Arnold & Porter

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October 5, 2018

VIA E-MAIL

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NC Department of Environmental Quality
217 West Jones Street
Raleigh, NC 27603
Bill.Lane@ncdenr.gov

Francisco Benzoni, Esq.
Assistant Attorney General
Environmental Division
NC Department of Justice
114 West Edenton Street
Raleigh, NC 27603
fbenzoni@ncdoj.gov

Re: Chemours - Health and Toxicity Studies: Ames Tests

Dear Mr. Lane and Mr. Benzoni:

On behalf of Chemours, this letter and the accompanying documents are submitted in further response to DEQ's requests seeking certain information related to Chemours' Fayetteville Works facility, which Chemours has been providing on a rolling basis since July 2017.

Please find enclosed at Attachment 1 (Bates labeled CH-FW-DEQ-0058854 to CH-FW-DEQ-0059339) additional documents responsive to DEQ's request for information provided to the U.S. Environmental Protection Agency regarding either HFPO Dimer Acid (CAS No. 13252-13-6) or its ammonium salt (CAS No. 62037-80-3). This attachment contains the final reports from bacterial reverse mutation assays, commonly referred to as Ames assays, which Chemours recently conducted for 10 Table 3 PFAS compounds following OECD Guideline Number 471. The purpose of this assay

is to determine whether a test substance can cause mutations resulting from damage to the bacterial cell's DNAs. In mammals, mutations in DNA could potentially result in cancer, so this assay is useful as a predictor of carcinogenic potential of a substance. The results of these assays on the Table 3 compounds tested indicate that these 10 test substances were negative for the ability to induce reverse mutations (*i.e.*, they are not mutagenic) at selected loci of several strains of Salmonella typhimurium and at the tryptophan locus of Escherichia coli strain WP2 uvrA in the presence and absence of an exogenous metabolic activation system.[1]

These documents supplement those Chemours has already produced in response to this request from DEQ. *See* Chemours's August 25, 2017 Production to DEQ (Bates labeled CH-FW-DEQ-0004100 to CH-FW-DEQ-0057599); Chemours's November 29, 2017 Production to DEQ (Bates labeled CH-FW-DEQ-0057900 to CH-FW-DEQ-0057965); Chemours's February 6, 2018 Production to DEQ (Bates labeled CH-FW-DEQ-0058402 to CH-FW-DEQ-0058853).

If you have any questions about this submission, please let me know.

Sincerely,

Brian D. Israel

Enclosure

cc: Linda Culpepper,
DEQ

John Savarese
Wachtell, Lipton, Rosen & Katz

Ralph Levene
Wachtell, Lipton, Rosen & Katz

[1] We understand the study reports note the chemical names and CAS numbers of the neutral acid form of the test substance. These generally correspond to the names and CAS numbers appearing in Table 3. Also enclosed is a summary of the study results that was shared with the US Environmental Protection Agency as a "FYI" submission. In the FYI submission, chemical names and CAS numbers listed for certain substances may not correspond directly to those identified in the study reports. This occurs in a limited number of cases when the sodium salt was identified in the FYI report as the test substance. Both the neutral acid and the salt readily disassociate into the same anion when dissolved in water, thus making the salt and the neutral acid toxicologically equivalent in this assay.

Joel Gross
Arnold & Porter Kaye Scholer

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

Sodium salt of Hydrolyzed N=0 TAF

Author

Emily Dakoulas, BS

Study Completion Date

29 August 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF28PF.503.BTL

Sponsor

The Chemours Company
1007 Market Street D-3008
Wilmington, DE 19899

Sponsor Number

C30049

1. STATEMENT OF COMPLIANCE

Study No. AF28PF.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

29 Nov 2018
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF28PF.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
13-Jun-2018	13-Jun-2018	Protocol Review	13-Jun-2018	13-Jun-2018
13-Jun-2018	13-Jun-2018	Scoring	13-Jun-2018	13-Jun-2018
02-Jul-2018	02-Jul-2018	Data/Draft Report	02-Jul-2018	02-Jul-2018
23-Aug-2018	23-Aug-2018	Final Report	23-Aug-2018	23-Aug-2018
23-Aug-2018	23-Aug-2018	Protocol Amendment Review	23-Aug-2018	23-Aug-2018
28-Aug-2018	28-Aug-2018	Protocol Amendment Review	28-Aug-2018	28-Aug-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Carlos Bonilla 28-Aug-2018 6:48 pm GMT

Reason for signature: QA Approval

Printed by:Carlos Bonilla

Printed on:28-Aug-18

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4. STUDY INFORMATION

Study Conduct

Sponsor:	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor's Authorized Representative:	Shawn Gannon, Ph.D., DABT
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF28PF.503.BTL
Sponsor No.:	C30049

Test Substance

Identification:	Sodium salt of Hydrolyzed N=0 TAF
CAS No.:	674-13-5
Purity:	99.9% (per protocol)
Molecular Weight:	202.02 g/mol
Description:	White powder
Storage Conditions:	Room temperature, protected from light
Receipt Date:	02 May 2018

Study Dates

Study Initiation Date:	23 May 2018
Experimental Starting Date (first day of data collection):	23 May 2018
Experimental Start Date (first day test substance administered to test system):	25 May 2018
Experimental Completion Date:	13 June 2018

Key Personnel

Study Director:	Emily Dakoulas, BS

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, Sodium salt of Hydrolyzed N=0 TAF, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Water was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Based upon these results, the maximum dose tested in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, the dose levels tested were 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate Sodium salt of Hydrolyzed N=0 TAF was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendments are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization using the information below.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

The vehicle used to deliver Sodium salt of Hydrolyzed N=0 TAF to the test system was water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Water	7732-18-5	Sigma-Aldrich	RNBF9658	Sterile-filtered	Mar 2019

To achieve a solution, the most concentrated dilution was sonicated at 31.3ºC for 7 minutes in the mutagenicity assay. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Water was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in water at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3925, Exp. Date: 21 Feb 2020) was purchased commercially from MolTox (Boone, NC).

Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and five dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	1.2	1.0	1.4	1.4	2.8
B2	1.4	1.0	1.3	1.5	3.4

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in water are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

Neither precipitate nor toxicity was observed.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 50.0, 150, 500, 1500 and 5000 µg per plate.

Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

A copy of the Common Technical Document Tables is included in Appendix III.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Sodium salt of Hydrolyzed N=0 TAF did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PF.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PF
Date Plated: 5/25/2018
Evaluation Period: 5/29/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*11*	*1*	*0.8*	10[A], 11[A]
		1500 µg	*17*	*1*	*1.3*	18[A], 16[A]
		500 µg	*13*	*2*	*1.0*	11[A], 14[A]
		150 µg	*15*	*3*	*1.2*	17[A], 13[A]
		50.0 µg	*15*	*0*	*1.2*	15[A], 15[A]
		15.0 µg	*16*	*2*	*1.2*	17[A], 14[A]
		5.00 µg	*12*	*1*	*0.9*	11[A], 13[A]
		1.50 µg	*16*	*2*	*1.2*	17[A], 14[A]
	Water	100 µL	*13*	*3*		15[A], 11[A]
TA100	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*97*	*11*	*1.1*	89[A], 104[A]
		1500 µg	*100*	*4*	*1.1*	103[A], 97[A]
		500 µg	*86*	*13*	*0.9*	95[A], 76[A]
		150 µg	*89*	*13*	*1.0*	98[A], 79[A]
		50.0 µg	*96*	*14*	*1.1*	86[A], 106[A]
		15.0 µg	*102*	*1*	*1.1*	101[A], 103[A]
		5.00 µg	*80*	*23*	*0.9*	64[A], 96[A]
		1.50 µg	*100*	*1*	*1.1*	99[A], 101[A]
	Water	100 µL	*91*	*2*		92[A], 89[A]
TA1535	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*12*	*4*	*1.2*	9[A], 15[A]
		1500 µg	*9*	*2*	*0.9*	7[A], 10[A]
		500 µg	*10*	*1*	*1.0*	11[A], 9[A]
		150 µg	*11*	*4*	*1.1*	13[A], 8[A]
		50.0 µg	*13*	*0*	*1.3*	13[A], 13[A]
		15.0 µg	*9*	*0*	*0.9*	9[A], 9[A]
		5.00 µg	*11*	*4*	*1.1*	14[A], 8[A]
		1.50 µg	*9*	*1*	*0.9*	9[A], 8[A]
	Water	100 µL	*10*	*1*		10[A], 9[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PF.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PF
Date Plated: 5/25/2018
Evaluation Period: 5/29/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*9*	*3*	*1.1*	11[A], 7[A]
		1500 µg	*3*	*0*	*0.4*	3[A], 3[A]
		500 µg	*5*	*2*	*0.6*	3[A], 6[A]
		150 µg	*8*	*4*	*1.0*	5[A], 11[A]
		50.0 µg	*10*	*1*	*1.3*	10[A], 9[A]
		15.0 µg	*9*	*1*	*1.1*	10[A], 8[A]
		5.00 µg	*9*	*3*	*1.1*	7[A], 11[A]
		1.50 µg	*7*	*1*	*0.9*	6[A], 8[A]
	Water	100 µL	*8*	*3*		6[A], 10[A]
WP2uvrA	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*28*	*6*	*1.0*	23[A], 32[A]
		1500 µg	*31*	*0*	*1.1*	31[A], 31[A]
		500 µg	*32*	*4*	*1.1*	35[A], 29[A]
		150 µg	*33*	*0*	*1.2*	33[A], 33[A]
		50.0 µg	*34*	*11*	*1.2*	41[A], 26[A]
		15.0 µg	*38*	*4*	*1.4*	40[A], 35[A]
		5.00 µg	*28*	*5*	*1.0*	24[A], 31[A]
		1.50 µg	*24*	*1*	*0.9*	23[A], 24[A]
	Water	100 µL	*28*	*7*		23[A], 33[A]
TA98	**2NF**	1.00 µg	*55*	*0*	*4.2*	55[A], 55[A]
TA100	**SA**	1.00 µg	*582*	*6*	*6.4*	586[A], 577[A]
TA1535	**SA**	1.00 µg	*600*	*16*	*60.0*	588[A], 611[A]
TA1537	**9AAD**	75.0 µg	*505*	*28*	*63.1*	485[A], 524[A]
WP2uvrA	**MMS**	1000 µg	*414*	*14*	*14.8*	404[A], 424[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PF.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PF
Date Plated: 5/25/2018
Evaluation Period: 5/29/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*23*	*2*	*1.2*	21[A], 24[A]
		1500 µg	*22*	*6*	*1.1*	17[A], 26[A]
		500 µg	*21*	*8*	*1.1*	15[A], 27[A]
		150 µg	*19*	*4*	*1.0*	22[A], 16[A]
		50.0 µg	*22*	*6*	*1.1*	26[A], 17[A]
		15.0 µg	*26*	*9*	*1.3*	19[A], 32[A]
		5.00 µg	*18*	*2*	*0.9*	16[A], 19[A]
		1.50 µg	*22*	*4*	*1.1*	19[A], 25[A]
	Water	100 µL	*20*	*4*		23[A], 17[A]
TA100	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*108*	*2*	*1.1*	106[A], 109[A]
		1500 µg	*117*	*1*	*1.2*	116[A], 117[A]
		500 µg	*104*	*6*	*1.1*	108[A], 99[A]
		150 µg	*108*	*11*	*1.1*	115[A], 100[A]
		50.0 µg	*108*	*18*	*1.1*	121[A], 95[A]
		15.0 µg	*90*	*15*	*0.9*	100[A], 79[A]
		5.00 µg	*101*	*0*	*1.0*	101[A], 101[A]
		1.50 µg	*98*	*3*	*1.0*	96[A], 100[A]
	Water	100 µL	*99*	*10*		92[A], 106[A]
TA1535	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*10*	*2*	*0.8*	11[A], 8[A]
		1500 µg	*13*	*3*	*1.0*	15[A], 11[A]
		500 µg	*14*	*8*	*1.1*	8[A], 19[A]
		150 µg	*16*	*0*	*1.2*	16[A], 16[A]
		50.0 µg	*13*	*3*	*1.0*	11[A], 15[A]
		15.0 µg	*19*	*0*	*1.5*	19[A], 19[A]
		5.00 µg	*11*	*6*	*0.8*	7[A], 15[A]
		1.50 µg	*15*	*2*	*1.2*	16[A], 13[A]
	Water	100 µL	*13*	*7*		8[A], 18[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PF.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PF
Date Plated: 5/25/2018
Evaluation Period: 5/29/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*8*	*4*	*0.9*	5[A], 10[A]
		1500 µg	*7*	*0*	*0.8*	7[A], 7[A]
		500 µg	*12*	*2*	*1.3*	10[A], 13[A]
		150 µg	*6*	*5*	*0.7*	2[A], 9[A]
		50.0 µg	*10*	*4*	*1.1*	13[A], 7[A]
		15.0 µg	*11*	*5*	*1.2*	14[A], 7[A]
		5.00 µg	*7*	*0*	*0.8*	7[A], 7[A]
		1.50 µg	*10*	*1*	*1.1*	11[A], 9[A]
	Water	100 µL	*9*	*4*		6[A], 11[A]
WP2uvrA	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*34*	*3*	*1.2*	36[A], 32[A]
		1500 µg	*34*	*3*	*1.2*	36[A], 32[A]
		500 µg	*35*	*6*	*1.2*	39[A], 31[A]
		150 µg	*37*	*2*	*1.3*	35[A], 38[A]
		50.0 µg	*36*	*5*	*1.2*	39[A], 32[A]
		15.0 µg	*32*	*4*	*1.1*	35[A], 29[A]
		5.00 µg	*42*	*1*	*1.4*	41[A], 43[A]
		1.50 µg	*31*	*11*	*1.1*	38[A], 23[A]
	Water	100 µL	*29*	*4*		32[A], 26[A]
TA98	**2AA**	1.00 µg	*215*	*11*	*10.8*	207[A], 223[A]
TA100	**2AA**	2.00 µg	*536*	*33*	*5.4*	559[A], 513[A]
TA1535	**2AA**	1.00 µg	*83*	*6*	*6.4*	79[A], 87[A]
TA1537	**2AA**	2.00 µg	*56*	*21*	*6.2*	41[A], 70[A]
WP2uvrA	**2AA**	15.0 µg	*314*	*25*	*10.8*	332[A], 296[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PF.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PF
Date Plated: 6/6/2018
Evaluation Period: 6/13/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*14*	*3*	*0.9*	14^{A}, 17^{A}, 11^{A}
		1500 µg	*17*	*4*	*1.1*	21^{A}, 16^{A}, 14^{A}
		500 µg	*15*	*0*	*1.0*	15^{A}, 15^{A}, 15^{A}
		150 µg	*14*	*4*	*0.9*	11^{A}, 13^{A}, 19^{A}
		50.0 µg	*13*	*7*	*0.9*	19^{A}, 13^{A}, 6^{A}
	Water	100 µL	*15*	*5*		19^{A}, 17^{A}, 10^{A}
TA100	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*91*	*10*	*0.9*	84^{A}, 103^{A}, 86^{A}
		1500 µg	*87*	*14*	*0.9*	78^{A}, 81^{A}, 103^{A}
		500 µg	*99*	*2*	*1.0*	96^{A}, 100^{A}, 100^{A}
		150 µg	*87*	*10*	*0.9*	89^{A}, 96^{A}, 76^{A}
		50.0 µg	*89*	*4*	*0.9*	86^{A}, 93^{A}, 88^{A}
	Water	100 µL	*97*	*11*		109^{A}, 87^{A}, 95^{A}
TA1535	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*12*	*4*	*0.9*	10^{A}, 10^{A}, 17^{A}
		1500 µg	*13*	*6*	*1.0*	19^{A}, 13^{A}, 8^{A}
		500 µg	*13*	*2*	*1.0*	11^{A}, 13^{A}, 14^{A}
		150 µg	*9*	*1*	*0.7*	9^{A}, 8^{A}, 10^{A}
		50.0 µg	*13*	*4*	*1.0*	15^{A}, 15^{A}, 8^{A}
	Water	100 µL	*13*	*2*		13^{A}, 15^{A}, 11^{A}
TA1537	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*7*	*2*	*1.0*	6^{A}, 6^{A}, 10^{A}
		1500 µg	*8*	*2*	*1.1*	7^{A}, 7^{A}, 11^{A}
		500 µg	*7*	*2*	*1.0*	7^{A}, 9^{A}, 6^{A}
		150 µg	*8*	*6*	*1.1*	2^{A}, 14^{A}, 9^{A}
		50.0 µg	*7*	*1*	*1.0*	8^{A}, 7^{A}, 7^{A}
	Water	100 µL	*7*	*2*		6^{A}, 6^{A}, 10^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PF.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PF
Date Plated: 6/6/2018
Evaluation Period: 6/13/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*29*	*4*	*1.0*	26[A], 27[A], 33[A]
		1500 µg	*30*	*1*	*1.0*	29[A], 29[A], 31[A]
		500 µg	*33*	*10*	*1.1*	25[A], 44[A], 29[A]
		150 µg	*29*	*4*	*1.0*	26[A], 33[A], 27[A]
		50.0 µg	*26*	*9*	*0.9*	23[A], 36[A], 19[A]
	Water	100 µL	*29*	*3*		26[A], 30[A], 32[A]
TA98	**2NF**	1.00 µg	*64*	*4*	*4.3*	65[A], 67[A], 59[A]
TA100	**SA**	1.00 µg	*754*	*44*	*7.8*	804[A], 735[A], 723[A]
TA1535	**SA**	1.00 µg	*697*	*19*	*53.6*	698[A], 677[A], 715[A]
TA1537	**9AAD**	75.0 µg	*612*	*130*	*87.4*	536[A], 539[A], 762[A]
WP2uvrA	**MMS**	1000 µg	*482*	*11*	*16.6*	494[A], 473[A], 478[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PF.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PF
Date Plated: 6/6/2018
Evaluation Period: 6/13/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*20*	*3*	*1.0*	23^{A}, 21^{A}, 17^{A}
		1500 µg	*23*	*1*	*1.2*	22^{A}, 23^{A}, 24^{A}
		500 µg	*18*	*4*	*0.9*	19^{A}, 14^{A}, 21^{A}
		150 µg	*26*	*5*	*1.3*	24^{A}, 32^{A}, 22^{A}
		50.0 µg	*22*	*2*	*1.1*	19^{A}, 23^{A}, 23^{A}
	Water	100 µL	*20*	*6*		23^{A}, 24^{A}, 14^{A}
TA100	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*114*	*5*	*1.1*	109^{A}, 119^{A}, 115^{A}
		1500 µg	*114*	*8*	*1.1*	119^{A}, 117^{A}, 105^{A}
		500 µg	*118*	*4*	*1.1*	114^{A}, 121^{A}, 120^{A}
		150 µg	*124*	*12*	*1.2*	133^{A}, 111^{A}, 128^{A}
		50.0 µg	*99*	*5*	*0.9*	99^{A}, 95^{A}, 104^{A}
	Water	100 µL	*106*	*5*		101^{A}, 109^{A}, 109^{A}
TA1535	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*12*	*3*	*0.8*	11^{A}, 16^{A}, 10^{A}
		1500 µg	*15*	*1*	*0.9*	15^{A}, 16^{A}, 14^{A}
		500 µg	*14*	*7*	*0.9*	10^{A}, 9^{A}, 22^{A}
		150 µg	*16*	*4*	*1.0*	17^{A}, 11^{A}, 19^{A}
		50.0 µg	*11*	*4*	*0.7*	8^{A}, 10^{A}, 15^{A}
	Water	100 µL	*16*	*1*		15^{A}, 16^{A}, 17^{A}
TA1537	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*8*	*6*	*1.0*	3^{A}, 15^{A}, 5^{A}
		1500 µg	*7*	*3*	*0.9*	9^{A}, 8^{A}, 3^{A}
		500 µg	*11*	*6*	*1.4*	7^{A}, 7^{A}, 18^{A}
		150 µg	*10*	*2*	*1.3*	8^{A}, 11^{A}, 10^{A}
		50.0 µg	*8*	*2*	*1.0*	10^{A}, 7^{A}, 8^{A}
	Water	100 µL	*8*	*0*		8^{A}, 8^{A}, 8^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 4 (CONT.)
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PF.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PF
Date Plated: 6/6/2018
Evaluation Period: 6/13/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Sodium salt of Hydrolyzed N=0 TAF**	5000 µg	*38*	*5*	*1.1*	33^{A}, 43^{A}, 38^{A}
		1500 µg	*37*	*5*	*1.0*	32^{A}, 39^{A}, 41^{A}
		500 µg	*40*	*2*	*1.1*	41^{A}, 40^{A}, 38^{A}
		150 µg	*43*	*3*	*1.2*	46^{A}, 42^{A}, 41^{A}
		50.0 µg	*29*	*6*	*0.8*	27^{A}, 24^{A}, 36^{A}
	Water	100 µL	*36*	*3*		35^{A}, 40^{A}, 34^{A}
TA98	**2AA**	1.00 µg	*207*	*10*	*10.4*	198^{A}, 207^{A}, 217^{A}
TA100	**2AA**	2.00 µg	*768*	*47*	*7.2*	719^{A}, 813^{A}, 771^{A}
TA1535	**2AA**	1.00 µg	*67*	*10*	*4.2*	59^{A}, 64^{A}, 78^{A}
TA1537	**2AA**	2.00 µg	*42*	*20*	*5.3*	35^{A}, 65^{A}, 27^{A}
WP2uvrA	**2AA**	15.0 µg	*157*	*49*	*4.4*	162^{A}, 105^{A}, 203^{A}

