) or S (With S-9) which will be followed by the dose and strain identification numbers. The doses will be identified by the numbers 1, 2, 3, ... indicating the highest to the lowest dose. The strain identification numbers will be as follows:

Salmonella typhimurium	Escherichia coli
1 = TA98	5 = WP2uvrA
2 = TA100	
3 = TA1535	
4 = TA1537	

An example of a plate label follows:

0074B1-S-1-3

0074	=	SITEK Test Article Number
B1	=	First Mutation Assay
S	=	With S-9
1	=	Highest Test Article Dose
3	=	Strain TA1535

In addition to the above, the Range Finding Test and Mutation Assay viability plates that contain 10X (0.5mM) histidine biotin or 10X (0.5mM) tryptophan will be designated with the prefix "T".

16.0 CONTROL SUBSTANCES

16.1 Positive Controls

The positive control chemicals that will be used for the tester strains in the presence and absence of exogenous metabolic activation are presented below. The abbreviations are defined in Section 11.0.

	Strain	S-9	Chemical	Dose (µg/plate)
Salmonella typhimurium				
	TA98	-	2-NF	2.5-7.5
	TA98	+	2-AA	1.25-5.0
	TA100	-	NaAz	0.5-2.0
	TA100	+	2-AA	1.25-5.0
	TA1535	-	NaAz	0.5-2.0
	TA1535	+	2-AA	1.25-5.0
	TA1537	-	9-AA	25-75
	TA1537	+	2-AA	1.25-5.0
Escherichia coli				
	WP2uvrA	-	MMS	2000-4000
	WP2uvrA	+	2-AA	10-20

DMSO will be used to solubilize the positive controls 2-AA, 2-NF and 9-AA. H_2O will be used to dissolve NaAz and MMS

If necessary, other appropriate positive controls can be used with the approval of the Sponsor.

16.2 Solvent Control

The solvent used for dissolving the test article will be used as the solvent control. Deionized, distilled water, dimethyl sulfoxide (CAS #67-68-5), ethanol (CAS #64-17-5) and acetone (CAS #67-64-1) are some of the solvents which are compatible with this test system. If there is a need to use other solvents, the approval of the Sponsor will be obtained prior to their use.

17.0 DOCUMENTATION

All procedures, results, significant observations, and methods used for analysis of results will be documented in a study notebook. The study notebook will also include copies of the protocol, all protocol amendments and protocol deviations, study reports, and all relevant communications with the Sponsor.

18.0 EXPERIMENTAL PROCEDURE

18.1 Determination of Solubility/Miscibility

In order to determine the optimal vehicle for delivering the test article to the test system or to determine the maximum achievable concentration in the solvent requested by the Sponsor, a solubility/miscibility test will be performed. The solvents of choice for this system are water, DMSO,acetone and ethanol. If the test article is not sufficiently soluble in any of these solvents, additional solvents will be screened.

For solid and viscous test articles, the solubility test will consist of weighing out 20- to 100-mg aliquots of test article and adding solvent in 0.1 mL increments, with thorough mixing between additions, until the test article is dissolved as determined by visual inspection or until 5.0 mL of solvent has been added to the vessel. The volume of solvent required for complete dissolution and any additional observations will be recorded in the study notebook. Test articles that do not dissolve in 5.0 mL of solvent will be visually inspected and recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

For liquid test articles, a miscibility test will be conducted. 0.5 mL of solvent will be added to 0.5 mL aliquots of the test article. The resulting solution will be thoroughly mixed and observed for miscibility. The test article will be rated by visual inspection as either "not miscible," "partially miscible," or "completely miscible" in each of the four preferred solvents. The miscibility rating and any additional observations will be recorded in the study notebook.

18.2 Preparation of Test Cultures

The strains of Salmonella typhimurium and Escherichia coli will be prepared from cultures that were started from scrapes placed in Oxoid Nutrient Broth No. 2. The cultures will be placed on the shaker, and a timer turns on the incubator approximately 8-12 or 4-6

hours for Salmonella typhimurium or Escherichia coli, respectively, prior to sampling the cultures for growth determination. The incubator will be set at 120 rpm and 37 ±1°C. Samples from each culture will be checked for Percent Transmittance (%T) at 650 nm.

Only cultures that have a %T of between 25% (O.D. 0.6) and 10% (O.D. 1.0) will be used. These cultures will have approximately between $5.0x10^8$ and $1.0x10^9$ cells per mL.

18.3 Preparation of S-9 Metabolic Activation Mix

For the portion of the Range Finding Test or the Mutation Assay in which the cells are exposed to the test article in conjunction with an exogenous metabolic activation system, induced rat liver S-9 plus cofactors (S-9 mix) will be used as the activation system. The components of the standard S-9 mix will be 8mM $MgCl_2$, 33mM KCl, 5mM glucose-6-phosphate, 4mM NADP, 100mM sodium phosphate buffer (pH 7.4), and 10% rat liver S-9.

18.4 Preparation of Test Article

The desired amount of the test article as specified in the dilution scheme will be weighed or measured just prior to use in either the Range Finding Test or the Mutation Assay. The dosing solutions will be prepared by adding the appropriate volume of solvent to the test article and thoroughly mixing the resulting solution until the test article goes completely into solution or a homogeneous suspension is achieved. The remaining doses specified in the dilution scheme will be prepared by either performing a serial dilution or by varying the volume delivered from the stock concentration to the cultures. In all treatments the amount of solvent delivered to the target cultures will be limited to a level which has no cytotoxic effect on the cells. If necessary, the test article may be added directly to the top agar.