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values
2016
revertants per plate

Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	

SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control

14. APPENDIX II: Study Protocol and Amendments

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PF.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 1500, 500, 150 and 50.0 µg per plate.

   Reason: To specify the dose levels to be used for the confirmatory mutagenicity assay based on the toxicity and precipitate profiles observed in the initial toxicity-mutation assay.

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PF.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, Ph.D., DABT
Sponsor Representative

14-Aug-2018
Date

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PF.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS
BioReliance Study Director

05 JUN 2018
Date

BioReliance Study Management

05-JUN-2018
Date

PROTOCOL AMENDMENT 2

Sponsor: The Chemours Company

BioReliance Study No.: AF28PF.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 3, Section 6, Test Substance Information - CAS Number

   Effective: Date of Study Director signature on this amendment

   Original:

   CAS Number: 674-13-4

   Replace with:

   CAS Number 674-13-5

   Reason: To correct CAS Number of the test substance

PROTOCOL AMENDMENT 2

Sponsor: The Chemours Company

BioReliance Study No.: AF28PF.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, Ph.D., DABT
Sponsor Representative

28-Aug-2018
Date

PROTOCOL AMENDMENT 2

Sponsor: The Chemours Company

BioReliance Study No.: AF28PF.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS — Date
BioReliance Study Director

BioReliance Study Management — Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF28PF.503.BTL

1. KEY PERSONNEL

Sponsor Information:

Sponsor	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor Number	C30049
Sponsor's Authorized Representative	Shawn Gannon, Ph.D., DABT The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899 Phone: 302-773-1376 Email: SHAWN.A.GANNON@chemours.com

Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	25-May-2018
Proposed Experimental Completion Date	21-June-2018
Proposed Report Date	06-July-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST SUBSTANCE INFORMATION

Identification	Sodium salt of Hydrolyzed N=0 TAF
CAS No.	674-13-4
Storage Conditions	Room Temperature Protect from light (Per BioReliance SOP)
Purity	99.9% (no correction factor will be used for dose formulations)
Molecular Weight	202.02 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused Test Substance will be returned to the sponsor prior to report finalization using the information below; unless the test substance is used on another study.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

Residual dose formulations will be discarded after use.

7. **TEST SYSTEM**
The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitoneal with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values
Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of

revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains

- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

Shawn Gannon, Ph.D., DABT
Sponsor Representative

8 May 2018
Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 23 MAY 2018

BioReliance Study Management — Date: 22-MAY-18

15. APPENDIX III: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test for Induction of: Reverse mutation in bacterial cells

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

Metabolizing System: Aroclor-induced rat liver S9

Vehicle for Test Substance: Water

Treatment: Plate incorporation

Cytotoxic Effects: None

Genotoxic Effects: None

No. of Independent Assays: 2

No. of Replicate Cultures: 2 (B1) and 3 (B2)

Test Substance: Sodium salt of Hydrolyzed N=0 TAF

Study No.: AF28PF.503.BTL

No. Cells Analyzed/Culture: 1.0 to 3.4 x 10^8 cells per plate

GLP Compliance: Yes

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Date(s) of Treatment: 25 May 2018 (B1) and 06 June 2018 (B2)

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B1: Initial toxicity-mutation assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	13 ± 3	91 ± 2	10 ± 1	8 ± 3	28 ± 7
	Sodium salt of Hydrolyzed N=0 TAF	1.50	16 ± 2	100 ± 1	9 ± 1	7 ± 1	24 ± 1
		5.00	12 ± 1	80 ± 23	11 ± 4	9 ± 3	28 ± 5
		15.0	16 ± 2	102 ± 1	9 ± 0	9 ± 1	38 ± 4
		50.0	15 ± 0	96 ± 14	13 ± 0	10 ± 1	34 ± 11
		150	15 ± 3	89 ± 13	11 ± 4	8 ± 4	33 ± 0
		500	13 ± 2	86 ± 13	10 ± 1	5 ± 2	32 ± 4
		1500	17 ± 1	100 ± 4	9 ± 2	3 ± 0	31 ± 0
		5000	11 ± 1	97 ± 11	12 ± 4	9 ± 3	28 ± 6
	2NF	1.00	55 ± 0				
	SA	1.00		582 ± 6	600 ± 16		
	9AAD	75.0				505 ± 28	
	MMS	1000					414 ± 14
With Activation	Water	100 µL/plate	20 ± 4	99 ± 10	13 ± 7	9 ± 4	29 ± 4
	Sodium salt of Hydrolyzed N=0 TAF	1.50	22 ± 4	98 ± 3	15 ± 2	10 ± 1	31 ± 11
		5.00	18 ± 2	101 ± 0	11 ± 6	7 ± 0	42 ± 1
		15.0	26 ± 9	90 ± 15	19 ± 0	11 ± 5	32 ± 4
		50.0	22 ± 6	108 ± 18	13 ± 3	10 ± 4	36 ± 5
		150	19 ± 4	108 ± 11	16 ± 0	6 ± 5	37 ± 2
		500	21 ± 8	104 ± 6	14 ± 8	12 ± 2	35 ± 6
		1500	22 ± 6	117 ± 1	13 ± 3	7 ± 0	34 ± 3
		5000	23 ± 2	108 ± 2	10 ± 2	8 ± 4	34 ± 3
	2AA	1.00	215 ± 11		83 ± 6		
	2AA	2.00		536 ± 33		56 ± 21	
	2AA	15.0					314 ± 25

Key to Positive Controls

SA	sodium azide	2NF	2-nitrofluorene
2AA	2-aminoanthracene	MMS	methyl methanesulfonate
9AAD	9-Aminoacridine		

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B2: Confirmatory Mutagenicity Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	15 ± 5	97 ± 11	13 ± 2	7 ± 2	29 ± 3
	Sodium salt of Hydrolyzed N=0 TAF	50.0	13 ± 7	89 ± 4	13 ± 4	7 ± 1	26 ± 9
		150	14 ± 4	87 ± 10	9 ± 1	8 ± 6	29 ± 4
		500	15 ± 0	99 ± 2	13 ± 2	7 ± 2	33 ± 10
		1500	17 ± 4	87 ± 14	13 ± 6	8 ± 2	30 ± 1
		5000	14 ± 3	91 ± 10	12 ± 4	7 ± 2	29 ± 4
	2NF	1.00	64 ± 4				
	SA	1.00		754 ± 44	697 ± 19		
	9AAD	75.0				612 ± 130	
	MMS	1000					482 ± 11
With Activation	Water	100 µL/plate	20 ± 6	106 ± 5	16 ± 1	8 ± 0	36 ± 3
	Sodium salt of Hydrolyzed N=0 TAF	50.0	22 ± 2	99 ± 5	11 ± 4	8 ± 2	29 ± 6
		150	26 ± 5	124 ± 12	16 ± 4	10 ± 2	43 ± 3
		500	18 ± 4	118 ± 4	14 ± 7	11 ± 6	40 ± 2
		1500	23 ± 1	114 ± 8	15 ± 1	7 ± 3	37 ± 5
		5000	20 ± 3	114 ± 5	12 ± 3	8 ± 6	38 ± 5
	2AA	1.00	207 ± 10		67 ± 10		
	2AA	2.00		768 ± 47		42 ± 20	
	2AA	15.0					157 ± 49

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

PFECA F

Author

Emily Dakoulas, BS

Study Completion Date

22 August 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF28PG.503.BTL

Sponsor

The Chemours Company
1007 Market Street D-3008
Wilmington, DE 19899

Sponsor Number

C30049

1. STATEMENT OF COMPLIANCE

Study No. AF28PG.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF28PG.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
19-Jun-2018	19-Jun-2018	Strain Characterization	19-Jun-2018	19-Jun-2018
19-Jun-2018	25-Jun-2018	Protocol Review	25-Jun-2018	25-Jun-2018
12-Jul-2018	12-Jul-2018	Data/Draft Report	12-Jul-2018	12-Jul-2018
12-Jul-2018	12-Jul-2018	Protocol Amendment Review	12-Jul-2018	12-Jul-2018
20-Aug-2018	20-Aug-2018	Final Report	20-Aug-2018	20-Aug-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Jeannie Eberle 21-Aug-2018 5:40 pm GMT

Reason for signature: QA Approval

Printed by:Jeannie Eberle
Printed on:21-Aug-18

3. TABLE OF CONTENTS

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4. STUDY INFORMATION

Study Conduct

Sponsor:	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor's Authorized Representative:	Shawn Gannon, Ph.D., DABT
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF28PG.503.BTL
Sponsor No.:	C30049

Test Substance

Identification:	PFECA F
Lot No.:	AS473550
CAS No.:	377-73-1
Purity:	98.9% (per protocol)
Molecular Weight:	230.04 g/mol
Description:	Clear colorless liquid
Storage Conditions:	Room temperature, protected from light and under argon
Receipt Date:	29 May 2018

Study Dates

Study Initiation Date:	06 June 2018
Experimental Starting Date (first day of data collection):	07 June 2018
Experimental Start Date (first day test substance administered to test system):	08 June 2018
Experimental Completion Date:	21 June 2018

Key Personnel

Study Director:	Emily Dakoulas, BS

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, PFECA F, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Water was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. A non-dose responsive increase of 1.5-fold, maximum increase was observed with tester strain WP2 *uvr*A in the presence of S9 activation. This response is not considered mutagenic, since the increase was not dose responsive and was within the 99% historical control limit. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Based upon these results, the maximum dose tested in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, the dose levels tested were 33.3, 100, 333, 1000, 3333 and 5000 µg per plate. Neither precipitate nor toxicity was observed. The increase in WP2 *uvr*A in the presence of S9 activation did not replicate. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate PFECA F was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization using the information below.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

The vehicle used to deliver PFECA F to the test system was water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Water	7732-18-5	Sigma-Aldrich	RNBF9658	Sterile-filtered	Mar 2019
			RNBG4913		Dec 2019

Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Water was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in water at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3925, Exp. Date: 21 Feb 2020; Lot No. 3961, Exp. Date: 15 May 2020) was purchased

commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and six dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	1.5	1.0	1.4	1.5	3.5
B2	1.2	1.1	1.5	1.9	2.8

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in water are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

Neither precipitate nor toxicity was observed. A non-dose responsive increase of 1.5-fold, maximum increase was observed with tester strain WP2 *uvr*A in the presence of S9 activation. This response is not considered mutagenic, since the increase was not dose responsive and was within the 99% historical control limit.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 33.3, 100, 333, 1000, 3333 and 5000 µg per plate.