18.5 Range Finding Test

In order to determine the test article concentrations that will produce from 0-100% toxicity, a Range Finding Test will be performed with and without S-9 activation using tester strains TA100 and WP2uvrA only. The test article will be weighed or measured, and a serial dilution will be prepared. If there are no solubility/miscibility limitations, prior knowledge of cytotoxicity indicates differently, or the Sponsor specifies differently, the treatment concentrations for solid and viscous test articles will be 5000, 1000, 500, 100, 50, 10 and 5.0 µg/plate. If the results based on the dosing regimen indicate that the threshold level of complete toxicity is below 5.0 µg/plate an additional Range Finding Test will be performed. Only one plate per concentration condition will be used.

18.5.1 Treatment

2 mL aliquots of molten top agar, to which trace amounts of histidine and biotin have been added, will be dispensed to a series of culture tubes maintained at 45 ±1°C. Treatment will be performed by adding 0.5 mL of S-9 mix or 0.5 mL of sterile, distilled, deionized water, 0.1 mL of tester strain TA100 or WP2uvrA, and 0.1 mL of test article to the top agar. Appropriate solvent controls will also be prepared.

In addition, plates for determining viability will be prepared by plating the test article doses with a $2.0x10^5$ dilution of tester strain TA100 or WP2uvrA in top agar containing 10X (0.5mM) histidine-biotin or 10X (0.5mM) tryptophan, respectively.

The contents will be mixed by vortexing the tube, and then the contents will be poured onto a bottom agar plate and evenly distributed by gently tilting and rotating the plate. The plate will be placed on a flat, level surface until solidified. After all treatment is performed, the plates will be inverted and incubated at 37 ±1°C for 48-72 hours.

18.5.2 Determination of Toxicity

After 48-72 hours of incubation, the plates will be removed from the incubator and evaluated or placed in cold storage (1-5°C) until evaluated.

Evaluation of test article toxicity on the tester strain will be based on three end points:

1. Viability of cells plated on minimal medium plates supplemented with excess histidine-biotin or tryptophan. Toxicity will be measured as a decrease in the number of colonies per plate with increasing test article concentration.

2. The number of revertant colonies on minimal medium plates supplemented with trace amounts of histidine-biotin or tryptophan. Toxicity will be measured as a reduction in the number of revertant colonies per plate with increasing test article concentration.

3. The integrity of the background microcolony lawn. Toxicity will be measured as a thinning or disappearance of the background lawn usually occurring with an increase in the size of the remaining microcolonies relative to the control plates.

The number of revertants per plate and the number of viable colonies per plate will be determined by counting them with an automatic colony counter or by hand as described in Sections 18.6.5.1 and 18.6.5.2.

The counts will be entered directly in the Lotus 123 computer program 2140A.WK3, and the calculations will be performed. The computer printouts will be included in the study notebook.

18.6 Mutation Assay

The maximum concentration of nontoxic test articles that is tested will be 5 mg per plate, unless the Sponsor requests otherwise or precipitation of the test article on the plate warrants the use of a lower concentration. Test articles that produce a toxic effect will be tested at a maximum dose that significantly reduces the number of revertants per plate and/or causes thinning of the background lawn. Four lower doses will be selected that should not produce toxicity. Test articles that are insoluble at concentrations of 5 mg per plate or lower will be tested at a maximum dose that produces precipitate. A concentration that produces precipitate in the test system will be considered to be beyond the limits of solubility. The actual dose levels for the assay, once determined, will be added to the protocol in the form of an amendment. Each test article dose, the positive controls and solvent controls will be plated in triplicate.

18.6.1 Test Culture Preparation and Exposure

18.6.1.1 Plate Incorporation Method

Cultures of Salmonella typhimurium, TA98, TA100, TA1535 and TA1537, and Escherichia coli WP2uvrA for use in the Mutation Assay will be prepared as described in Section 18.2. The test article will be weighed or measured, and a serial dilution will be performed as previously described in Section 18.4. 2 mL aliquots of molten top agar to which histidine and biotin or tryptophan have been added will be dispensed to a series of culture tubes maintained at 45 ±1°C. Treatment will be performed by adding 0.5 mL of S-9 mix or 0.5 mL of sterile, distilled, deionized water, 0.1 mL of tester strain, and 0.1 mL of test article to the top agar. Appropriate solvent and positive controls will also be prepared. The contents will be mixed by vortexing the tube, and then the contents will be poured onto a bottom agar plate and evenly distributed by gently tilting and rotating the plate. The plate will be placed on a flat, level surface until solidified. After all treatment will be performed, the plates will be inverted and incubated at 37 ±1°C for 48-72 hours.

18.6.1.2 Preincubation Method

Cultures of Salmonella typhimurium, TA98, TA100, TA1535 and TA1537, and Escherichia coli WP2uvrA for use in the Mutation Assay will be prepared as described in Section 18.2. The test article will be weighed or measured, and a serial dilution will be performed as previously described in Section 18.4. 0.5 mL of sterile, deionized, distilled water or S-9 mix will be dispensed into a series of labeled culture tubes, and treatment will be performed by adding 0.1 mL of tester strain and 0.1 mL of test article. The tubes will be vortexed gently and preincubated at 37 ±1°C for 20 minutes. The tubes are shaken at a moderate speed during the preincubation. Appropriate solvent and positive controls will also be prepared. 2.0 mL of top agar containing 1X histidine-biotin or 1X tryptophan will be added to each culture tube after the preincubation period. The contents of each tube will be mixed by vortexing, poured onto a bottom agar plate, and evenly distributed by gently tilting and rotating the plates. All of the plates will be placed on a flat, level surface until solidified. After all treatments have been performed, the plates will be inverted and incubated at 37 ±1°C for 48-72 hours.