Neither precipitate nor toxicity was observed. The increase in WP2 *uvr*A in the presence of S9 activation did not replicate.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

A copy of the Common Technical Document Tables is included in Appendix III.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, PFECA F did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PG.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PG
Date Plated: 6/8/2018
Evaluation Period: 6/12/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA F**	5000 µg	*16*	*4*	*0.9*	13[A], 18[A]
		1500 µg	*13*	*6*	*0.8*	9[A], 17[A]
		500 µg	*12*	*2*	*0.7*	10[A], 13[A]
		150 µg	*16*	*2*	*0.9*	17[A], 14[A]
		50.0 µg	*16*	*1*	*0.9*	15[A], 16[A]
		15.0 µg	*19*	*4*	*1.1*	21[A], 16[A]
		5.00 µg	*12*	*6*	*0.7*	16[A], 8[A]
		1.50 µg	*18*	*5*	*1.1*	21[A], 14[A]
	Water	100 µL	*17*	*1*		16[A], 18[A]
TA100	**PFECA F**	5000 µg	*78*	*9*	*0.8*	71[A], 84[A]
		1500 µg	*86*	*18*	*0.9*	98[A], 73[A]
		500 µg	*98*	*8*	*1.1*	92[A], 103[A]
		150 µg	*96*	*6*	*1.0*	100[A], 92[A]
		50.0 µg	*95*	*8*	*1.0*	89[A], 101[A]
		15.0 µg	*101*	*6*	*1.1*	105[A], 96[A]
		5.00 µg	*85*	*2*	*0.9*	86[A], 83[A]
		1.50 µg	*88*	*28*	*0.9*	107[A], 68[A]
	Water	100 µL	*93*	*3*		91[A], 95[A]
TA1535	**PFECA F**	5000 µg	*10*	*0*	*0.8*	10[A], 10[A]
		1500 µg	*12*	*1*	*1.0*	11[A], 13[A]
		500 µg	*12*	*1*	*1.0*	13[A], 11[A]
		150 µg	*9*	*1*	*0.8*	8[A], 10[A]
		50.0 µg	*15*	*3*	*1.3*	17[A], 13[A]
		15.0 µg	*12*	*3*	*1.0*	14[A], 10[A]
		5.00 µg	*15*	*1*	*1.3*	14[A], 15[A]
		1.50 µg	*10*	*1*	*0.8*	9[A], 11[A]
	Water	100 µL	*12*	*1*		13[A], 11[A]
TA1537	**PFECA F**	5000 µg	*7*	*6*	*0.9*	3[A], 11[A]
		1500 µg	*10*	*1*	*1.3*	11[A], 9[A]
		500 µg	*7*	*1*	*0.9*	8[A], 6[A]
		150 µg	*11*	*0*	*1.4*	11[A], 11[A]
		50.0 µg	*8*	*2*	*1.0*	9[A], 6[A]
		15.0 µg	*5*	*3*	*0.6*	7[A], 3[A]
		5.00 µg	*9*	*1*	*1.1*	8[A], 9[A]
		1.50 µg	*7*	*2*	*0.9*	8[A], 5[A]
	Water	100 µL	*8*	*4*		5[A], 10[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PG.503.BTL
Study Code: AF28PG
Experiment: B1
Date Plated: 6/8/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/12/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**PFECA F**	5000 µg	*27*	*8*	*1.0*	32[A], 21[A]
		1500 µg	*26*	*7*	*1.0*	31[A], 21[A]
		500 µg	*22*	*6*	*0.8*	17[A], 26[A]
		150 µg	*34*	*1*	*1.3*	33[A], 34[A]
		50.0 µg	*27*	*3*	*1.0*	25[A], 29[A]
		15.0 µg	*34*	*1*	*1.3*	33[A], 35[A]
		5.00 µg	*23*	*9*	*0.9*	29[A], 16[A]
		1.50 µg	*22*	*10*	*0.8*	15[A], 29[A]
	Water	100 µL	*26*	*1*		26[A], 25[A]
TA98	**2NF**	1.00 µg	*87*	*16*	*5.1*	76[A], 98[A]
TA100	**SA**	1.00 µg	*639*	*49*	*6.9*	604[A], 674[A]
TA1535	**SA**	1.00 µg	*654*	*13*	*54.5*	645[A], 663[A]
TA1537	**9AAD**	75.0 µg	*656*	*14*	*82.0*	666[A], 646[A]
WP2uvrA	**MMS**	1000 µg	*431*	*27*	*16.6*	412[A], 450[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PG.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PG
Date Plated: 6/8/2018
Evaluation Period: 6/12/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA F**	5000 µg	*23*	*9*	*1.0*	29[A], 16[A]
		1500 µg	*21*	*6*	*1.0*	17[A], 25[A]
		500 µg	*22*	*1*	*1.0*	23[A], 21[A]
		150 µg	*19*	*6*	*0.9*	23[A], 15[A]
		50.0 µg	*18*	*0*	*0.8*	18[A], 18[A]
		15.0 µg	*16*	*8*	*0.7*	21[A], 10[A]
		5.00 µg	*12*	*4*	*0.5*	15[A], 9[A]
		1.50 µg	*17*	*3*	*0.8*	19[A], 15[A]
	Water	100 µL	*22*	*4*		24[A], 19[A]
TA100	**PFECA F**	5000 µg	*94*	*13*	*1.0*	84[A], 103[A]
		1500 µg	*92*	*19*	*1.0*	105[A], 78[A]
		500 µg	*93*	*4*	*1.0*	96[A], 90[A]
		150 µg	*97*	*1*	*1.1*	97[A], 96[A]
		50.0 µg	*129*	*1*	*1.4*	130[A], 128[A]
		15.0 µg	*105*	*11*	*1.1*	97[A], 113[A]
		5.00 µg	*79*	*1*	*0.9*	79[A], 78[A]
		1.50 µg	*91*	*2*	*1.0*	92[A], 89[A]
	Water	100 µL	*92*	*7*		87[A], 97[A]
TA1535	**PFECA F**	5000 µg	*11*	*5*	*0.9*	14[A], 7[A]
		1500 µg	*15*	*1*	*1.3*	14[A], 15[A]
		500 µg	*12*	*2*	*1.0*	10[A], 13[A]
		150 µg	*13*	*3*	*1.1*	11[A], 15[A]
		50.0 µg	*13*	*0*	*1.1*	13[A], 13[A]
		15.0 µg	*10*	*1*	*0.8*	9[A], 10[A]
		5.00 µg	*9*	*1*	*0.8*	8[A], 9[A]
		1.50 µg	*12*	*2*	*1.0*	13[A], 10[A]
	Water	100 µL	*12*	*1*		11[A], 13[A]
TA1537	**PFECA F**	5000 µg	*8*	*4*	*0.9*	5[A], 10[A]
		1500 µg	*8*	*2*	*0.9*	9[A], 6[A]
		500 µg	*8*	*4*	*0.9*	10[A], 5[A]
		150 µg	*9*	*6*	*1.0*	5[A], 13[A]
		50.0 µg	*8*	*1*	*0.9*	8[A], 7[A]
		15.0 µg	*11*	*1*	*1.2*	11[A], 10[A]
		5.00 µg	*8*	*2*	*0.9*	6[A], 9[A]
		1.50 µg	*9*	*6*	*1.0*	5[A], 13[A]
	Water	100 µL	*9*	*1*		9[A], 8[A]

Key to Automatic & Manual Count Flags

[M]: Manual count [A]: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PG.503.BTL
Study Code: AF28PG
Experiment: B1
Date Plated: 6/8/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/12/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**PFECA F**	5000 µg	*34*	*1*	*1.2*	33[A], 34[A]
		1500 µg	*42*	*8*	*1.5*	36[A], 47[A]
		500 µg	*32*	*1*	*1.1*	32[A], 31[A]
		150 µg	*31*	*1*	*1.1*	30[A], 32[A]
		50.0 µg	*33*	*11*	*1.2*	40[A], 25[A]
		15.0 µg	*35*	*4*	*1.3*	38[A], 32[A]
		5.00 µg	*31*	*3*	*1.1*	33[A], 29[A]
		1.50 µg	*32*	*6*	*1.1*	27[A], 36[A]
	Water	100 µL	*28*	*7*		33[A], 23[A]
TA98	**2AA**	1.00 µg	*268*	*12*	*12.2*	259[A], 276[A]
TA100	**2AA**	2.00 µg	*645*	*9*	*7.0*	638[A], 651[A]
TA1535	**2AA**	1.00 µg	*77*	*9*	*6.4*	83[A], 70[A]
TA1537	**2AA**	2.00 µg	*44*	*11*	*4.9*	52[A], 36[A]
WP2uvrA	**2AA**	15.0 µg	*364*	*24*	*13.0*	347[A], 381[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PG.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PG
Date Plated: 6/19/2018
Evaluation Period: 6/21/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA F**	5000 µg	*9*	*3*	*0.5*	7^{A}, 13^{A}, 7^{A}
		3333 µg	*14*	*2*	*0.8*	15^{A}, 11^{A}, 15^{A}
		1000 µg	*16*	*4*	*0.9*	11^{A}, 18^{A}, 18^{A}
		333 µg	*14*	*1*	*0.8*	13^{A}, 15^{A}, 15^{A}
		100 µg	*14*	*6*	*0.8*	7^{A}, 17^{A}, 18^{A}
		33.3 µg	*14*	*7*	*0.8*	21^{A}, 14^{A}, 8^{A}
	Water	100 µL	*17*	*1*		16^{A}, 17^{A}, 17^{A}
TA100	**PFECA F**	5000 µg	*81*	*10*	*0.9*	72^{A}, 79^{A}, 91^{A}
		3333 µg	*83*	*8*	*0.9*	89^{A}, 74^{A}, 86^{A}
		1000 µg	*90*	*24*	*1.0*	66^{A}, 91^{A}, 114^{A}
		333 µg	*85*	*6*	*1.0*	82^{A}, 81^{A}, 91^{A}
		100 µg	*78*	*13*	*0.9*	78^{A}, 65^{A}, 91^{A}
		33.3 µg	*81*	*14*	*0.9*	86^{A}, 92^{A}, 65^{A}
	Water	100 µL	*89*	*6*		95^{A}, 83^{A}, 88^{A}
TA1535	**PFECA F**	5000 µg	*9*	*4*	*0.9*	5^{A}, 13^{A}, 9^{A}
		3333 µg	*9*	*3*	*0.9*	9^{A}, 6^{A}, 11^{A}
		1000 µg	*8*	*2*	*0.8*	7^{A}, 10^{A}, 7^{A}
		333 µg	*8*	*3*	*0.8*	11^{A}, 8^{A}, 6^{A}
		100 µg	*8*	*1*	*0.8*	7^{A}, 7^{A}, 9^{A}
		33.3 µg	*8*	*2*	*0.8*	6^{A}, 10^{A}, 8^{A}
	Water	100 µL	*10*	*3*		10^{A}, 13^{A}, 7^{A}
TA1537	**PFECA F**	5000 µg	*6*	*3*	*1.0*	7^{A}, 9^{A}, 3^{A}
		3333 µg	*5*	*3*	*0.8*	7^{A}, 7^{A}, 1^{A}
		1000 µg	*5*	*3*	*0.8*	7^{A}, 7^{A}, 2^{A}
		333 µg	*6*	*5*	*1.0*	6^{A}, 11^{A}, 2^{A}
		100 µg	*7*	*2*	*1.2*	9^{A}, 5^{A}, 6^{A}
		33.3 µg	*7*	*3*	*1.2*	5^{A}, 10^{A}, 7^{A}
	Water	100 µL	*6*	*0*		6^{A}, 6^{A}, 6^{A}
WP2uvrA	**PFECA F**	5000 µg	*27*	*2*	*0.8*	25^{A}, 26^{A}, 29^{A}
		3333 µg	*37*	*3*	*1.1*	41^{A}, 35^{A}, 35^{A}
		1000 µg	*31*	*7*	*0.9*	38^{A}, 25^{A}, 30^{A}
		333 µg	*32*	*7*	*0.9*	39^{A}, 26^{A}, 32^{A}
		100 µg	*34*	*7*	*1.0*	30^{A}, 42^{A}, 30^{A}
		33.3 µg	*39*	*7*	*1.1*	46^{A}, 38^{A}, 33^{A}
	Water	100 µL	*34*	*1*		35^{A}, 34^{A}, 33^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PG.503.BTL
Study Code: AF28PG
Experiment: B2
Date Plated: 6/19/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/21/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**2NF**	1.00 µg	*68*	*19*	*4.0*	46[A], 76[A], 81[A]
TA100	**SA**	1.00 µg	*607*	*15*	*6.8*	593[A], 622[A], 606[A]
TA1535	**SA**	1.00 µg	*611*	*19*	*61.1*	593[A], 610[A], 630[A]
TA1537	**9AAD**	75.0 µg	*771*	*71*	*128.5*	809[A], 815[A], 690[A]
WP2uvrA	**MMS**	1000 µg	*463*	*4*	*13.6*	458[A], 466[A], 465[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PG.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PG
Date Plated: 6/19/2018
Evaluation Period: 6/21/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA F**	5000 µg	*14*	*3*	*1.0*	15^{A}, 11^{A}, 16^{A}
		3333 µg	*13*	*6*	*0.9*	14^{A}, 7^{A}, 19^{A}
		1000 µg	*15*	*3*	*1.1*	13^{A}, 18^{A}, 15^{A}
		333 µg	*16*	*4*	*1.1*	21^{A}, 13^{A}, 14^{A}
		100 µg	*16*	*5*	*1.1*	21^{A}, 16^{A}, 11^{A}
		33.3 µg	*15*	*5*	*1.1*	9^{A}, 19^{A}, 16^{A}
	Water	100 µL	*14*	*1*		14^{A}, 14^{A}, 13^{A}
TA100	**PFECA F**	5000 µg	*107*	*7*	*1.1*	100^{A}, 114^{A}, 108^{A}
		3333 µg	*97*	*12*	*1.0*	83^{A}, 101^{A}, 107^{A}
		1000 µg	*104*	*9*	*1.1*	106^{A}, 112^{A}, 95^{A}
		333 µg	*107*	*18*	*1.1*	97^{A}, 97^{A}, 128^{A}
		100 µg	*104*	*16*	*1.1*	101^{A}, 121^{A}, 90^{A}
		33.3 µg	*93*	*8*	*0.9*	93^{A}, 101^{A}, 86^{A}
	Water	100 µL	*99*	*21*		95^{A}, 81^{A}, 122^{A}
TA1535	**PFECA F**	5000 µg	*11*	*2*	*1.0*	10^{A}, 13^{A}, 9^{A}
		3333 µg	*11*	*4*	*1.0*	8^{A}, 15^{A}, 9^{A}
		1000 µg	*15*	*3*	*1.4*	13^{A}, 18^{A}, 14^{A}
		333 µg	*11*	*4*	*1.0*	8^{A}, 15^{A}, 10^{A}
		100 µg	*12*	*6*	*1.1*	6^{A}, 15^{A}, 16^{A}
		33.3 µg	*8*	*2*	*0.7*	8^{A}, 6^{A}, 10^{A}
	Water	100 µL	*11*	*3*		13^{A}, 11^{A}, 8^{A}
TA1537	**PFECA F**	5000 µg	*6*	*3*	*1.2*	5^{A}, 9^{A}, 3^{A}
		3333 µg	*7*	*3*	*1.4*	5^{A}, 10^{A}, 7^{A}
		1000 µg	*7*	*4*	*1.4*	3^{A}, 10^{A}, 7^{A}
		333 µg	*7*	*1*	*1.4*	6^{A}, 8^{A}, 6^{A}
		100 µg	*7*	*1*	*1.4*	7^{A}, 8^{A}, 6^{A}
		33.3 µg	*5*	*1*	*1.0*	6^{A}, 5^{A}, 5^{A}
	Water	100 µL	*5*	*0*		5^{A}, 5^{A}, 5^{A}
WP2uvrA	**PFECA F**	5000 µg	*31*	*4*	*1.0*	36^{A}, 29^{A}, 29^{A}
		3333 µg	*34*	*7*	*1.1*	36^{A}, 26^{A}, 39^{A}
		1000 µg	*31*	*4*	*1.0*	34^{A}, 33^{A}, 26^{A}
		333 µg	*35*	*1*	*1.1*	36^{A}, 34^{A}, 34^{A}
		100 µg	*28*	*4*	*0.9*	30^{A}, 23^{A}, 30^{A}
		33.3 µg	*36*	*4*	*1.2*	41^{A}, 33^{A}, 34^{A}
	Water	100 µL	*31*	*6*		24^{A}, 33^{A}, 36^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 4 (CONT.)
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PG.503.BTL
Study Code: AF28PG
Experiment: B2
Date Plated: 6/19/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/21/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**2AA**	1.00 µg	*223*	*19*	*15.9*	212[A], 213[A], 245[A]
TA100	**2AA**	2.00 µg	*831*	*60*	*8.4*	846[A], 882[A], 765[A]
TA1535	**2AA**	1.00 µg	*75*	*2*	*6.8*	75[A], 73[A], 76[A]
TA1537	**2AA**	2.00 µg	*40*	*2*	*8.0*	43[A], 39[A], 39[A]
WP2uvrA	**2AA**	15.0 µg	*307*	*18*	*9.9*	318[A], 286[A], 318[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values 2016 revertants per plate											
Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	
SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control											

14. APPENDIX II: Study Protocol and Amendment

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PG.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 3333, 1000, 333, 100 and 33.3 µg per plate

   Reason: To specify the dose levels to be used for the confirmatory mutagenicity assay based on the toxicity and precipitate profiles observed in the initial toxicity-mutation assay.

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PG.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, PhD, DABT
Sponsor Representative

18 June 2018
Date

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PG.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS
BioReliance Study Director

15 JUN 2018
Date

BioReliance Study Management

15-JUN-18
Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF28PG.503.BTL

1. KEY PERSONNEL

Sponsor Information:

Sponsor	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor Number	C30049
Sponsor's Authorized Representative	Shawn Gannon, Ph.D., DABT The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899 Phone: 302-773-1376 Email: SHAWN.A.GANNON@chemours.com

Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	31-May-2018
Proposed Experimental Completion Date	27-June-2018
Proposed Report Date	12-July-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST SUBSTANCE INFORMATION

Identification	PFECA F
CAS No.	377-73-1
Storage Conditions	Room Temperature Protect from light (Per BioReliance SOP) With argon
Purity	98.9% (no correction factor will be used for dose formulations)
Molecular Weight	230.04 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused Test Substance will be returned to the sponsor prior to report finalization using the information below; unless the test substance is used on another study.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

Residual dose formulations will be discarded after use.

7. TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values
Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of

revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains

- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

Shawn Gannon, Ph.D., DABT
Sponsor Representative

8 May 2018
Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 06 JUN 2018

BioReliance Study Management — Date: 05-JUN-18

15. APPENDIX III: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test for Induction of: Reverse mutation in bacterial cells

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

Metabolizing System: Aroclor-induced rat liver S9

Vehicle for Test Substance: Water

Treatment: Plate incorporation

Cytotoxic Effects: None

Genotoxic Effects: None

No. of Independent Assays: 2

No. of Replicate Cultures: 2 (B1) and 3 (B2)

Test Substance: PFECA F

Study No.: AF28PG.503.BTL

No. Cells Analyzed/Culture: 1.0 to 3.5 x 10^8 cells per plate

GLP Compliance: Yes

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Date(s) of Treatment: 08 June 2018 (B1) and 19 June 2018 (B2)

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B1: Initial Toxicity-Mutation Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	17 ± 1	93 ± 3	12 ± 1	8 ± 4	26 ± 1
	PFECA F	1.50	18 ± 5	88 ± 28	10 ± 1	7 ± 2	22 ± 10
		5.00	12 ± 6	85 ± 2	15 ± 1	9 ± 1	23 ± 9
		15.0	19 ± 4	101 ± 6	12 ± 3	5 ± 3	34 ± 1
		50.0	16 ± 1	95 ± 8	15 ± 3	8 ± 2	27 ± 3
		150	16 ± 2	96 ± 6	9 ± 1	11 ± 0	34 ± 1
		500	12 ± 2	98 ± 8	12 ± 1	7 ± 1	22 ± 6
		1500	13 ± 6	86 ± 18	12 ± 1	10 ± 1	26 ± 7
		5000	16 ± 4	78 ± 9	10 ± 0	7 ± 6	27 ± 8
	2NF	1.00	87 ± 16				
	SA	1.00		639 ± 49	654 ± 13		
	9AAD	75.0				656 ± 14	
	MMS	1000					431 ± 27
With Activation	Water	100 µL/plate	22 ± 4	92 ± 7	12 ± 1	9 ± 1	28 ± 7
	PFECA F	1.50	17 ± 3	91 ± 2	12 ± 2	9 ± 6	32 ± 6
		5.00	12 ± 4	79 ± 1	9 ± 1	8 ± 2	31 ± 3
		15.0	16 ± 8	105 ± 11	10 ± 1	11 ± 1	35 ± 4
		50.0	18 ± 0	129 ± 1	13 ± 0	8 ± 1	33 ± 11
		150	19 ± 6	97 ± 1	13 ± 3	9 ± 6	31 ± 1
		500	22 ± 1	93 ± 4	12 ± 2	8 ± 4	32 ± 1
		1500	21 ± 6	92 ± 19	15 ± 1	8 ± 2	42 ± 8
		5000	23 ± 9	94 ± 13	11 ± 5	8 ± 4	34 ± 1
	2AA	1.00	268 ± 12		77 ± 9		
	2AA	2.00		645 ± 9		44 ± 11	
	2AA	15.0					364 ± 24

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B2: Confirmatory Mutagenicity Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	17 ± 1	89 ± 6	10 ± 3	6 ± 0	34 ± 1
	PFECA F	33.3	14 ± 7	81 ± 14	8 ± 2	7 ± 3	39 ± 7
		100	14 ± 6	78 ± 13	8 ± 1	7 ± 2	34 ± 7
		333	14 ± 1	85 ± 6	8 ± 3	6 ± 5	32 ± 7
		1000	16 ± 4	90 ± 24	8 ± 2	5 ± 3	31 ± 7
		3333	14 ± 2	83 ± 8	9 ± 3	5 ± 3	37 ± 3
		5000	9 ± 3	81 ± 10	9 ± 4	6 ± 3	27 ± 2
	2NF	1.00	68 ± 19				
	SA	1.00		607 ± 15	611 ± 19		
	9AAD	75.0				771 ± 71	
	MMS	1000					463 ± 4
With Activation	Water	100 µL/plate	14 ± 1	99 ± 21	11 ± 3	5 ± 0	31 ± 6
	PFECA F	33.3	15 ± 5	93 ± 8	8 ± 2	5 ± 1	36 ± 4
		100	16 ± 5	104 ± 16	12 ± 6	7 ± 1	28 ± 4
		333	16 ± 4	107 ± 18	11 ± 4	7 ± 1	35 ± 1
		1000	15 ± 3	104 ± 9	15 ± 3	7 ± 4	31 ± 4
		3333	13 ± 6	97 ± 12	11 ± 4	7 ± 3	34 ± 7
		5000	14 ± 3	107 ± 7	11 ± 2	6 ± 3	31 ± 4
	2AA	1.00	223 ± 19		75 ± 2		
	2AA	2.00		831 ± 60		40 ± 2	
	2AA	15.0					307 ± 18

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

PFECA A

Author

Emily Dakoulas, BS

Study Completion Date

21 August 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF28PH.503.BTL

Sponsor

The Chemours Company
1007 Market Street D-3008
Wilmington, DE 19899

Sponsor Number

C30049

1. STATEMENT OF COMPLIANCE

Study No. AF28PH.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

21 Nov 2018
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF28PH.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
18-Jun-2018	18-Jun-2018	Protocol Review	18-Jun-2018	18-Jun-2018
18-Jun-2018	19-Jun-2018	Scoring	19-Jun-2018	19-Jun-2018
05-Jul-2018	05-Jul-2018	Protocol Amendment Review	05-Jul-2018	05-Jul-2018
06-Jul-2018	06-Jul-2018	Data/Draft Report	06-Jul-2018	06-Jul-2018
17-Aug-2018	17-Aug-2018	Final Report	17-Aug-2018	17-Aug-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Carlos Bonilla 21-Aug-2018 3:40 pm GMT

Reason for signature: QA Approval

Printed by:Carlos Bonilla
Printed on:21-Aug-18

3. TABLE OF CONTENTS

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4. STUDY INFORMATION

Study Conduct

Sponsor:	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor's Authorized Representative:	Shawn Gannon, Ph.D., DABT
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF28PH.503.BTL
Sponsor No.:	C30049

Test Substance

Identification:	PFECA A
CAS No.:	863090-89-5
Purity:	98% (per protocol)
Molecular Weight:	280.05 g/mol
Description:	Clear colorless liquid
Storage Conditions:	Room temperature, protected from light
Receipt Date:	03 Apr 2018

Study Dates

Study Initiation Date:	29 May 2018
Experimental Starting Date (first day of data collection):	30 May 2018
Experimental Start Date (first day test substance administered to test system):	31 May 2018
Experimental Completion Date:	18 June 2018

Key Personnel

Study Director:	Emily Dakoulas, BS

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, PFECA A, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Water was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Based upon these results, the maximum dose tested in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, the dose levels tested were 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate PFECA A was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization using the information below.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

The vehicle used to deliver PFECA A to the test system was water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Water	7732-18-5	Sigma-Aldrich	RNBF9658	Sterile-filtered	Mar 2019

Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Water was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in water at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3925, Exp. Date: 21 Feb 2020; Lot No. 3961, Exp. Date: 15 May 2020) was purchased

commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and five dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers (see Deviations).

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

Deviations

Following deviation from assay-method SOPs occurred during the conduct of this study.

Event No. 326527: Laboratory technician recorded the wrong lot number in the raw data for oxoid nutrient broth used for confirmation of tester strain genotype. The lot number recorded was for bottom agar. The Study Director determined no Impact as the strain characterization results were within the historical control range for the titers.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	1.3	1.4	1.1	1.4	3.1
B2	1.3	1.2	1.3	1.7	2.9

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in water are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

Neither precipitate nor toxicity was observed.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 50.0, 150, 500, 1500 and 5000 µg per plate.

Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

A copy of the Common Technical Document Tables is included in Appendix III.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, PFECA A did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

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Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PH.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PH
Date Plated: 5/31/2018
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA A**	5000 µg	*13*	*2*	*0.8*	14[A], 11[A]
		1500 µg	*13*	*0*	*0.8*	13[A], 13[A]
		500 µg	*10*	*2*	*0.6*	8[A], 11[A]
		150 µg	*14*	*5*	*0.8*	17[A], 10[A]
		50.0 µg	*13*	*6*	*0.8*	9[A], 17[A]
		15.0 µg	*13*	*5*	*0.8*	9[A], 16[A]
		5.00 µg	*19*	*1*	*1.1*	18[A], 19[A]
		1.50 µg	*8*	*1*	*0.5*	7[A], 9[A]
	Water	100 µL	*17*	*8*		11[A], 22[A]
TA100	**PFECA A**	5000 µg	*76*	*11*	*1.0*	68[A], 84[A]
		1500 µg	*66*	*2*	*0.9*	64[A], 67[A]
		500 µg	*78*	*3*	*1.1*	80[A], 76[A]
		150 µg	*85*	*7*	*1.1*	90[A], 80[A]
		50.0 µg	*76*	*18*	*1.0*	63[A], 89[A]
		15.0 µg	*68*	*11*	*0.9*	76[A], 60[A]
		5.00 µg	*63*	*15*	*0.9*	73[A], 52[A]
		1.50 µg	*68*	*3*	*0.9*	70[A], 66[A]
	Water	100 µL	*74*	*8*		68[A], 80[A]
TA1535	**PFECA A**	5000 µg	*12*	*6*	*1.2*	8[A], 16[A]
		1500 µg	*7*	*1*	*0.7*	6[A], 7[A]
		500 µg	*14*	*0*	*1.4*	14[A], 14[A]
		150 µg	*11*	*6*	*1.1*	15[A], 6[A]
		50.0 µg	*12*	*7*	*1.2*	17[A], 7[A]
		15.0 µg	*7*	*1*	*0.7*	8[A], 6[A]
		5.00 µg	*12*	*3*	*1.2*	10[A], 14[A]
		1.50 µg	*8*	*4*	*0.8*	5[A], 11[A]
	Water	100 µL	*10*	*0*		10[A], 10[A]
TA1537	**PFECA A**	5000 µg	*7*	*3*	*0.9*	5[A], 9[A]
		1500 µg	*9*	*1*	*1.1*	10[A], 8[A]
		500 µg	*7*	*0*	*0.9*	7[A], 7[A]
		150 µg	*7*	*0*	*0.9*	7[A], 7[A]
		50.0 µg	*8*	*2*	*1.0*	9[A], 6[A]
		15.0 µg	*6*	*4*	*0.8*	8[A], 3[A]
		5.00 µg	*9*	*0*	*1.1*	9[A], 9[A]
		1.50 µg	*6*	*0*	*0.8*	6[A], 6[A]
	Water	100 µL	*8*	*2*		6[A], 9[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PH.503.BTL
Study Code: AF28PH
Experiment: B1
Date Plated: 5/31/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**PFECA A**	5000 µg	*25*	*3*	*1.0*	23[A], 27[A]
		1500 µg	*21*	*3*	*0.9*	23[A], 19[A]
		500 µg	*23*	*3*	*1.0*	25[A], 21[A]
		150 µg	*27*	*7*	*1.1*	32[A], 22[A]
		50.0 µg	*36*	*1*	*1.5*	35[A], 36[A]
		15.0 µg	*30*	*8*	*1.3*	35[A], 24[A]
		5.00 µg	*24*	*4*	*1.0*	21[A], 26[A]
		1.50 µg	*32*	*11*	*1.3*	24[A], 39[A]
	Water	100 µL	*24*	*8*		30[A], 18[A]
TA98	**2NF**	1.00 µg	*88*	*29*	*5.2*	108[A], 67[A]
TA100	**SA**	1.00 µg	*676*	*136*	*9.1*	580[A], 772[A]
TA1535	**SA**	1.00 µg	*669*	*2*	*66.9*	667[A], 670[A]
TA1537	**9AAD**	75.0 µg	*520*	*61*	*65.0*	477[A], 563[A]
WP2uvrA	**MMS**	1000 µg	*306*	*22*	*12.8*	321[A], 290[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PH.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PH
Date Plated: 5/31/2018
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA A**	5000 µg	*15*	*3*	*1.1*	13[A], 17[A]
		1500 µg	*17*	*2*	*1.2*	15[A], 18[A]
		500 µg	*12*	*2*	*0.9*	13[A], 10[A]
		150 µg	*18*	*1*	*1.3*	17[A], 18[A]
		50.0 µg	*14*	*1*	*1.0*	14[A], 13[A]
		15.0 µg	*20*	*6*	*1.4*	24[A], 15[A]
		5.00 µg	*14*	*4*	*1.0*	11[A], 17[A]
		1.50 µg	*18*	*2*	*1.3*	19[A], 16[A]
	Water	100 µL	*14*	*1*		13[A], 14[A]
TA100	**PFECA A**	5000 µg	*94*	*3*	*1.1*	92[A], 96[A]
		1500 µg	*94*	*6*	*1.1*	98[A], 90[A]
		500 µg	*84*	*17*	*1.0*	96[A], 72[A]
		150 µg	*87*	*7*	*1.0*	92[A], 82[A]
		50.0 µg	*88*	*5*	*1.0*	84[A], 91[A]
		15.0 µg	*98*	*14*	*1.2*	108[A], 88[A]
		5.00 µg	*99*	*18*	*1.2*	86[A], 111[A]
		1.50 µg	*84*	*8*	*1.0*	90[A], 78[A]
	Water	100 µL	*85*	*19*		98[A], 71[A]
TA1535	**PFECA A**	5000 µg	*16*	*3*	*1.0*	18[A], 14[A]
		1500 µg	*17*	*1*	*1.1*	17[A], 16[A]
		500 µg	*11*	*1*	*0.7*	10[A], 11[A]
		150 µg	*14*	*6*	*0.9*	18[A], 10[A]
		50.0 µg	*11*	*1*	*0.7*	10[A], 11[A]
		15.0 µg	*11*	*3*	*0.7*	9[A], 13[A]
		5.00 µg	*12*	*6*	*0.8*	16[A], 7[A]
		1.50 µg	*11*	*0*	*0.7*	11[A], 11[A]
	Water	100 µL	*16*	*1*		15[A], 17[A]
TA1537	**PFECA A**	5000 µg	*5*	*0*	*0.7*	5[A], 5[A]
		1500 µg	*6*	*0*	*0.9*	6[A], 6[A]
		500 µg	*12*	*3*	*1.7*	14[A], 10[A]
		150 µg	*10*	*2*	*1.4*	8[A], 11[A]
		50.0 µg	*9*	*2*	*1.3*	7[A], 10[A]
		15.0 µg	*9*	*1*	*1.3*	10[A], 8[A]
		5.00 µg	*8*	*1*	*1.1*	7[A], 8[A]
		1.50 µg	*7*	*1*	*1.0*	6[A], 8[A]
	Water	100 µL	*7*	*1*		7[A], 6[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PH.503.BTL
Study Code: AF28PH
Experiment: B1
Date Plated: 5/31/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**PFECA A**	5000 µg	*28*	*2*	*0.8*	29[A], 26[A]
		1500 µg	*29*	*6*	*0.9*	24[A], 33[A]
		500 µg	*24*	*0*	*0.7*	24[A], 24[A]
		150 µg	*35*	*4*	*1.0*	38[A], 32[A]
		50.0 µg	*29*	*5*	*0.9*	32[A], 25[A]
		15.0 µg	*31*	*1*	*0.9*	32[A], 30[A]
		5.00 µg	*33*	*8*	*1.0*	38[A], 27[A]
		1.50 µg	*36*	*18*	*1.1*	49[A], 23[A]
	Water	100 µL	*34*	*6*		38[A], 30[A]
TA98	**2AA**	1.00 µg	*352*	*81*	*25.1*	409[A], 295[A]
TA100	**2AA**	2.00 µg	*716*	*129*	*8.4*	624[A], 807[A]
TA1535	**2AA**	1.00 µg	*127*	*48*	*7.9*	93[A], 161[A]
TA1537	**2AA**	2.00 µg	*89*	*33*	*12.7*	112[A], 65[A]
WP2uvrA	**2AA**	15.0 µg	*180*	*20*	*5.3*	166[A], 194[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PH.503.BTL
Study Code: AF28PH
Experiment: B2
Date Plated: 6/12/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/18/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA A**	5000 µg	*17*	*1*	*1.3*	18^{A}, 16^{A}, 16^{A}
		1500 µg	*9*	*1*	*0.7*	8^{A}, 8^{A}, 10^{A}
		500 µg	*10*	*2*	*0.8*	8^{A}, 11^{A}, 11^{A}
		150 µg	*12*	*3*	*0.9*	16^{A}, 11^{A}, 10^{A}
		50.0 µg	*7*	*3*	*0.5*	7^{A}, 5^{A}, 10^{A}
	Water	100 µL	*13*	*6*		13^{A}, 8^{A}, 19^{A}
TA100	**PFECA A**	5000 µg	*92*	*2*	*1.1*	93^{A}, 90^{A}, 92^{A}
		1500 µg	*90*	*9*	*1.1*	99^{A}, 82^{A}, 88^{A}
		500 µg	*86*	*11*	*1.1*	88^{A}, 96^{A}, 75^{A}
		150 µg	*79*	*2*	*1.0*	79^{A}, 78^{A}, 81^{A}
		50.0 µg	*89*	*7*	*1.1*	83^{A}, 97^{A}, 88^{A}
	Water	100 µL	*81*	*13*		92^{A}, 84^{A}, 66^{A}
TA1535	**PFECA A**	5000 µg	*8*	*1*	*0.7*	9^{A}, 8^{A}, 7^{A}
		1500 µg	*9*	*1*	*0.8*	8^{A}, 10^{A}, 9^{A}
		500 µg	*10*	*4*	*0.8*	7^{A}, 9^{A}, 14^{A}
		150 µg	*13*	*4*	*1.1*	15^{A}, 16^{A}, 9^{A}
		50.0 µg	*8*	*2*	*0.7*	9^{A}, 6^{A}, 8^{A}
	Water	100 µL	*12*	*6*		8^{A}, 18^{A}, 9^{A}
TA1537	**PFECA A**	5000 µg	*6*	*2*	*1.0*	5^{A}, 5^{A}, 8^{A}
		1500 µg	*5*	*0*	*0.8*	5^{A}, 5^{A}, 5^{A}
		500 µg	*4*	*2*	*0.7*	5^{A}, 1^{A}, 5^{A}
		150 µg	*7*	*1*	*1.2*	7^{A}, 6^{A}, 7^{A}
		50.0 µg	*5*	*2*	*0.8*	3^{A}, 6^{A}, 6^{A}
	Water	100 µL	*6*	*2*		6^{A}, 5^{A}, 8^{A}
WP2uvrA	**PFECA A**	5000 µg	*29*	*7*	*1.0*	23^{A}, 36^{A}, 27^{A}
		1500 µg	*26*	*7*	*0.9*	30^{A}, 18^{A}, 29^{A}
		500 µg	*32*	*7*	*1.1*	25^{A}, 38^{A}, 33^{A}
		150 µg	*38*	*6*	*1.3*	31^{A}, 42^{A}, 40^{A}
		50.0 µg	*27*	*5*	*0.9*	22^{A}, 32^{A}, 27^{A}
	Water	100 µL	*29*	*5*		35^{A}, 26^{A}, 27^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PH.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PH
Date Plated: 6/12/2018
Evaluation Period: 6/18/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**2NF**	1.00 µg	*64*	*9*	*4.9*	55^{A}, 72^{A}, 65^{A}
TA100	**SA**	1.00 µg	*663*	*33*	*8.2*	633^{A}, 657^{A}, 698^{A}
TA1535	**SA**	1.00 µg	*626*	*75*	*52.2*	705^{A}, 619^{A}, 555^{A}
TA1537	**9AAD**	75.0 µg	*730*	*154*	*121.7*	903^{A}, 606^{A}, 682^{A}
WP2uvrA	**MMS**	1000 µg	*468*	*47*	*16.1*	446^{A}, 437^{A}, 522^{A}

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PH.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PH
Date Plated: 6/12/2018
Evaluation Period: 6/18/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA A**	5000 µg	*14*	*3*	*1.2*	16[A], 15[A], 11[A]
		1500 µg	*13*	*3*	*1.1*	13[A], 11[A], 16[A]
		500 µg	*18*	*4*	*1.5*	22[A], 15[A], 16[A]
		150 µg	*14*	*3*	*1.2*	15[A], 16[A], 11[A]
		50.0 µg	*11*	*4*	*0.9*	15[A], 8[A], 9[A]
	Water	100 µL	*12*	*3*		10[A], 10[A], 15[A]
TA100	**PFECA A**	5000 µg	*113*	*9*	*1.2*	103[A], 119[A], 116[A]
		1500 µg	*107*	*9*	*1.2*	104[A], 99[A], 117[A]
		500 µg	*98*	*2*	*1.1*	100[A], 96[A], 97[A]
		150 µg	*91*	*8*	*1.0*	89[A], 100[A], 84[A]
		50.0 µg	*92*	*7*	*1.0*	100[A], 88[A], 87[A]
	Water	100 µL	*92*	*3*		91[A], 90[A], 96[A]
TA1535	**PFECA A**	5000 µg	*12*	*5*	*0.9*	17[A], 10[A], 8[A]
		1500 µg	*13*	*6*	*1.0*	13[A], 7[A], 18[A]
		500 µg	*9*	*1*	*0.7*	9[A], 9[A], 8[A]
		150 µg	*17*	*3*	*1.3*	17[A], 14[A], 19[A]
		50.0 µg	*13*	*5*	*1.0*	9[A], 13[A], 18[A]
	Water	100 µL	*13*	*2*		11[A], 13[A], 14[A]
TA1537	**PFECA A**	5000 µg	*6*	*4*	*1.0*	2[A], 6[A], 10[A]
		1500 µg	*5*	*2*	*0.8*	6[A], 6[A], 3[A]
		500 µg	*7*	*3*	*1.2*	9[A], 9[A], 3[A]
		150 µg	*9*	*4*	*1.5*	13[A], 6[A], 7[A]
		50.0 µg	*4*	*2*	*0.7*	6[A], 3[A], 2[A]
	Water	100 µL	*6*	*2*		7[A], 7[A], 3[A]
WP2uvrA	**PFECA A**	5000 µg	*33*	*7*	*0.9*	29[A], 29[A], 41[A]
		1500 µg	*33*	*3*	*0.9*	32[A], 36[A], 30[A]
		500 µg	*34*	*5*	*1.0*	39[A], 30[A], 32[A]
		150 µg	*38*	*4*	*1.1*	42[A], 35[A], 36[A]
		50.0 µg	*35*	*5*	*1.0*	41[A], 33[A], 32[A]
	Water	100 µL	*35*	*1*		35[A], 35[A], 34[A]
TA98	**2AA**	1.00 µg	*216*	*3*	*18.0*	217[A], 218[A], 213[A]
TA100	**2AA**	2.00 µg	*844*	*225*	*9.2*	1100[A], 679[A], 754[A]
TA1535	**2AA**	1.00 µg	*78*	*11*	*6.0*	91[A], 72[A], 72[A]
TA1537	**2AA**	2.00 µg	*43*	*4*	*7.2*	39[A], 46[A], 44[A]
WP2uvrA	**2AA**	15.0 µg	*248*	*24*	*7.1*	274[A], 242[A], 228[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values
2016
revertants per plate

Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	

SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control

14. APPENDIX II: Study Protocol and Amendment

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PH.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 1500, 500, 150 and 50.0 µg per plate.

   Reason: To specify the dose levels to be used for the mutagenicity assay based on the toxicity and precipitate profiles observed in the initial toxicity-mutation assay.

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PH.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, Ph.D., DABT
Sponsor Representative

11 June 2018
Date

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PH.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS
BioReliance Study Director

08 JUN 2018
Date

BioReliance Study Management

08-JUN-18
Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF28PH.503.BTL

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Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	31-May-2018
Proposed Experimental Completion Date	26-June-2018
Proposed Report Date	11-July-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST SUBSTANCE INFORMATION

Identification	PFECA A
CAS No.	863090-89-5
Storage Conditions	Room Temperature Protect from light (Per BioReliance SOP)
Purity	98% (no correction factor will be used for dose formulations)
Molecular Weight	280.05 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused Test Substance will be returned to the sponsor prior to report finalization using the information below; unless the test substance is used on another study.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

Residual dose formulations will be discarded after use.

7. TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values
Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of

revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains

- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^{+} reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

Shawn Gannon, Ph.D., DABT
Sponsor Representative

8 May 2018
Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 27 MAY 2018

BioReliance Study Management — Date: 25 May 2018

15. APPENDIX III: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test for Induction of: Reverse mutation in bacterial cells

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

Metabolizing System: Aroclor-induced rat liver S9

Vehicle for Test Substance: Water

Treatment: Plate incorporation

Cytotoxic Effects: None

Genotoxic Effects: None

No. of Independent Assays: 2

No. of Replicate Cultures: 2 (B1) and 3 (B2)

Test Substance: PFECA A

Study No.: AF28PH.503.BTL

No. Cells Analyzed/Culture: 1.1 to 3.1 x 10^8 cells per plate

GLP Compliance: Yes

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Date(s) of Treatment: 31 May 2018 (B1) and 12 June 2018 (B2)

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B1: Initial Toxicity-Mutation Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	17 ± 8	74 ± 8	10 ± 0	8 ± 2	24 ± 8
	PFECA A	1.50	8 ± 1	68 ± 3	8 ± 4	6 ± 0	32 ± 11
		5.00	19 ± 1	63 ± 15	12 ± 3	9 ± 0	24 ± 4
		15.0	13 ± 5	68 ± 11	7 ± 1	6 ± 4	30 ± 8
		50.0	13 ± 6	76 ± 18	12 ± 7	8 ± 2	36 ± 1
		150	14 ± 5	85 ± 7	11 ± 6	7 ± 0	27 ± 7
		500	10 ± 2	78 ± 3	14 ± 0	7 ± 0	23 ± 3
		1500	13 ± 0	66 ± 2	7 ± 1	9 ± 1	21 ± 3
		5000	13 ± 2	76 ± 11	12 ± 6	7 ± 3	25 ± 3
	2NF	1.00	88 ± 29				
	SA	1.00		676 ± 136	669 ± 2		
	9AAD	75.0				520 ± 61	
	MMS	1000					306 ± 22
With Activation	Water	100 µL/plate	14 ± 1	85 ± 19	16 ± 1	7 ± 1	34 ± 6
	PFECA A	1.50	18 ± 2	84 ± 8	11 ± 0	7 ± 1	36 ± 18
		5.00	14 ± 4	99 ± 18	12 ± 6	8 ± 1	33 ± 8
		15.0	20 ± 6	98 ± 14	11 ± 3	9 ± 1	31 ± 1
		50.0	14 ± 1	88 ± 5	11 ± 1	9 ± 2	29 ± 5
		150	18 ± 1	87 ± 7	14 ± 6	10 ± 2	35 ± 4
		500	12 ± 2	84 ± 17	11 ± 1	12 ± 3	24 ± 0
		1500	17 ± 2	94 ± 6	17 ± 1	6 ± 0	29 ± 6
		5000	15 ± 3	94 ± 3	16 ± 3	5 ± 0	28 ± 2
	2AA	1.00	352 ± 81		127 ± 48		
	2AA	2.00		716 ± 129		89 ± 33	
	2AA	15.0					180 ± 20

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B2: Confirmatory mutagenicity assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	13 ± 6	81 ± 13	12 ± 6	6 ± 2	29 ± 5
	PFECA A	50.0	7 ± 3	89 ± 7	8 ± 2	5 ± 2	27 ± 5
		150	12 ± 3	79 ± 2	13 ± 4	7 ± 1	38 ± 6
		500	10 ± 2	86 ± 11	10 ± 4	4 ± 2	32 ± 7
		1500	9 ± 1	90 ± 9	9 ± 1	5 ± 0	26 ± 7
		5000	17 ± 1	92 ± 2	8 ± 1	6 ± 2	29 ± 7
	2NF	1.00	64 ± 9				
	SA	1.00		663 ± 33	626 ± 75		
	9AAD	75.0				730 ± 154	
	MMS	1000					468 ± 47
With Activation	Water	100 µL/plate	12 ± 3	92 ± 3	13 ± 2	6 ± 2	35 ± 1
	PFECA A	50.0	11 ± 4	92 ± 7	13 ± 5	4 ± 2	35 ± 5
		150	14 ± 3	91 ± 8	17 ± 3	9 ± 4	38 ± 4
		500	18 ± 4	98 ± 2	9 ± 1	7 ± 3	34 ± 5
		1500	13 ± 3	107 ± 9	13 ± 6	5 ± 2	33 ± 3
		5000	14 ± 3	113 ± 9	12 ± 5	6 ± 4	33 ± 7
	2AA	1.00	216 ± 3		78 ± 11		
	2AA	2.00		844 ± 225		43 ± 4	
	2AA	15.0					248 ± 24

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

PFECA G

Author

Emily Dakoulas, BS

Study Completion Date

21 August 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF28PJ.503.BTL

Sponsor

The Chemours Company
1007 Market Street D-3008
Wilmington, DE 19899

Sponsor Number

C30049

1. STATEMENT OF COMPLIANCE

Study No. AF28PJ.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

21 AUG 2018
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF28PJ.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
13-Jun-2018	13-Jun-2018	Strain Characterization	14-Jun-2018	14-Jun-2018
13-Jun-2018	09-Jul-2018	Protocol Review	09-Jul-2018	09-Jul-2018
06-Jul-2018	06-Jul-2018	Data/Draft Report	06-Jul-2018	06-Jul-2018
17-Aug-2018	17-Aug-2018	Final Report	17-Aug-2018	17-Aug-2018
17-Aug-2018	17-Aug-2018	Protocol Amendment Review	17-Aug-2018	17-Aug-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Alan Tarwater 21-Aug-2018 6:29 pm GMT

Reason for signature: QA Approval

Printed by:Alan Tarwater
Printed on:21-Aug-18

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4. STUDY INFORMATION

Study Conduct

Sponsor:	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor's Authorized Representative:	Shawn Gannon, Ph.D., DABT
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF28PJ.503.BTL
Sponsor No.:	C30049

Test Substance

Identification:	PFECA G
CAS No.:	801212-59-9
Purity:	99% (per protocol)
Molecular Weight:	380.06 g/mol
Description:	Clear colorless liquid
Storage Conditions:	Room temperature, protected from light
Receipt Date:	03 Apr 2018

Study Dates

Study Initiation Date:	24 May 2018
Experimental Starting Date (first day of data collection):	25 May 2018
Experimental Start Date (first day test substance administered to test system):	31 May 2018
Experimental Completion Date:	18 June 2018

Key Personnel

Study Director:	Emily Dakoulas, BS

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, PFECA G, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Water was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Based upon these results, the maximum dose tested in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, the dose levels tested were 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate PFECA G was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization using the information below.

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The vehicle used to deliver PFECA G to the test system was water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Water	7732-18-5	Sigma-Aldrich	RNBF9658	Sterile-filtered	Mar 2019

Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light. To achieve a solution, the most concentrated dilution was sonicated at 36.1 to 32.8ºC for 17 to 26 minutes in each assay.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Water was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in water at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3925, Exp. Date: 21 Feb 2020; Lot No. 3961, Exp. Date: 15 May 2020) was purchased

commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and five dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	1.3	1.4	1.1	1.4	3.1
B2	1.3	1.1	1.5	1.8	2.9

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in water are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

Neither precipitate nor toxicity was observed.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 50.0, 150, 500, 1500 and 5000 µg per plate.

Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

A copy of the Common Technical Document Tables is included in Appendix III.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, PFECA G did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PJ.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PJ
Date Plated: 5/31/2018
Evaluation Period: 6/4/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA G**	5000 µg	*9*	*1*	*0.8*	9^{A}, 8^{A}
		1500 µg	*10*	*4*	*0.8*	13^{A}, 7^{A}
		500 µg	*13*	*2*	*1.1*	14^{A}, 11^{A}
		150 µg	*13*	*2*	*1.1*	11^{A}, 14^{A}
		50.0 µg	*12*	*1*	*1.0*	11^{A}, 13^{A}
		15.0 µg	*14*	*6*	*1.2*	18^{A}, 10^{A}
		5.00 µg	*15*	*1*	*1.3*	14^{A}, 15^{A}
		1.50 µg	*16*	*4*	*1.3*	19^{A}, 13^{A}
	Water	100 µL	*12*	*8*		18^{A}, 6^{A}
TA100	**PFECA G**	5000 µg	*84*	*5*	*1.2*	80^{A}, 87^{A}
		1500 µg	*87*	*4*	*1.2*	89^{A}, 84^{A}
		500 µg	*86*	*6*	*1.2*	90^{A}, 82^{A}
		150 µg	*86*	*8*	*1.2*	80^{A}, 92^{A}
		50.0 µg	*81*	*1*	*1.1*	80^{A}, 81^{A}
		15.0 µg	*74*	*8*	*1.0*	79^{A}, 68^{A}
		5.00 µg	*74*	*13*	*1.0*	64^{A}, 83^{A}
		1.50 µg	*92*	*5*	*1.3*	95^{A}, 88^{A}
	Water	100 µL	*73*	*1*		73^{A}, 72^{A}
TA1535	**PFECA G**	5000 µg	*11*	*6*	*1.0*	15^{A}, 7^{A}
		1500 µg	*14*	*4*	*1.3*	17^{A}, 11^{A}
		500 µg	*19*	*12*	*1.7*	10^{A}, 27^{A}
		150 µg	*9*	*3*	*0.8*	7^{A}, 11^{A}
		50.0 µg	*14*	*4*	*1.3*	11^{A}, 17^{A}
		15.0 µg	*14*	*0*	*1.3*	14^{A}, 14^{A}
		5.00 µg	*7*	*1*	*0.6*	7^{A}, 6^{A}
		1.50 µg	*12*	*1*	*1.1*	11^{A}, 13^{A}
	Water	100 µL	*11*	*4*		8^{A}, 14^{A}
TA1537	**PFECA G**	5000 µg	*7*	*1*	*1.0*	7^{A}, 6^{A}
		1500 µg	*7*	*5*	*1.0*	3^{A}, 10^{A}
		500 µg	*10*	*1*	*1.4*	10^{A}, 9^{A}
		150 µg	*5*	*0*	*0.7*	5^{A}, 5^{A}
		50.0 µg	*8*	*4*	*1.1*	5^{A}, 10^{A}
		15.0 µg	*7*	*3*	*1.0*	9^{A}, 5^{A}
		5.00 µg	*8*	*1*	*1.1*	7^{A}, 9^{A}
		1.50 µg	*7*	*2*	*1.0*	5^{A}, 8^{A}
	Water	100 µL	*7*	*1*		6^{A}, 8^{A}