18.6.2 Confirmation of Tester Strain Genotypes

On the same day as the plating of the Mutation Assay, the genotypes of the tester strains will be confirmed. All of the Salmonella typhimurium strains will be tested for histidine dependence and the rfa mutation. Each Salmonella typhimurium strain will be tested for the uvrB deletion after cryopreservation of the stock ampules. The tester strains TA98 and TA100 will also be tested for the pKM101 plasmid. The Escherichia coli WP2uvrA strain will be tested for tryptophan dependence.

18.6.3 Tester Strain Viability Determination

After the Mutation Assay has been plated, a dilution of each tester strain will be prepared, and approximately 250-500 bacteria will be plated in top agar supplemented with 10X (0.5mM) histidine-biotin or 10X (0.5mM) tryptophan. These plates will be incubated for 48-72 hours, and then the total number of colonies that develop will be determined.

18.6.4 Background Lawn Evaluation

The integrity of the background microcolony lawn will be evaluated by viewing each plate with the aid of a 2X to 4X microscope. The lawns will be rated as normal, slightly reduced, markedly reduced, extremely reduced or absent.

18.6.5 Enumeration of Colonies

After 48-72 hours of incubation, the plates treated with the highest test article concentration will be observed for the presence of precipitate. If precipitate is absent, the entire assay will be counted using an automatic colony counter. If observation of the high dose plates reveals precipitate that interferes with accurate automatic counting, those plates will be counted by hand. The procedure will be repeated for each subsequent dose level or until no precipitate is evident.

18.6.5.1 Automatic Colony Counting

Each plate will be placed on the stage, and three counts are made with the automatic counter. The plate will be rotated on the stage approximately 120° between each count, and the median count will be entered directly into the computer.

18.6.5.2 Hand Counting

Hand counting of colonies will be performed by marking a dot over each colony on the bottom of the plate. The hand count will be entered directly into the computer.

The counts will be entered directly in the Lotus 123 spread sheet program 2140B or 2150B for the Plate Incorporation or Preincubation method, respectively. The computer printouts will be included in the study notebook.

18.7 Confirmatory Mutation Assay

If the first Mutation Assay or a portion thereof produces negative or equivocal results, a confirmatory Mutation Assay will be performed. The test article treatment concentrations may be altered based on the results obtained in the first Mutation Assay. On the other hand, if the results of the first Mutation Assay are clearly positive, a confirmatory Mutation Assay may or may not be performed depending on the Sponsor's instructions.

18.8 Criteria For a Valid Assay

The following criteria will be used as guidelines in determining the acceptability of the results. Since it is impossible to formulate criteria that would apply to every configuration of data generated by the Mutation Assay, the Study Director will be responsible for the ultimate decision regarding the acceptability of the results.

18.8.1 Solvent Control Cultures

The mean reversion frequency of the test article solvent control plates for each strain must fall within the range presented below:

Salmonella typhimurium		Escherichia coli	
TA98	20 ±15	WP2uvrA	15 ±10
TA100	100 ±70		
TA1535	20 ±15		
TA1537	15 ±12		

18.8.2 Positive Controls

The results for the positive control cultures will be considered acceptable if the treated strains have mean reversion frequencies that are three times or greater than the mean reversion frequencies of the test article solvent control plates.

18.8.3 Tester Strain Characterization

1. All of the Salmonella typhimurium strains will be confirmed positive for histidine dependence and the Escherichia coli strain for tryptophan dependence.

2. All of the Salmonella typhimurium strains will be confirmed positive for the rfa mutation as evidenced by sensitivity to crystal violet.

3. The R-factor strains, TA98 and TA100, will be confirmed positive for the pKM101 plasmid as evidenced by ampicillin resistance.

4. The titer of the stock cultures of each strain will indicate that the stock cultures contained approximately between 5.0×10^8 and 1.0×10^9 bacteria per mL.

18.9 Evaluation of Test Results

The following criteria will be used as guidelines in evaluating the results of the Mutation Assay for a negative, positive or equivocal response. Since it is impossible to write criteria that would apply to every configuration of data generated by the Mutation Assay, the Study Director will be responsible for the ultimate decision in the evaluation of the results. The factors considered in making the decision will be discussed in the report.

18.9.1 Criteria for a Negative Response

A response will be considered negative if all of the strains treated with the test article have mean reversion frequencies that are less than twice that of the mean reversion frequencies of the corresponding solvent control plates in TA98 and TA100 and less than three times in TA1535, TA1537 and WP2uvrA, and there is no evidence of a dose-dependent response.

18.9.2 Criteria for a Positive Response

A response will be considered positive if either strain TA98 or TA100 has a dose that produces a mean reversion frequency that is greater than or equal to two times the mean reversion frequency of the corresponding solvent control plates or if either strain TA1535, TA1537 or WP2uvrA has a dose producing a three-fold or greater increase in the mean reversion frequency compared to the solvent control frequency. In addition, the response must be dose dependent or increasing concentrations of the test article must show increasing mean reversion frequencies. In evaluating the results, consideration will be given to the degree of toxicity exhibited by the dose causing the two-fold/three-fold or greater increase in reversion frequency and the magnitude of the increase in reversion frequency.

18.9.3 Criteria for an Equivocal Response

A response will be considered equivocal if it does not fulfill the criteria of either a negative or a positive response and/or the Study Director does not consider the response to be either positive or negative. If an equivocal response is obtained, a repeat assay may be performed at the Sponsor's request.