Key to Automatic Count Flags

[A]: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PJ.503.BTL
Study Code: AF28PJ
Experiment: B1
Date Plated: 5/31/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/4/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**PFECA G**	5000 µg	*17*	*2*	*0.6*	18[A], 15[A]
		1500 µg	*28*	*6*	*0.9*	23[A], 32[A]
		500 µg	*30*	*4*	*1.0*	33[A], 27[A]
		150 µg	*34*	*1*	*1.1*	35[A], 33[A]
		50.0 µg	*41*	*11*	*1.4*	48[A], 33[A]
		15.0 µg	*39*	*0*	*1.3*	39[A], 39[A]
		5.00 µg	*41*	*8*	*1.4*	47[A], 35[A]
		1.50 µg	*35*	*7*	*1.2*	30[A], 40[A]
	Water	100 µL	*30*	*6*		34[A], 26[A]
TA98	**2NF**	1.00 µg	*85*	*18*	*7.1*	72[A], 97[A]
TA100	**SA**	1.00 µg	*756*	*55*	*10.4*	717[A], 795[A]
TA1535	**SA**	1.00 µg	*679*	*33*	*61.7*	656[A], 702[A]
TA1537	**9AAD**	75.0 µg	*362*	*156*	*51.7*	251[A], 472[A]
WP2uvrA	**MMS**	1000 µg	*513*	*43*	*17.1*	482[A], 543[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PJ.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PJ
Date Plated: 5/31/2018
Evaluation Period: 6/4/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA G**	5000 µg	*19*	*3*	*0.7*	21[A], 17[A]
		1500 µg	*26*	*13*	*1.0*	16[A], 35[A]
		500 µg	*24*	*2*	*0.9*	25[A], 22[A]
		150 µg	*17*	*0*	*0.7*	17[A], 17[A]
		50.0 µg	*22*	*1*	*0.8*	21[A], 22[A]
		15.0 µg	*20*	*4*	*0.8*	17[A], 22[A]
		5.00 µg	*19*	*7*	*0.7*	24[A], 14[A]
		1.50 µg	*18*	*1*	*0.7*	19[A], 17[A]
	Water	100 µL	*26*	*6*		30[A], 22[A]
TA100	**PFECA G**	5000 µg	*117*	*27*	*1.3*	136[A], 98[A]
		1500 µg	*107*	*11*	*1.2*	115[A], 99[A]
		500 µg	*97*	*5*	*1.1*	93[A], 100[A]
		150 µg	*97*	*20*	*1.1*	83[A], 111[A]
		50.0 µg	*103*	*6*	*1.1*	99[A], 107[A]
		15.0 µg	*84*	*23*	*0.9*	68[A], 100[A]
		5.00 µg	*87*	*9*	*1.0*	80[A], 93[A]
		1.50 µg	*95*	*6*	*1.0*	91[A], 99[A]
	Water	100 µL	*91*	*4*		88[A], 93[A]
TA1535	**PFECA G**	5000 µg	*14*	*4*	*1.4*	16[A], 11[A]
		1500 µg	*14*	*1*	*1.4*	13[A], 14[A]
		500 µg	*12*	*4*	*1.2*	9[A], 14[A]
		150 µg	*12*	*6*	*1.2*	16[A], 7[A]
		50.0 µg	*9*	*0*	*0.9*	9[A], 9[A]
		15.0 µg	*12*	*1*	*1.2*	11[A], 13[A]
		5.00 µg	*14*	*4*	*1.4*	11[A], 16[A]
		1.50 µg	*9*	*2*	*0.9*	7[A], 10[A]
	Water	100 µL	*10*	*1*		10[A], 9[A]
TA1537	**PFECA G**	5000 µg	*7*	*1*	*1.0*	8[A], 6[A]
		1500 µg	*8*	*4*	*1.1*	10[A], 5[A]
		500 µg	*8*	*4*	*1.1*	5[A], 11[A]
		150 µg	*12*	*4*	*1.7*	9[A], 15[A]
		50.0 µg	*7*	*6*	*1.0*	11[A], 3[A]
		15.0 µg	*10*	*4*	*1.4*	13[A], 7[A]
		5.00 µg	*3*	*3*	*0.4*	1[A], 5[A]
		1.50 µg	*6*	*4*	*0.9*	3[A], 8[A]
	Water	100 µL	*7*	*1*		6[A], 8[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PJ.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PJ
Date Plated: 5/31/2018
Evaluation Period: 6/4/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**PFECA G**	5000 µg	*34*	*2*	*1.0*	35[A], 32[A]
		1500 µg	*29*	*2*	*0.9*	27[A], 30[A]
		500 µg	*29*	*6*	*0.9*	33[A], 24[A]
		150 µg	*38*	*7*	*1.1*	33[A], 43[A]
		50.0 µg	*33*	*1*	*1.0*	32[A], 34[A]
		15.0 µg	*30*	*7*	*0.9*	35[A], 25[A]
		5.00 µg	*34*	*1*	*1.0*	34[A], 33[A]
		1.50 µg	*41*	*4*	*1.2*	38[A], 44[A]
	Water	100 µL	*34*	*12*		42[A], 25[A]
TA98	**2AA**	1.00 µg	*234*	*23*	*9.0*	250[A], 218[A]
TA100	**2AA**	2.00 µg	*709*	*320*	*7.8*	482[A], 935[A]
TA1535	**2AA**	1.00 µg	*90*	*11*	*9.0*	82[A], 97[A]
TA1537	**2AA**	2.00 µg	*53*	*16*	*7.6*	64[A], 41[A]
WP2uvrA	**2AA**	15.0 µg	*286*	*19*	*8.4*	272[A], 299[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PJ.503.BTL
Study Code: AF28PJ
Experiment: B2
Date Plated: 6/13/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/18/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA G**	5000 µg	*24*	*2*	*1.1*	24^{A}, 26^{A}, 22^{A}
		1500 µg	*34*	*8*	*1.5*	28^{A}, 30^{A}, 43^{A}
		500 µg	*28*	*2*	*1.3*	27^{A}, 30^{A}, 28^{A}
		150 µg	*20*	*4*	*0.9*	24^{A}, 21^{A}, 16^{A}
		50.0 µg	*20*	*4*	*0.9*	24^{A}, 17^{A}, 20^{A}
	Water	100 µL	*22*	*3*		19^{A}, 22^{A}, 24^{A}
TA100	**PFECA G**	5000 µg	*99*	*15*	*1.1*	87^{A}, 116^{A}, 93^{A}
		1500 µg	*98*	*11*	*1.1*	110^{A}, 88^{A}, 97^{A}
		500 µg	*92*	*6*	*1.0*	88^{A}, 99^{A}, 89^{A}
		150 µg	*96*	*10*	*1.0*	103^{A}, 100^{A}, 85^{A}
		50.0 µg	*95*	*13*	*1.0*	99^{A}, 105^{A}, 80^{A}
	Water	100 µL	*92*	*3*		94^{A}, 88^{A}, 93^{A}
TA1535	**PFECA G**	5000 µg	*17*	*5*	*1.5*	21^{A}, 12^{A}, 19^{A}
		1500 µg	*19*	*2*	*1.7*	19^{A}, 20^{A}, 17^{A}
		500 µg	*17*	*5*	*1.5*	14^{A}, 14^{A}, 22^{A}
		150 µg	*14*	*7*	*1.3*	17^{A}, 19^{A}, 6^{A}
		50.0 µg	*13*	*3*	*1.2*	17^{A}, 11^{A}, 12^{A}
	Water	100 µL	*11*	*6*		6^{A}, 10^{A}, 17^{A}
TA1537	**PFECA G**	5000 µg	*10*	*4*	*0.8*	11^{A}, 6^{A}, 14^{A}
		1500 µg	*11*	*3*	*0.8*	14^{A}, 10^{A}, 9^{A}
		500 µg	*12*	*3*	*0.9*	10^{A}, 10^{A}, 15^{A}
		150 µg	*9*	*2*	*0.7*	10^{A}, 6^{A}, 10^{A}
		50.0 µg	*11*	*4*	*0.8*	15^{A}, 11^{A}, 7^{A}
	Water	100 µL	*13*	*2*		15^{A}, 12^{A}, 12^{A}
WP2uvrA	**PFECA G**	5000 µg	*33*	*4*	*1.1*	30^{A}, 33^{A}, 37^{A}
		1500 µg	*32*	*4*	*1.0*	30^{A}, 30^{A}, 37^{A}
		500 µg	*32*	*1*	*1.0*	31^{A}, 32^{A}, 33^{A}
		150 µg	*30*	*3*	*1.0*	27^{A}, 30^{A}, 32^{A}
		50.0 µg	*29*	*5*	*0.9*	35^{A}, 28^{A}, 25^{A}
	Water	100 µL	*31*	*4*		33^{A}, 33^{A}, 26^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PJ.503.BTL
Study Code: AF28PJ
Experiment: B2
Date Plated: 6/13/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/18/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**2NF**	1.00 µg	*82*	*12*	*3.7*	69[A], 85[A], 92[A]
TA100	**SA**	1.00 µg	*498*	*11*	*5.4*	490[A], 493[A], 510[A]
TA1535	**SA**	1.00 µg	*768*	*44*	*69.8*	724[A], 768[A], 812[A]
TA1537	**9AAD**	75.0 µg	*727*	*56*	*55.9*	755[A], 763[A], 662[A]
WP2uvrA	**MMS**	1000 µg	*516*	*38*	*16.6*	508[A], 483[A], 557[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PJ.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PJ
Date Plated: 6/13/2018
Evaluation Period: 6/18/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**PFECA G**	5000 µg	*28*	*5*	*1.0*	22[A], 31[A], 31[A]
		1500 µg	*31*	*7*	*1.1*	37[A], 31[A], 24[A]
		500 µg	*31*	*3*	*1.1*	33[A], 33[A], 27[A]
		150 µg	*36*	*5*	*1.3*	36[A], 31[A], 41[A]
		50.0 µg	*39*	*8*	*1.4*	33[A], 48[A], 36[A]
	Water	100 µL	*27*	*5*		22[A], 28[A], 32[A]
TA100	**PFECA G**	5000 µg	*122*	*3*	*1.1*	121[A], 119[A], 125[A]
		1500 µg	*110*	*15*	*1.0*	93[A], 113[A], 123[A]
		500 µg	*125*	*8*	*1.1*	116[A], 130[A], 130[A]
		150 µg	*119*	*9*	*1.0*	111[A], 128[A], 118[A]
		50.0 µg	*106*	*4*	*0.9*	104[A], 110[A], 103[A]
	Water	100 µL	*115*	*15*		100[A], 129[A], 115[A]
TA1535	**PFECA G**	5000 µg	*12*	*2*	*1.0*	12[A], 14[A], 11[A]
		1500 µg	*13*	*2*	*1.1*	12[A], 15[A], 11[A]
		500 µg	*15*	*4*	*1.3*	17[A], 17[A], 10[A]
		150 µg	*14*	*2*	*1.2*	16[A], 12[A], 14[A]
		50.0 µg	*11*	*2*	*0.9*	11[A], 12[A], 9[A]
	Water	100 µL	*12*	*3*		15[A], 10[A], 12[A]
TA1537	**PFECA G**	5000 µg	*16*	*4*	*1.1*	19[A], 17[A], 12[A]
		1500 µg	*14*	*2*	*1.0*	14[A], 15[A], 12[A]
		500 µg	*17*	*2*	*1.2*	19[A], 16[A], 17[A]
		150 µg	*14*	*2*	*1.0*	16[A], 15[A], 12[A]
		50.0 µg	*14*	*3*	*1.0*	12[A], 12[A], 17[A]
	Water	100 µL	*14*	*4*		17[A], 10[A], 14[A]
WP2uvrA	**PFECA G**	5000 µg	*37*	*5*	*1.1*	42[A], 37[A], 32[A]
		1500 µg	*36*	*6*	*1.1*	41[A], 36[A], 30[A]
		500 µg	*37*	*3*	*1.1*	35[A], 37[A], 40[A]
		150 µg	*38*	*4*	*1.2*	43[A], 36[A], 35[A]
		50.0 µg	*38*	*3*	*1.2*	41[A], 37[A], 35[A]
	Water	100 µL	*33*	*6*		32[A], 40[A], 28[A]
TA98	**2AA**	1.00 µg	*246*	*33*	*9.1*	279[A], 244[A], 214[A]
TA100	**2AA**	2.00 µg	*680*	*19*	*5.9*	702[A], 672[A], 666[A]
TA1535	**2AA**	1.00 µg	*83*	*16*	*6.9*	84[A], 98[A], 67[A]
TA1537	**2AA**	2.00 µg	*72*	*9*	*5.1*	62[A], 77[A], 78[A]
WP2uvrA	**2AA**	15.0 µg	*333*	*20*	*10.1*	311[A], 338[A], 349[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values
2016
revertants per plate

Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	

SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control

14. APPENDIX II: Study Protocol and Amendment

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PJ.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 1500, 500, 150 and 50.0 µg per plate

   Reason: To specify the dose levels to be used for the confirmatory mutagenicity assay based on the toxicity and precipitate profiles observed in the initial toxicity-mutation assay.

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PJ.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, Ph.D., DABT
Sponsor Representative

11 June 2018
Date

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PJ.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS
BioReliance Study Director

08 JUN 2018
Date

BioReliance Study Management

08- JUN- 2018
Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF28PJ.503.BTL

1. KEY PERSONNEL

Sponsor Information:

Sponsor	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor Number	C30049
Sponsor's Authorized Representative	Shawn Gannon, Ph.D., DABT The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899 Phone: 302-773-1376 Email: SHAWN.A.GANNON@chemours.com

Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	31-May-2018
Proposed Experimental Completion Date	27-June-2018
Proposed Report Date	12-July-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST SUBSTANCE INFORMATION

Identification	PFECA G
CAS No.	801212-59-9
Storage Conditions	Room Temperature Protect from light (Per BioReliance SOP)
Purity	99% (no correction factor will be used for dose formulations)
Molecular Weight	380.06 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused Test Substance will be returned to the sponsor prior to report finalization using the information below; unless the test substance is used on another study.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

Residual dose formulations will be discarded after use.

7. TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles witb no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values
Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of

revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains

- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^{+} reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

Shawn Gannon, Ph.D., DABT
Sponsor Representative

8 May 2018
Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 24 MAY 2018

BioReliance Study Management — Date: 24-MAY-18

15. APPENDIX III: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test for Induction of: Reverse mutation in bacterial cells

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

Metabolizing System: Aroclor-induced rat liver S9

Vehicle for Test Substance: Water

Treatment: Plate incorporation

Cytotoxic Effects: None

Genotoxic Effects: None

No. of Independent Assays: 2

No. of Replicate Cultures: 2 (B1) and 3 (B2)

Test Substance: PFECA G

Study No.: AF28PJ.503.BTL

No. Cells Analyzed/Culture: 1.1 to 3.1 x 10^8 cells per plate

GLP Compliance: Yes

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Date(s) of Treatment: 31 May 2018 (B1) and 13 June 2018 (B2)

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B1: Initial Toxicity-Mutation Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	12 ± 8	73 ± 1	11 ± 4	7 ± 1	30 ± 6
	PFECA G	1.50	16 ± 4	92 ± 5	12 ± 1	7 ± 2	35 ± 7
		5.00	15 ± 1	74 ± 13	7 ± 1	8 ± 1	41 ± 8
		15.0	14 ± 6	74 ± 8	14 ± 0	7 ± 3	39 ± 0
		50.0	12 ± 1	81 ± 1	14 ± 4	8 ± 4	41 ± 11
		150	13 ± 2	86 ± 8	9 ± 3	5 ± 0	34 ± 1
		500	13 ± 2	86 ± 6	19 ± 12	10 ± 1	30 ± 4
		1500	10 ± 4	87 ± 4	14 ± 4	7 ± 5	28 ± 6
		5000	9 ± 1	84 ± 5	11 ± 6	7 ± 1	17 ± 2
	2NF	1.00	85 ± 18				
	SA	1.00		756 ± 55	679 ± 33		
	9AAD	75.0				362 ± 156	
	MMS	1000					513 ± 43
With Activation	Water	100 µL/plate	26 ± 6	91 ± 4	10 ± 1	7 ± 1	34 ± 12
	PFECA G	1.50	18 ± 1	95 ± 6	9 ± 2	6 ± 4	41 ± 4
		5.00	19 ± 7	87 ± 9	14 ± 4	3 ± 3	34 ± 1
		15.0	20 ± 4	84 ± 23	12 ± 1	10 ± 4	30 ± 7
		50.0	22 ± 1	103 ± 6	9 ± 0	7 ± 6	33 ± 1
		150	17 ± 0	97 ± 20	12 ± 6	12 ± 4	38 ± 7
		500	24 ± 2	97 ± 5	12 ± 4	8 ± 4	29 ± 6
		1500	26 ± 13	107 ± 11	14 ± 1	8 ± 4	29 ± 2
		5000	19 ± 3	117 ± 27	14 ± 4	7 ± 1	34 ± 2
	2AA	1.00	234 ± 23		90 ± 11		
	2AA	2.00		709 ± 320		53 ± 16	
	2AA	15.0					286 ± 19