19.0 PROTOCOL AMENDMENTS AND DEVIATIONS

If changes in the approved protocol are necessary, such changes will be documented in the form of protocol amendments and protocol deviations. Protocol amendments will be generated when changes in the protocol are made prior to performing a study or part of a study affected by the changes. In such cases, a verbal agreement to make such changes will be made between the Study Director and the Sponsor. These changes and the reasons for them will be documented and attached to the protocol as an addendum. Protocol deviations will be generated when the procedures used to perform the study do not conform to the approved protocol. The Sponsor will be informed of these deviations, and as soon as practical, such changes along with their reasons or explanations will be documented and kept in the study notebook.

20.0 REPORT OF RESULTS

20.1 Content

The results of the study will be submitted to the Sponsor in the form of a final report. A draft report will be submitted before the final report is issued. The report will include, but not be limited to, the following:

1. Name and address of the testing facility and the dates on which the study was initiated and completed, terminated or discontinued.

2. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

3. Methods used to analyze the data.

4. The test and control substances.

5. Description of the methods used to perform the study.

6. The data, mean plate counts, +/- SD, and any observations regarding toxicity and precipitate

7. The name and signature of the Study Director and the names of other technical personnel who participated in performing the study.

8. The location where the raw data and reports are to be stored.

9. A statement from the Quality Assurance Unit.

20.2 Changes and Corrections to the Final Report

All changes to the final report will be in the form of report amendments which will include the reason(s) for the change, and these amendments will be added to the final report as an addendum.

21.0 ARCHIVES

The raw data, electronic file containing the data tables, documentation, protocol and final report of the study will be maintained in the SITEK Research Laboratories Archives, 15235 Shady Grove Road, Suite 303, Rockville, Maryland, according to the terms and conditions of the study.

22.0 REFERENCES

1. Ames, B. N., J. McCann and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian-microsome mutagenicity test. Mut. Res., 31:347-367, 1975.

2. Maron, D., and B. N. Ames. Revised methods for the Salmonella mutagenicity test. Mut. Res., 113:173-215, 1983.

3. Green, M. H. L., and W. J. Muriel. Mutagen testing using trp+ reversion in Escherichia coli. In: B. J. Kilbey, et al. (eds.), Handbook of Mutagenicity Test Procedures, pp. 65-94, Elsevier North Holland Biomedical Press, Amsterdam, 1977.

4. Venitt, S., and J. M. Parry (eds.). Mutagenicity testing: A practical approach. IRL Press, Oxford, England and Washington, D.C., 1984.

PROTOCOL AMENDMENTS

Amendment Nos.:	1 - 2
Sponsor:	Primedica Redfield 100 E. Boone Street Redfield, AR 72132
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	0623-2140
Sponsor's Study No.:	132-008
Test Article ID:	Potassium Perfluorobutane Sulfonate
Protocol Title:	Evaluation of a Test Article in the *Salmonella typhimurium/Escherichia coli* Plate Incorporation/Preincubation Mutation Assay in the Presence and Absence of Induced Rat Liver S-9

Amendment No. 1: Protocol Page 6, Section 12.1, Source, The *Escherichia coli* strain, WP2uvrA, that will be used in the assay was received from Ms. Judy Mayo of Pharmacia and Upjohn Co., Kalamazoo, Michigan.

Reason for Amendment No. 1: A new strain of *Escherichia coli* has been obtained and is currently in use at SITEK Research Laboratories.

Amendment No. 2: Protocol Page 11, Section 18.6, Mutation Assay, The test article was tested in the Mutation Assays at the following concentrations:

50, 100, 500, 1000 and 5000 μg/plate.

Reason for Amendment No. 2: As stated in the protocol, the test article concentrations will be included in the form of an amendment, once determined.

Study Number: 0623-2140
Protocol Amendment Nos. 1 - 2

APPROVAL:

Kamala Pant — 11.8.00

Kamala J. Pant, M.S. — Date
Study Director

John S Seng — 11/13/00

John Seng, Ph.D. — Date
Sponsor's Study Coordinator

APPENDIX IV

HISTORICAL POSITIVE AND

SOLVENT CONTROL DATA

SITEK RESEARCH LABORATORIES

HISTORICAL POSITIVE CONTROL DATA FOR SALMONELLA TYPHIMURIUM/
ESCHERICHIA COLI MUTATION ASSAY
MUTANTS EXPRESSED IN 10^6 SURVIVING CELLS
WITH AND WITHOUT ACTIVATION

WITHOUT ACTIVATION	TA98 (2NF)	TA100 (NaAz)	TA1535 (NaAz)	TA1537 (9AA)	E.coli (MMS)
AVERAGE	724	578	134	701	742
STANDARD DEVIATION (±)	301	174	141	238	391
MINIMUM VALUE	189	272	22	187	173
MAXIMUM VALUE	2108	1433	846	1410	1528
N*	180	178	160	144	34
WITH ACTIVATION	**TA98 (2AA)**	**TA100 (2AA)**	**TA1535 (2AA)**	**TA1537 (2AA)**	**E.coli (2AA)**
AVERAGE	811	888	179	81	226
STANDARD DEVIATION (±)	319	302	129	48	148
MINIMUM VALUE	151	194	43	18	19
MAXIMUM VALUE	1699	1618	1336	349	719
N*	178	175	156	157	145

SITEK Research Laboratories

HISTORICAL NEGATIVE CONTROL DATA FOR SALMONELLA TYPHIMURIUM/
ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
MUTANTS EXPRESSED IN 10^6 SURVIVING CELLS
NON–ACTIVATED

TA98	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	24.3	26.4	26.0	25.8	24.7
STANDARD DEVIATION (±)	3.9	5.3	3.1	3.5	5.9
MINIMUM VALUE	16	11	21	17	17
MAXIMUM VALUE	33	32	31	33	35
N*	72	16	12	57	14
TA100	**DMSO**	**ACET**	**CORN OIL**	**H_2O**	**SALINE**
AVERAGE	89.5	101.6	90.6	103.9	91.1
STANDARD DEVIATION (±)	24.1	36.4	16.5	22.4	18.8
MINIMUM VALUE	23	26	59	66	70
MAXIMUM VALUE	144	174	118	159	132
N*	73	17	12	57	14
TA1535	**DMSO**	**ACET**	**CORN OIL**	**H_2O**	**SALINE**
AVERAGE	18.3	19.3	16.7	18.0	16.7
STANDARD DEVIATION (±)	5.8	4.6	3.8	4.0	3.6
MINIMUM VALUE	10	12	11	11	11
MAXIMUM VALUE	46	33	23	28	22
N*	68	16	12	40	14
TA1537	**DMSO**	**ACET**	**CORN OIL**	**H_2O**	**SALINE**
AVERAGE	9.5	9.6	10.6	9.1	8.0
STANDARD DEVIATION (±)	3.1	2.7	5.8	2.5	2.8
MINIMUM VALUE	3	6	3	5	4
MAXIMUM VALUE	17	16	23	17	13
N*	66	18	12	41	14
E. COLI	**DMSO**	**ACET**	**CORN OIL**	**H_2O**	**SALINE**
AVERAGE	14.6	15.3	15.3	17.6	15.6
STANDARD DEVIATION (±)	3.2	4.6	3.0	3.5	3.7
MINIMUM VALUE	9	8	10	12	10
MAXIMUM VALUE	24	25	19	25	25
N*	56	16	12	40	14

N* = Number of data points.

SITEK Research Laboratories

HISTORICAL NEGATIVE CONTROL DATA FOR SALMONELLA TYPHIMURIUM/
ESCHERICHIA COLI PLATE INCORPORATION MUTATION ASSAY
MUTANTS EXPRESSED IN 10^6 SURVIVING CELLS
WITH S-9 ACTIVATION

TA98	DMSO	ACET	CORN OIL	H_2O	SALINE
AVERAGE	30.8	31.9	31.1	31.5	31.4
STANDARD DEVIATION (±)	4.9	3.7	3.8	3.7	7.2
MINIMUM VALUE	15	26	22	22	20
MAXIMUM VALUE	45	37	35	38	52
N*	74	16	12	56	14
TA100	**DMSO**	**ACET**	**CORN OIL**	**H_2O**	**SALINE**
AVERAGE	89.0	88.4	88.5	96.9	81.7
STANDARD DEVIATION (±)	25.9	32.7	18.5	26.7	21.1
MINIMUM VALUE	48	52	67	45	55
MAXIMUM VALUE	149	174	121	159	115
N*	73	16	12	58	14
TA1535	**DMSO**	**ACET**	**CORN OIL**	**H_2O**	**SALINE**
AVERAGE	16.4	16.4	18.8	16.7	15.4
STANDARD DEVIATION (±)	4.8	4.8	3.0	3.7	3.6
MINIMUM VALUE	8	8	15	8	9
MAXIMUM VALUE	31	31	25	27	23
N*	71	71	12	41	14
TA1537	**DMSO**	**ACET**	**CORN OIL**	**H_2O**	**SALINE**
AVERAGE	10.2	10.3	10.4	9.8	7.1
STANDARD DEVIATION (±)	4.2	2.7	3.6	2.8	2.2
MINIMUM VALUE	3	7	6	6	3
MAXIMUM VALUE	28	14	18	17	11
N*	70	16	12	41	14
E. COLI	**DMSO**	**ACET**	**CORN OIL**	**H_2O**	**SALINE**
AVERAGE	15.2	16.9	17.2	17.5	16.8
STANDARD DEVIATION (±)	3.4	3.4	5.2	4.2	3.5
MINIMUM VALUE	8	8	9	10	10
MAXIMUM VALUE	25	25	26	30	23
N*	60	60	12	43	14

N* = Number of data points.

APPENDIX V

DOSING SOLUTION ANALYSIS

DOSE FORMULAION ANALYSIS OF PERFLUOROBUTANESULFONATE IN DMSO BY HPLC/MS

STUDY ID: A239.1

SPONSOR STUDY NO. 132-008

Southern Research Institute
2000 Ninth Avenue South
P.O. Box 55537
Birmingham, AL 35255-5537

Quality Assurance Statement

Final Report On

Dose Formulation Analysis of Perfluorobutane Sulfonate (PFBS) In DMSO

A239.1
Sponsor Study No. 132-008

This study was performed using a method validated for accuracy, precision (repeatability), linearity, range and specificity. No audits were performed during the course of the study, either as the analyses were performed, of the resulting data, or the report.

C.L. Marsh, Manager, Quality Assurance/Quality Control

7/3/01
Date

SUMMARY

A total of 12 formulated dose samples including blanks ranging in concentration from 0.5 to 50 mg/mL were analyzed by analytical method BACG 3533 to determine the concentration of perfluorobutanesulfonate (PFBS) in the formulated mixture. All dose formulations were found to be within ± 10% of the reported concentration.