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B2: Confirmatory Mutagenicity Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	22 ± 3	92 ± 3	11 ± 6	13 ± 2	31 ± 4
	PFECA G	50.0	20 ± 4	95 ± 13	13 ± 3	11 ± 4	29 ± 5
		150	20 ± 4	96 ± 10	14 ± 7	9 ± 2	30 ± 3
		500	28 ± 2	92 ± 6	17 ± 5	12 ± 3	32 ± 1
		1500	34 ± 8	98 ± 11	19 ± 2	11 ± 3	32 ± 4
		5000	24 ± 2	99 ± 15	17 ± 5	10 ± 4	33 ± 4
	2NF	1.00	82 ± 12				
	SA	1.00		498 ± 11	768 ± 44		
	9AAD	75.0				727 ± 56	
	MMS	1000					516 ± 38
With Activation	Water	100 µL/plate	27 ± 5	115 ± 15	12 ± 3	14 ± 4	33 ± 6
	PFECA G	50.0	39 ± 8	106 ± 4	11 ± 2	14 ± 3	38 ± 3
		150	36 ± 5	119 ± 9	14 ± 2	14 ± 2	38 ± 4
		500	31 ± 3	125 ± 8	15 ± 4	17 ± 2	37 ± 3
		1500	31 ± 7	110 ± 15	13 ± 2	14 ± 2	36 ± 6
		5000	28 ± 5	122 ± 3	12 ± 2	16 ± 4	37 ± 5
	2AA	1.00	246 ± 33		83 ± 16		
	2AA	2.00		680 ± 19		72 ± 9	
	2AA	15.0					333 ± 20

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

Sodium salt of Hydrolyzed TAF n=4

Author

Emily Dakoulas, BS

Study Completion Date

22 August 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF28PK.503.BTL

Sponsor

The Chemours Company
1007 Market Street D-3008
Wilmington, DE 19899

Sponsor Number

C30049

1. STATEMENT OF COMPLIANCE

Study No. AF28PK.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

23AUG2018
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF28PK.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
20-Jun-2018	20-Jun-2018	Scoring	20-Jun-2018	20-Jun-2018
20-Jun-2018	27-Jun-2018	Protocol Review	28-Jun-2018	28-Jun-2018
09-Jul-2018	09-Jul-2018	Data/Draft Report	09-Jul-2018	09-Jul-2018
17-Aug-2018	17-Aug-2018	Final Report	17-Aug-2018	17-Aug-2018
17-Aug-2018	17-Aug-2018	Protocol Amendment Review	17-Aug-2018	17-Aug-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Jeannie Eberle 21-Aug-2018 5:34 pm GMT

Reason for signature: QA Approval

Printed by:Jeannie Eberle
Printed on:21-Aug-18

3. TABLE OF CONTENTS

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4. STUDY INFORMATION

Study Conduct

Sponsor:	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor's Authorized Representative:	Shawn Gannon, Ph.D., DABT
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF28PK.503.BTL
Sponsor No.:	C30049

Test Substance

Identification:	Sodium salt of Hydrolyzed TAF n=4
CAS No.:	39492-91-6
Purity:	99% (per protocol)
Molecular Weight:	466.04 g/mol
Description:	Off-white solid
Storage Conditions:	Room temperature, protected from light
Receipt Date:	02 May 2018

Study Dates

Study Initiation Date:	30 May 2018
Experimental Starting Date (first day of data collection):	30 May 2018
Experimental Start Date (first day test substance administered to test system):	01 June 2018
Experimental Completion Date:	20 June 2018

Key Personnel

Study Director:	Emily Dakoulas, BS

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, Sodium salt of Hydrolyzed TAF n=4, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Water was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. No precipitate was observed. Toxicity was observed at 5000 µg per plate with tester strains TA100 and TA1535 in the presence and absence of S9 activation. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Based upon these results, the maximum dose tested in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, the dose levels tested were 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. No precipitate was observed. Toxicity was observed at 5000 µg per plate with tester strains TA100 and TA1535 in the presence and absence of S9 activation. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate Sodium salt of Hydrolyzed TAF n=4 was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization using the information below.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

The vehicle used to deliver Sodium salt of Hydrolyzed TAF n=4 to the test system was water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Water	7732-18-5	Sigma-Aldrich	RNBF9658	Sterile-filtered	Mar 2019

To achieve a solution, the most concentrated dilution was sonicated at 20.9 to 33.8ºC for 2 minutes in each assay. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Water was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in water at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to $0.3x10^9$ cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3925, Exp. Date: 21 Feb 2020; Lot No. 3961, Exp. Date: 15 May 2020) was purchased

commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and six dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

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Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	1.2	1.1	1.5	1.6	2.8
B2	1.3	1.0	1.3	1.0	2.7

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in water are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

No precipitate was observed. Toxicity was observed at 5000 µg per plate with tester strains TA100 and TA1535 in the presence and absence of S9 activation.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate.

No precipitate was observed. Toxicity was observed at 5000 µg per plate with tester strains TA100 and TA1535 in the presence and absence of S9 activation. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

A copy of the Common Technical Document Tables is included in Appendix III.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Sodium salt of Hydrolyzed TAF n=4 did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PK.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PK
Date Plated: 6/1/2018
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*11*	*4*	*0.8*	13[A], 8[A]
		1500 µg	*15*	*2*	*1.1*	13[A], 16[A]
		500 µg	*14*	*6*	*1.0*	18[A], 10[A]
		150 µg	*15*	*5*	*1.1*	18[A], 11[A]
		50.0 µg	*14*	*5*	*1.0*	10[A], 17[A]
		15.0 µg	*17*	*1*	*1.2*	16[A], 18[A]
		5.00 µg	*17*	*0*	*1.2*	17[A], 17[A]
		1.50 µg	*12*	*6*	*0.9*	16[A], 7[A]
	Water	100 µL	*14*	*6*		18[A], 10[A]
TA100	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*73*	*15*	*0.9*	83[A] 3, 62[A] 3
		1500 µg	*72*	*23*	*0.9*	88[A], 56[A]
		500 µg	*85*	*16*	*1.0*	74[A], 96[A]
		150 µg	*86*	*4*	*1.0*	83[A], 89[A]
		50.0 µg	*94*	*10*	*1.1*	101[A], 87[A]
		15.0 µg	*79*	*18*	*0.9*	92[A], 66[A]
		5.00 µg	*87*	*11*	*1.0*	79[A], 95[A]
		1.50 µg	*84*	*0*	*1.0*	84[A], 84[A]
	Water	100 µL	*84*	*11*		76[A], 92[A]
TA1535	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*7*	*0*	*0.5*	7[A] 3, 7[A] 3
		1500 µg	*8*	*0*	*0.6*	8[A], 8[A]
		500 µg	*15*	*0*	*1.1*	15[A], 15[A]
		150 µg	*11*	*1*	*0.8*	11[A], 10[A]
		50.0 µg	*10*	*0*	*0.7*	10[A], 10[A]
		15.0 µg	*9*	*1*	*0.6*	8[A], 10[A]
		5.00 µg	*8*	*1*	*0.6*	7[A], 9[A]
		1.50 µg	*15*	*1*	*1.1*	15[A], 14[A]
	Water	100 µL	*14*	*4*		16[A], 11[A]

Key to Plate Postfix Codes

3 Moderately reduced background

Key to Automatic Count Flags

[A]: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PK.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PK
Date Plated: 6/1/2018
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*7*	*1*	*1.0*	8^{A} 2, 6^{A} 2
		1500 µg	*6*	*0*	*0.9*	6^{A}, 6^{A}
		500 µg	*11*	*6*	*1.6*	7^{A}, 15^{A}
		150 µg	*7*	*2*	*1.0*	8^{A}, 5^{A}
		50.0 µg	*6*	*4*	*0.9*	3^{A}, 9^{A}
		15.0 µg	*6*	*0*	*0.9*	6^{A}, 6^{A}
		5.00 µg	*9*	*1*	*1.3*	8^{A}, 9^{A}
		1.50 µg	*7*	*3*	*1.0*	5^{A}, 9^{A}
	Water	100 µL	*7*	*1*		6^{A}, 8^{A}
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*17*	*8*	*0.5*	11^{A}, 22^{A}
		1500 µg	*23*	*5*	*0.7*	26^{A}, 19^{A}
		500 µg	*29*	*6*	*0.9*	24^{A}, 33^{A}
		150 µg	*30*	*6*	*0.9*	25^{A}, 34^{A}
		50.0 µg	*36*	*4*	*1.1*	39^{A}, 33^{A}
		15.0 µg	*29*	*5*	*0.9*	32^{A}, 25^{A}
		5.00 µg	*21*	*4*	*0.6*	24^{A}, 18^{A}
		1.50 µg	*33*	*1*	*1.0*	32^{A}, 34^{A}
	Water	100 µL	*34*	*4*		36^{A}, 31^{A}
TA98	**2NF**	1.00 µg	*71*	*4*	*5.1*	73^{A}, 68^{A}
TA100	**SA**	1.00 µg	*701*	*46*	*8.3*	668^{A}, 733^{A}
TA1535	**SA**	1.00 µg	*679*	*11*	*48.5*	671^{A}, 687^{A}
TA1537	**9AAD**	75.0 µg	*591*	*56*	*84.4*	630^{A}, 551^{A}
WP2uvrA	**MMS**	1000 µg	*419*	*8*	*12.3*	413^{A}, 425^{A}

Key to Positive Controls	
2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Plate Postfix Codes	
2	Slightly reduced background

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PK.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PK
Date Plated: 6/1/2018
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*20*	*9*	*1.0*	26^{A}, 13^{A}
		1500 µg	*20*	*3*	*1.0*	22^{A}, 18^{A}
		500 µg	*25*	*9*	*1.3*	31^{A}, 18^{A}
		150 µg	*21*	*4*	*1.1*	24^{A}, 18^{A}
		50.0 µg	*23*	*6*	*1.2*	19^{A}, 27^{A}
		15.0 µg	*16*	*1*	*0.8*	15^{A}, 17^{A}
		5.00 µg	*21*	*2*	*1.1*	22^{A}, 19^{A}
		1.50 µg	*26*	*1*	*1.3*	25^{A}, 26^{A}
	Water	100 µL	*20*	*4*		17^{A}, 22^{A}
TA100	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*94*	*7*	*0.9*	89^{A} 3, 99^{A} 3
		1500 µg	*112*	*17*	*1.1*	124^{A}, 100^{A}
		500 µg	*119*	*2*	*1.2*	117^{A}, 120^{A}
		150 µg	*122*	*2*	*1.2*	123^{A}, 120^{A}
		50.0 µg	*96*	*6*	*1.0*	100^{A}, 91^{A}
		15.0 µg	*99*	*9*	*1.0*	105^{A}, 92^{A}
		5.00 µg	*119*	*8*	*1.2*	113^{A}, 124^{A}
		1.50 µg	*105*	*20*	*1.1*	91^{A}, 119^{A}
	Water	100 µL	*99*	*1*		98^{A}, 99^{A}
TA1535	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*7*	*2*	*0.5*	5^{A} 3, 8^{A} 3
		1500 µg	*13*	*0*	*0.9*	13^{A}, 13^{A}
		500 µg	*15*	*2*	*1.1*	13^{A}, 16^{A}
		150 µg	*9*	*1*	*0.6*	10^{A}, 8^{A}
		50.0 µg	*13*	*2*	*0.9*	11^{A}, 14^{A}
		15.0 µg	*16*	*2*	*1.1*	17^{A}, 14^{A}
		5.00 µg	*16*	*4*	*1.1*	18^{A}, 13^{A}
		1.50 µg	*19*	*7*	*1.4*	24^{A}, 14^{A}
	Water	100 µL	*14*	*1*		13^{A}, 15^{A}

Key to Plate Postfix Codes

3 Moderately reduced background

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PK.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PK
Date Plated: 6/1/2018
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*8*	*1*	*0.9*	8[A], 7[A]
		1500 µg	*8*	*4*	*0.9*	5[A], 10[A]
		500 µg	*11*	*3*	*1.2*	13[A], 9[A]
		150 µg	*7*	*1*	*0.8*	6[A], 7[A]
		50.0 µg	*7*	*1*	*0.8*	6[A], 8[A]
		15.0 µg	*7*	*2*	*0.8*	5[A], 8[A]
		5.00 µg	*10*	*4*	*1.1*	13[A], 7[A]
		1.50 µg	*6*	*6*	*0.7*	2[A], 10[A]
	Water	100 µL	*9*	*0*		9[A], 9[A]
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*28*	*7*	*0.8*	23[A], 33[A]
		1500 µg	*23*	*12*	*0.6*	31[A], 14[A]
		500 µg	*31*	*8*	*0.9*	25[A], 36[A]
		150 µg	*40*	*0*	*1.1*	40[A], 40[A]
		50.0 µg	*31*	*6*	*0.9*	35[A], 26[A]
		15.0 µg	*32*	*15*	*0.9*	42[A], 21[A]
		5.00 µg	*35*	*6*	*1.0*	39[A], 30[A]
		1.50 µg	*35*	*8*	*1.0*	40[A], 29[A]
	Water	100 µL	*36*	*3*		34[A], 38[A]
TA98	**2AA**	1.00 µg	*232*	*9*	*11.6*	225[A], 238[A]
TA100	**2AA**	2.00 µg	*949*	*40*	*9.6*	920[A], 977[A]
TA1535	**2AA**	1.00 µg	*69*	*3*	*4.9*	67[A], 71[A]
TA1537	**2AA**	2.00 µg	*36*	*8*	*4.0*	30[A], 42[A]
WP2uvrA	**2AA**	15.0 µg	*287*	*17*	*8.0*	299[A], 275[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PK.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PK
Date Plated: 6/14/2018
Evaluation Period: 6/20/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*13*	*5*	*0.8*	17^{A}, 15^{A}, 8^{A}
		1500 µg	*16*	*2*	*1.0*	17^{A}, 14^{A}, 16^{A}
		500 µg	*18*	*4*	*1.1*	15^{A}, 17^{A}, 23^{A}
		150 µg	*19*	*7*	*1.2*	27^{A}, 15^{A}, 14^{A}
		50.0 µg	*15*	*4*	*0.9*	19^{A}, 15^{A}, 11^{A}
		15.0 µg	*17*	*5*	*1.1*	13^{A}, 23^{A}, 15^{A}
	Water	100 µL	*16*	*2*		18^{A}, 15^{A}, 16^{A}
TA100	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*69*	*13*	*0.8*	73^{A} 3, 80^{A} 3, 54^{A} 3
		1500 µg	*88*	*4*	*1.0*	84^{A}, 88^{A}, 91^{A}
		500 µg	*87*	*16*	*1.0*	97^{A}, 68^{A}, 96^{A}
		150 µg	*82*	*9*	*0.9*	91^{A}, 74^{A}, 81^{A}
		50.0 µg	*89*	*3*	*1.0*	89^{A}, 87^{A}, 92^{A}
		15.0 µg	*89*	*13*	*1.0*	80^{A}, 103^{A}, 83^{A}
	Water	100 µL	*89*	*6*		83^{A}, 95^{A}, 90^{A}
TA1535	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*12*	*3*	*1.1*	14^{A} 3, 9^{A} 3, 14^{A} 3
		1500 µg	*13*	*2*	*1.2*	14^{A}, 11^{A}, 14^{A}
		500 µg	*8*	*1*	*0.7*	8^{A}, 9^{A}, 8^{A}
		150 µg	*9*	*1*	*0.8*	10^{A}, 10^{A}, 8^{A}
		50.0 µg	*14*	*3*	*1.3*	10^{A}, 15^{A}, 16^{A}
		15.0 µg	*9*	*5*	*0.8*	8^{A}, 5^{A}, 14^{A}
	Water	100 µL	*11*	*6*		6^{A}, 10^{A}, 17^{A}
TA1537	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*8*	*3*	*1.1*	7^{A} 2, 11^{A} 2, 6^{A} 2
		1500 µg	*6*	*1*	*0.9*	6^{A}, 6^{A}, 5^{A}
		500 µg	*6*	*2*	*0.9*	8^{A}, 6^{A}, 5^{A}
		150 µg	*8*	*4*	*1.1*	10^{A}, 3^{A}, 11^{A}
		50.0 µg	*6*	*1*	*0.9*	7^{A}, 5^{A}, 5^{A}
		15.0 µg	*5*	*3*	*0.7*	6^{A}, 7^{A}, 1^{A}
	Water	100 µL	*7*	*2*		6^{A}, 10^{A}, 6^{A}