KEY PERSONNEL

James D. Johnson, M.S., MBA
Manager
Bioanalytical Chemistry Group

Gregory S. Gorman, Ph.D.
Staff Chemist
Bioanalytical Chemistry Group

1. OBJECTIVE

The objective of this study was to determine the dose concentration of PFBS in the supplied dosing solutions received from the study director.

2. SAFETY

All necessary procedures to ensure safety of the analysts were based on information contained in the Material Safety and Data Sheets (MSDS), provided by the study director for the test article used in this study.

3. Compliance

This work was performed using a validated analytical method (BACG 3533), which along with the validation report is included in the appendix of this report. While this work was not audited in compliance with GLP regulations, it was performed in the spirit of the regulations using calibrated and validated instrumentation.

4. EXPERIMENTAL

4.1 Analytical Procedures

The sample preparation and analysis procedures as described in the analytical method BACG 3533 were employed for all analyses. Each sample was allowed to warm to room temperature and was then vortexed well before an aliquot was taken. Duplicated aliquots were taken from each sample and diluted as described in the method. Two standard curves was prepared over a concentration range of 500 to 10,000 ng/mL and analyzed along with the samples. A single composite curve with a correlation coefficient of 0.9991 was used to quantitative the samples. No outliers in the calibration standards were noted and subsequently no standards were dropped from either of the calibration curves.

4.2 Results

The results of the analysis are presented in the Table I at the end of the report.

5.0 Conclusion

A total of 12 dose formulation samples ranging in concentration from 0.5 to 500 mg/mL were analyzed by BACG 3533. All samples were found to be within ± 10 % of the reported concentration.

Table I
Dose Formulation Analysis of PFBS in DMSO

Sample	Date Prepared	Measured Conc. (mg/mL)*	% of Target
0 mg/mL B2	9/20/00	ND	NA
0 mg/mL B1	8/15/00	ND	NA
0.5 mg/Ml B2	9/20/00	0.49	98.0
0.5 mg/mL B1	8/15/00	0.48	96.0
1 mg/mL B1	8/15/00	1.03	103
1 mg/mL B2	9/20/00	1.03	103
5 mg/mL B1	8/15/00	4.83	96.6
5 mg/mL B2	9/20/00	4.99	99.8
10 mg/mL B2	9/20/00	10.1	101
10 mg/mL B1	8/15/00	10.0	100
50 mg/mL B2	9/20/00	49.7	99.4
50 mg/mL B1	8/15/00	51.3	103

ND = not detected
NA = not applicable
* = average of duplicate analysis

6.0 Approvals

7-3-01

Gregory S. Gorman, Ph.D.
Staff Chemist
Bioanalytical Chemistry Group

Date

7/3/01

James D. Johnson, M.S., MBA
Manager
Bioanalytical Chemistry Group

Date

METHOD VALIDATION REPORT

VALIDATION OF ANALYTICAL METHOD FOR DOSE FORMULATION ANALYSIS OF PERFLUOROBUTANE SULFONATE (PFBS) IN 1% CARBOXYMETHYL CELLULOSE (CMC)

STUDY ID: A098.1

Southern Research Institute
2000 Ninth Avenue South
P.O. Box 55537
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SUMMARY

Southern Research Institute has successfully validated for 3M an analytical method (BACG 3533) entitled "Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC Mass Spectrometry". Calibration standards were prepared by spiking solvent solutions with known amounts of test article, PFBS, and internal standard, perfluoropentanoic acid. The calibration standards were prepared over a concentration range of 500 to 10,000 ng/mL and the carboxymethyl cellulose (CMC) concentration was adjusted to equal that of each diluted sample in each set. Additionally, three standard curves were prepared and analyzed which contained no CMC as controls. A total of 6 calibration curves were generated during the study which produced correlation coefficients ranging from 0.9992 to 0.9999.

KEY PERSONNEL

James D. Johnson, M.S.
Manager
Bioanalytical Chemistry Group

Gregory S. Gorman, Ph.D.
Research Chemist III
Bioanalytical Chemistry Group

Lester Williams, B.S.
Associate Chemist II
Bioanalytical Chemistry Group

1. OBJECTIVE

The objective of this study was to provide a validated analytical method for the determination of PFBS concentration in dose formulation samples containing 1% CMC.

2. SAFETY

All necessary procedures to ensure safety of the analysts were based on information contained in the Material Safety and Data Sheets (MSDS), provided by 3M for the test article used in this study.

3. EXPERIMENTAL

3.1 Analytical Procedures

The sample preparation and analysis procedures as described in the analytical method BACG 3533 were employed for all analyses. For the preparation of the calibration standards, a known volume of a solvent (e.g., 1 mL) containing CMC was spiked with a known amount of test article and internal standard and vortexed briefly to ensure mixing. Each formulation sample was diluted to a final concentration of 3200 ng/mL and an aliquot of this was placed into autosampler vials for analysis.

3.2 Method Validation

Validation for BACG 3533 "Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC Mass Spectrometry" consisted of analyzing three standard curves containing CMC at the concentration present in the diluted 4 mg/mL dose formulation samples (0.001%) and three curves prepared without CMC. The concentration range for both sets ranged from 500 to 10,000 ng/mL. A total of 7 calibration levels in each curve containing CMC and 6 in each of the three without CMC were evaluated. The summaries are given below:

<u>Calibration Results 0% CMC</u>

A total of three standard curves each containing 6 individual standards encompassing a range of 500 to 10,000 ng/mL were analyzed in duplicate. The correlation coefficient for the composite curve comprising a total of 36 data points was found to be 0.9992. No data points were dropped from the analysis. A statistical summary for the composite curve is shown below:

STANDARD COMPOSITE CURVE PFBS (0% CMC)

Concen. (ng/mL)	500	1000	3000	5000	8000	10,000
# of Standards	6	6	6	6	6	6
Mean % Accur.	97.71	102.9	99.73	99.52	100.1	100.2
Std. Deviation	19.47	55.85	110.6	187.8	288.2	356.4

Calibration Results (CMC = 0.001%)

Three standards curves containing a total of 21 single standards encompassing a range of 500 to 10,000 ng/mL were analyzed. The correlation coefficient for the calibration curve was found to be 0.9997. No data points were dropped from the analysis. A statistical summary for the composite curve is shown below:

STANDARD COMPOSITE CURVE PFBS (0.001% CMC)

Concen. (ng/mL)	500	1000	3000	3200	5000	8000	10,000
# of Standards	3	3	3	3	3	3	3
Mean % Accur.	98.11	101.5	101	101	98.44	99.31	100.7
Std. Deviation	18.19	17.72	48.33	49.67	81.61	76.38	203.4

3.3 Calculations

Calculations were performed using TurboQuan (Version 1.0). The amount of analyte in the diluted dose formulation samples (ng/mL) was back calculated using a calibration curve generated from a set of calibration standards containing the equivalent amount of CMC as in the diluted samples. The calibration curve was generated by a regression analysis to determine the best fit curve (e.g., linear, quadratic etc.) and amount of weighting. A quadratic fit with 1/X weighting was determined to be the best fit :

$$y = ax^2 + bx + c$$

where:

y = Peak area response of test article

x = Concentration of the test article in standards.

a, b, c = Constants derived from the regression analysis.

4.0 Conclusion

A quantitative method (BAGC 3533) has been developed and validated for the determination of PFBS in dose formulation solutions containing 1% CMC. Quantitation for this method is based on internal standard which produces standard curves with a correlation coefficients of 0.9992 or greater over a concentration range of 500 to 10,000 ng/mL.

5.0 Approvals

Gregory S. Gorman, Ph.D.
Staff Chemist
Bioanalytical Chemistry Group

7-5-01
Date

James D. Johnson, MS, MBA
Manager
Bioanalytical Chemistry Group

7/5/01
Date

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

1.0 PRINCIPLE

Dose formulation samples of perfluorobutane sulfonate (PFBS) in 1 % carboxymethyl cellulose (CMC) are diluted down to 3200 ng/mL and analyzed by HPLC/MS. Quantitation is based on an internal standard using back calculated values from a calibration curve encompassing a concentration range from 500 to 10,000 ng/mL and containing the same concentration of CMC as the dose formulations in the diluted form.

2.0 REAGENTS AND SOLUTIONS

The listed reagents or their equivalents may be used.

2.1 Neat Reagents

2.1.1 Water, deionized and organic free (from in-house purification system; e.g., Ingalls 210N)

2.1.2 Methanol, HPLC grade

2.1.3 Perfluorobutanesulfonate (analyte), as provided by the client

2.1.4 Perfluoropentanoic acid (internal standard), 97%

2.1.5 Ammonium acetate, HPLC grade

2.2 Prepared Solutions

Appropriate changes in the solutions may be made at the discretion of the analyst

2.2.1 5 mM Ammonium acetate in organic free water

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

2.2.1.1 For example, to prepare 4 liters, measure out ammonium acetate (e.g., 1.542 g) and add in organic-free water (e.g., 4 L). Mix well and filter through HPLC mobile phase filtration apparatus.

3.0 INSTRUMENTS, MATERIALS, AND APPARATUS

The following or their equivalents may be used.

3.1 HPLC pump(s), autosampler, and single quadrupole mass spectrometer

3.2 Autosampler vials with inserts

3.3 Vortex mixers (e.g., touch mixer and IKA-Vibrax ® platform mixer)

3.4 HPLC mobile phase filtration apparatus

3.5 Filters for HPLC mobile phase filtration apparatus (e.g., Nylon-66, 0.20 μm)

3.7 Analytical balance

3.8 Volumetric flasks (e.g., 10 and 25 mL)

3.9 Disposable Pasteur pipets

3.10 Micropipettor(s) with tips

3.11 Culture tubes with teflon-lined caps

3.12 Assorted glassware and syringes

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

4.0 PREPARATION OF STOCKS AND WORKING STOCKS

Appropriate changes in the concentrations of the solutions may be made at the discretion of the analyst. Actual dilutions will be documented on the preparation sheets.

4.1 Main Stock Solution of PFBS ~1000 µg/mL

4.1.1 Prepare an ~1000 µg/mL solution of PFBS in mobile phase (e.g., accurately weigh about 10 mg PFBS into a 10-mL volumetric flask). Add mobile phase to dissolve. Dilute to the mark. Alternatively, weigh the compound into an appropriate vessel (e.g., culture tube) and add 10 mL of mobile phase. Mix well. Transfer the solution to a clean vessel if desired.

4.2 Spiking Stock Solution of PFBS ~50 µg/mL

4.2.1 Prepare an ~50 µg/mL solution of PFBS in mobile phase (e.g., measure about 500 µL of ~1000 µg/mL PFBS into a 10-mL volumetric flask). Add mobile phase to dissolve. Dilute to the mark. Alternatively, weigh the compound into an appropriate vessel (e.g., culture tube) and add 10 mL of mobile phase. Mix well. Transfer the solution to a clean vessel if desired.

4.3 Stock Solution of Internal Standard (PFPA), ~200 µg/mL

4.3.1 Prepare an ~200 µg/mL solution of PFPA in deionized organic-free water (e.g., accurately weigh about 10 mg into a 50-mL volumetric flask). Add deionized organic-free water to dissolve and dilute to the mark with deionized organic-free water. Alternatively, weigh the compound into a an appropriate vessel (e.g., culture tube) and add 50 mL of deionized organic-free water. Mix well. Transfer the solution to a clean vessel if desired.

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

4.3 **Working Stock Solutions of PFBS**

4.3.1 To prepare working stock solutions, make the proper dilutions as shown in the following table. Prepare in 10-mL volumetric flasks or other appropriate glassware. If desired a modified dilution scheme can be used and documented in the study records.

Working Stock Level (WSL) Approximate Concentration (ng/mL)	Volume of Internal Standard (µL)	Volume of PFBS spiking solution (µL)	Volume of mobile phase (µL)
500	200	10	790
1000	200	20	780
3000	200	60	740
3200	200	64	736
5000	200	100	700
8000	200	160	640
10000	200	200	600

Note: The mobile phase used for each standard curve should contain the same concentration of CMC as the samples. For example, the dilution factor of a 4 mg/mL sample is 1000 (prior to addition of IS). The mobile phase for a 4 mg/mL sample prepared in 1% CMC should contain 1/1000% CMC to account for the dilution.

5.0 PREPARATION OF SPIKED STANDARDS AND BLANKS

Appropriate changes in the concentrations of the solutions may be made at the discretion of the analyst.

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

5.1 Multiple (e.g., about three) sets of standards blanks (blank + IS) are analyzed with each set of unknown samples. A double blank (blank-IS) may also be analyzed if desired. Standards may be prepared as shown in the table in 4.3.1.

6.0 PREPARATION OF SAMPLES

6.1 All samples are to be diluted to approximately the middle of the curve (e.g. 3200 ng/mL). Dilutions are to be made with mobile phase. For example, for a 4 mg/mL sample, take 1 mL and dilute with mobile phase in a 10 mL volumetric flask. Then take 100 µL of the diluted sample and dilute that with mobile phase in a 10 mL volumetric flask. Take 800 µL of the second dilution, place in an autosampler vial, add 200 µL of IS, mix, and analyze.

7.0 ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY/MASS SPECTROMETRY (HPLC/MS/MS)

7.1 Conditions are to be optimized if necessary.

7.1.1 HPLC Conditions

Analytical Column:	none
Guard Column:	Fluofix 120E 10 mm x 2 mm
Elution Flow rate:	1000 µL/min.
Injection volume:	5 µL
Mobile phase:	A: 5mM ammonium acetate buffer B: methanol
Elution Profile:	Isocratic 30% A : 70% B
Temperature:	Ambient

7.1.2 PE Sciex API 150EX Single Quadrupole Mass Spectrometer Conditions

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

Software: PE Sciex TurboQuan

Turboion Spray Source
Note: Values listed under "MS/MS Acquisition Conditions" override parameters in this table.

Auxiliary Gas: Air (e.g., Grade 0.1) at 85 pounds per square inch

Parameter	Value
IS	-5000
NC	0
TEM	450
OR	-25
RNG	-170
Q0	10
IQ1	11
ST	16
RO1	11
DF	300
CEM	2400
NEB	15
CUR	8
CAD	0
QPE	0
POL	0
VCM	0
IPE	0

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

Masses requested:

PFBS:

Q1 Mass (amu)	Dwell Time (ms)
299.2	200

PFPA (IS)

Q1 Mass (amu)	Dwell Time (ms)
218.9	200

8.0 CALCULATIONS

8.1 At the end of the analytical run, review each chromatogram to ensure the retention time, peak shape, and peak height and peak area determination of the test article and the IS are acceptable. The data may be smoothed as appropriate. For quantitation, use the ion profiles at the following mass-to-charge ratios:

Analyte	Ion Profile
PFBS	299.2
PFPA	218.9

8.2 Plot the peak area response of PFBS divided by the peak area response of the IS (PFPA) from all standards versus the concentration of the test article in the standards. Alternatively, the peak heights may be used instead of peak areas. Obtain the best curve fit of the data (e.g., quadratic fit weighted with 1/concentration of the test article or a quadratic fit). Note: The best curve fit may be dependent on the range of the standard curve and it may be necessary to have more than one standard curve for various concentration ranges using the following:

$$y = ax^2 + bx + c$$

where y = Peak height response of PFBS divided by peak height response of the IS (PFPA) in standards.

x = Concentration of the PFBS in standards.

ANALYTICAL METHOD

Method No.: BACG-3533

Title: Dose Formulation Analysis of Perfluorobutane Sulfonate in 1% Carboxymethyl Cellulose by HPLC/Mass Spectrometry (HPLC/MS)

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a, b, c = Constants derived from the regression analysis.

8.3 Using the standard curve, calculate the level of PFBS in each unknown sample. Correct the results of samples for any dilutions.

9.0 ACCEPTANCE AND REJECTION CRITERIA

9.1 Refer to SOP SRI 91-3 for acceptance/rejection criteria except acceptable accuracy for standards is 80-120% of theoretical.

10.0 REPORTING

10.1 Results of all analyses are tabulated, and the raw data, original chromatograms, and reports are to be filed in the appropriate study file.

Authors: Gregory A Gorman 7-17-2000

Gregory S.Gorman, Ph.D., Research Chemist III Date

Approved by: James D Johnson 7-17-2000

James D. Johnson MS, MBA, Manager Date
Bioanalytical Chemistry Group