Key to Plate Postfix Codes

3	Moderately reduced background
2	Slightly reduced background

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PK.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PK
Date Plated: 6/14/2018
Evaluation Period: 6/20/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*19*	*6*	*0.6*	25[A], 13[A], 18[A]
		1500 µg	*30*	*2*	*1.0*	32[A], 30[A], 29[A]
		500 µg	*36*	*3*	*1.2*	39[A], 35[A], 33[A]
		150 µg	*32*	*8*	*1.0*	39[A], 24[A], 33[A]
		50.0 µg	*32*	*9*	*1.0*	34[A], 39[A], 22[A]
		15.0 µg	*35*	*2*	*1.1*	35[A], 36[A], 33[A]
	Water	100 µL	*31*	*3*		34[A], 30[A], 29[A]
TA98	**2NF**	1.00 µg	*84*	*18*	*5.3*	99[A], 64[A], 88[A]
TA100	**SA**	1.00 µg	*717*	*39*	*8.1*	727[A], 674[A], 750[A]
TA1535	**SA**	1.00 µg	*721*	*23*	*65.5*	694[A], 732[A], 736[A]
TA1537	**9AAD**	75.0 µg	*834*	*174*	*119.1*	1035[A], 749[A], 719[A]
WP2uvrA	**MMS**	1000 µg	*503*	*15*	*16.2*	507[A], 486[A], 516[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PK.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PK
Date Plated: 6/14/2018
Evaluation Period: 6/20/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*16*	*3*	*0.6*	19[A], 14[A], 16[A]
		1500 µg	*25*	*3*	*1.0*	27[A], 25[A], 22[A]
		500 µg	*25*	*2*	*1.0*	23[A], 27[A], 25[A]
		150 µg	*22*	*9*	*0.9*	32[A], 21[A], 14[A]
		50.0 µg	*28*	*4*	*1.1*	31[A], 23[A], 30[A]
		15.0 µg	*21*	*4*	*0.8*	18[A], 25[A], 21[A]
	Water	100 µL	*25*	*8*		22[A], 18[A], 34[A]
TA100	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*93*	*13*	*1.0*	105[A] 3, 96[A] 3, 79[A] 3
		1500 µg	*114*	*11*	*1.2*	127[A], 107[A], 109[A]
		500 µg	*106*	*8*	*1.1*	107[A], 113[A], 97[A]
		150 µg	*107*	*7*	*1.1*	101[A], 106[A], 115[A]
		50.0 µg	*91*	*11*	*0.9*	103[A], 88[A], 82[A]
		15.0 µg	*102*	*8*	*1.1*	105[A], 108[A], 93[A]
	Water	100 µL	*97*	*9*		104[A], 100[A], 86[A]
TA1535	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*11*	*4*	*0.9*	8[A] 3, 15[A] 3, 11[A] 3
		1500 µg	*14*	*5*	*1.2*	16[A], 17[A], 8[A]
		500 µg	*9*	*2*	*0.8*	7[A], 9[A], 11[A]
		150 µg	*12*	*6*	*1.0*	6[A], 15[A], 16[A]
		50.0 µg	*11*	*0*	*0.9*	11[A], 11[A], 11[A]
		15.0 µg	*15*	*7*	*1.3*	21[A], 7[A], 16[A]
	Water	100 µL	*12*	*3*		11[A], 10[A], 16[A]
TA1537	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*11*	*5*	*1.0*	15[A], 6[A], 13[A]
		1500 µg	*10*	*4*	*0.9*	7[A], 15[A], 9[A]
		500 µg	*8*	*2*	*0.7*	8[A], 7[A], 10[A]
		150 µg	*6*	*2*	*0.5*	8[A], 6[A], 5[A]
		50.0 µg	*8*	*2*	*0.7*	7[A], 10[A], 7[A]
		15.0 µg	*10*	*1*	*0.9*	9[A], 9[A], 11[A]
	Water	100 µL	*11*	*3*		14[A], 11[A], 9[A]

Key to Plate Postfix Codes

3 Moderately reduced background

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4 (CONT.)
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PK.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PK
Date Plated: 6/14/2018
Evaluation Period: 6/20/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=4**	5000 µg	*30*	*5*	*0.9*	35[A], 29[A], 25[A]
		1500 µg	*30*	*0*	*0.9*	30[A], 30[A], 30[A]
		500 µg	*30*	*5*	*0.9*	35[A], 31[A], 25[A]
		150 µg	*33*	*1*	*1.0*	34[A], 34[A], 32[A]
		50.0 µg	*26*	*4*	*0.8*	24[A], 24[A], 31[A]
		15.0 µg	*38*	*3*	*1.2*	35[A], 40[A], 38[A]
	Water	100 µL	*33*	*2*		34[A], 31[A], 34[A]
TA98	**2AA**	1.00 µg	*228*	*20*	*9.1*	205[A], 241[A], 237[A]
TA100	**2AA**	2.00 µg	*827*	*43*	*8.5*	797[A], 808[A], 876[A]
TA1535	**2AA**	1.00 µg	*98*	*16*	*8.2*	117[A], 91[A], 87[A]
TA1537	**2AA**	2.00 µg	*47*	*2*	*4.3*	46[A], 46[A], 50[A]
WP2uvrA	**2AA**	15.0 µg	*380*	*4*	*11.5*	384[A], 376[A], 381[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values 2016 revertants per plate											
Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	
SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control											

14. APPENDIX II: Study Protocol and Amendment

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PK.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 1500, 500, 150, 50.0 and 15.0 µg per plate.

   Reason: To specify the dose levels to be used for the confirmatory mutagenicity assay based on the toxicity and precipitate profiles observed in the initial toxicity mutation assay.

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PK.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, Ph.D., DABT
Sponsor Representative

13 June 2018
Date

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PK.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS — 12 JUN 2018 — Date
BioReliance Study Director

BioReliance Study Management — 12-JUN-2018 — Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF28PK.503.BTL

1. KEY PERSONNEL

Sponsor Information:

Sponsor	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor Number	C30049
Sponsor's Authorized Representative	Shawn Gannon, Ph.D., DABT The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899 Phone: 302-773-1376 Email: SHAWN.A.GANNON@chemours.com

Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	01-June-2018
Proposed Experimental Completion Date	28-June-2018
Proposed Report Date	13-July-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will he audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST SUBSTANCE INFORMATION

Identification	Sodium salt of Hydrolyzed TAF n=4
CAS No.	39492-91-6
Storage Conditions	Room Temperature Protect from light (Per BioReliance SOP)
Purity	99% (no correction factor will be used for dose formulations)
Molecular Weight	466.04 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused Test Substance will be returned to the sponsor prior to report finalization using the information below: unless the test substance is used on another study.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

Residual dose formulations will be discarded after use.

7. **TEST SYSTEM**
The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test article, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test article.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test article, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test article, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test article, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values
Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of

revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test article
- Vehicle
- Strains

- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test article, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

Shawn Gannon, Ph.D., DABT
Sponsor Representative

8 May 2018
Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 30 MAY 2018

BioReliance Study Management — Date: 21-May-18

15. APPENDIX III: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test Substance: Sodium salt of Hydrolyzed TAF n=4

Test for Induction of: Reverse mutation in bacterial cells

No. of Independent Assays: 2

Study No.: AF28PK.503.BTL

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

No. of Replicate Cultures: 2 (B1) and 3 (B2)

No. Cells Analyzed/Culture: 1.0 to 2.8 x 10^8 cells per plate

Metabolizing System: Aroclor-induced rat liver S9

GLP Compliance: Yes

Vehicle for Test Substance: Water

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Treatment: Plate incorporation

Date(s) of Treatment: 01 June 2018 (B1) and 14 June 2018 (B2)

Cytotoxic Effects: Toxicity was observed at 5000 µg per plate with tester strains TA100 and TA1535 in the presence and absence of S9 activation

Genotoxic Effects: None

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B1: Initial Toxicity-Mutation Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	14 ± 6	84 ± 11	14 ± 4	7 ± 1	34 ± 4
	Sodium salt of Hydrolyzed TAF n=4	1.50	12 ± 6	84 ± 0	15 ± 1	7 ± 3	33 ± 1
		5.00	17 ± 0	87 ± 11	8 ± 1	9 ± 1	21 ± 4
		15.0	17 ± 1	79 ± 18	9 ± 1	6 ± 0	29 ± 5
		50.0	14 ± 5	94 ± 10	10 ± 0	6 ± 4	36 ± 4
		150	15 ± 5	86 ± 4	11 ± 1	7 ± 2	30 ± 6
		500	14 ± 6	85 ± 16	15 ± 0	11 ± 6	29 ± 6
		1500	15 ± 2	72 ± 23	8 ± 0	6 ± 0	23 ± 5
		5000	11 ± 4	73 ± 15	7 ± 0	7 ± 1	17 ± 8
	2NF	1.00	71 ± 4				
	SA	1.00		701 ± 46	679 ± 11		
	9AAD	75.0				591 ± 56	
	MMS	1000					419 ± 8
With Activation	Water	100 µL/plate	20 ± 4	99 ± 1	14 ± 1	9 ± 0	36 ± 3
	Sodium salt of Hydrolyzed TAF n=4	1.50	26 ± 1	105 ± 20	19 ± 7	6 ± 6	35 ± 8
		5.00	21 ± 2	119 ± 8	16 ± 4	10 ± 4	35 ± 6
		15.0	16 ± 1	99 ± 9	16 ± 2	7 ± 2	32 ± 15
		50.0	23 ± 6	96 ± 6	13 ± 2	7 ± 1	31 ± 6
		150	21 ± 4	122 ± 2	9 ± 1	7 ± 1	40 ± 0
		500	25 ± 9	119 ± 2	15 ± 2	11 ± 3	31 ± 8
		1500	20 ± 3	112 ± 17	13 ± 0	8 ± 4	23 ± 12
		5000	20 ± 9	94 ± 7	7 ± 2	8 ± 1	28 ± 7
	2AA	1.00	232 ± 9		69 ± 3		
	2AA	2.00		949 ± 40		36 ± 8	
	2AA	15.0					287 ± 17

Key to Positive Controls

SA	sodium azide	2NF	2-nitrofluorene
2AA	2-aminoanthracene	MMS	methyl methanesulfonate
9AAD	9-Aminoacridine		

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B2: Confirmatory Mutagenicity Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	16 ± 2	89 ± 6	11 ± 6	7 ± 2	31 ± 3
	Sodium salt of Hydrolyzed TAF n=4	15.0	17 ± 5	89 ± 13	9 ± 5	5 ± 3	35 ± 2
		50.0	15 ± 4	89 ± 3	14 ± 3	6 ± 1	32 ± 9
		150	19 ± 7	82 ± 9	9 ± 1	8 ± 4	32 ± 8
		500	18 ± 4	87 ± 16	8 ± 1	6 ± 2	36 ± 3
		1500	16 ± 2	88 ± 4	13 ± 2	6 ± 1	30 ± 2
		5000	13 ± 5	69 ± 13	12 ± 3	8 ± 3	19 ± 6
	2NF	1.00	84 ± 18				
	SA	1.00		717 ± 39	721 ± 23		
	9AAD	75.0				834 ± 174	
	MMS	1000					503 ± 15
With Activation	Water	100 µL/plate	25 ± 8	97 ± 9	12 ± 3	11 ± 3	33 ± 2
	Sodium salt of Hydrolyzed TAF n=4	15.0	21 ± 4	102 ± 8	15 ± 7	10 ± 1	38 ± 3
		50.0	28 ± 4	91 ± 11	11 ± 0	8 ± 2	26 ± 4
		150	22 ± 9	107 ± 7	12 ± 6	6 ± 2	33 ± 1
		500	25 ± 2	106 ± 8	9 ± 2	8 ± 2	30 ± 5
		1500	25 ± 3	114 ± 11	14 ± 5	10 ± 4	30 ± 0
		5000	16 ± 3	93 ± 13	11 ± 4	11 ± 5	30 ± 5
	2AA	1.00	228 ± 20		98 ± 16		
	2AA	2.00		827 ± 43		47 ± 2	
	2AA	15.0					380 ± 4

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

Sodium salt of Hydrolyzed TAF n=3

Author

Emily Dakoulas, BS

Study Completion Date

29 August 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF28PL.503.BTL

Sponsor

The Chemours Company
1007 Market Street D-3008
Wilmington, DE 19899

Sponsor Number

C30049

1. STATEMENT OF COMPLIANCE

Study No. AF28PL.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

27AUG.2018
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF28PL.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
12-Jun-2018	12-Jun-2018	Protocol Review	12-Jun-2018	12-Jun-2018
20-Jun-2018	20-Jun-2018	Scoring	20-Jun-2018	20-Jun-2018
09-Jul-2018	09-Jul-2018	Data/Draft Report	09-Jul-2018	09-Jul-2018
21-Aug-2018	21-Aug-2018	Final Report	21-Aug-2018	21-Aug-2018
21-Aug-2018	21-Aug-2018	Protocol Amendment Review	21-Aug-2018	21-Aug-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Luleayenwa Aberra-Degu 28-Aug-2018 8:12 pm GMT

Reason for signature: QA Approval

Printed by:Luleayenwa Aberra-Degu
Printed on:28-Aug-18

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4. STUDY INFORMATION

Study Conduct

Sponsor:	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor's Authorized Representative:	Shawn Gannon, Ph.D., DABT
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF28PL.503.BTL
Sponsor No.:	C30049

Test Substance

Identification:	Sodium salt of Hydrolyzed TAF n=3
CAS No.:	39492-90-5
Purity:	99.4% (per protocol)
Molecular Weight:	400.03 g/mol
Description:	Off-white solid
Storage Conditions:	Room temperature, protected from light
Receipt Date:	02 May 2018

Study Dates

Study Initiation Date:	30 May 2018
Experimental Starting Date (first day of data collection):	30 May 2018
Experimental Start Date (first day test substance administered to test system):	01 June 2018
Experimental Completion Date:	20 June 2018

Key Personnel

Study Director:	Emily Dakoulas, BS

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, Sodium salt of Hydrolyzed TAF n=3, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Water was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Based upon these results, the maximum dose tested in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, the dose levels tested were 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate Sodium salt of Hydrolyzed TAF n=3 was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization using the information below.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

The vehicle used to deliver Sodium salt of Hydrolyzed TAF n=3 to the test system was water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Water	7732-18-5	Sigma-Aldrich	RNBF9658	Sterile-filtered	Mar 2019

To achieve a solution, the most concentrated dilution was sonicated at 22.9ºC for 4 minutes in the initial toxicity-mutation assay. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Water was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in water at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3925, Exp. Date: 21 Feb 2020; Lot No. 3961, Exp. Date: 15 May 2020) was purchased

commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and five dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	1.2	1.1	1.5	1.6	2.8
B2	1.3	1.0	1.3	1.0	2.7

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in water are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

Neither precipitate nor toxicity was observed.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 50.0, 150, 500, 1500 and 5000 µg per plate.

Neither precipitate nor toxicity was observed.

A copy of the Common Technical Document Tables is included in Appendix III.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Sodium salt of Hydrolyzed TAF n=3 did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PL.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PL
Date Plated: 6/1/2018
Evaluation Period: 6/4/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*13*	*6*	*0.8*	17[A], 8[A]
		1500 µg	*18*	*0*	*1.1*	18[A], 18[A]
		500 µg	*18*	*1*	*1.1*	18[A], 17[A]
		150 µg	*12*	*7*	*0.8*	17[A], 7[A]
		50.0 µg	*11*	*1*	*0.7*	11[A], 10[A]
		15.0 µg	*17*	*9*	*1.1*	10[A], 23[A]
		5.00 µg	*14*	*0*	*0.9*	14[A], 14[A]
		1.50 µg	*14*	*1*	*0.9*	15[A], 13[A]
	Water	100 µL	*16*	*3*		14[A], 18[A]
TA100	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*86*	*3*	*1.1*	88[A], 84[A]
		1500 µg	*91*	*4*	*1.2*	88[A], 93[A]
		500 µg	*88*	*18*	*1.2*	75[A], 100[A]
		150 µg	*76*	*4*	*1.0*	78[A], 73[A]
		50.0 µg	*80*	*12*	*1.1*	71[A], 88[A]
		15.0 µg	*83*	*2*	*1.1*	81[A], 84[A]
		5.00 µg	*78*	*5*	*1.0*	74[A], 81[A]
		1.50 µg	*69*	*10*	*0.9*	76[A], 62[A]
	Water	100 µL	*76*	*16*		87[A], 65[A]
TA1535	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*8*	*1*	*0.9*	9[A], 7[A]
		1500 µg	*13*	*5*	*1.4*	9[A], 16[A]
		500 µg	*12*	*2*	*1.3*	10[A], 13[A]
		150 µg	*9*	*1*	*1.0*	9[A], 8[A]
		50.0 µg	*10*	*2*	*1.1*	8[A], 11[A]
		15.0 µg	*12*	*1*	*1.3*	11[A], 13[A]
		5.00 µg	*11*	*1*	*1.2*	11[A], 10[A]
		1.50 µg	*8*	*3*	*0.9*	6[A], 10[A]
	Water	100 µL	*9*	*2*		7[A], 10[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PL.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PL
Date Plated: 6/1/2018
Evaluation Period: 6/4/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*6*	*1*	*1.2*	7[A], 5[A]
		1500 µg	*7*	*1*	*1.4*	7[A], 6[A]
		500 µg	*7*	*1*	*1.4*	6[A], 7[A]
		150 µg	*5*	*4*	*1.0*	2[A], 8[A]
		50.0 µg	*7*	*3*	*1.4*	9[A], 5[A]
		15.0 µg	*5*	*0*	*1.0*	5[A], 5[A]
		5.00 µg	*8*	*1*	*1.6*	9[A], 7[A]
		1.50 µg	*5*	*2*	*1.0*	3[A], 6[A]
	Water	100 µL	*5*	*3*		3[A], 7[A]
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*26*	*4*	*0.9*	23[A], 29[A]
		1500 µg	*33*	*8*	*1.2*	27[A], 38[A]
		500 µg	*28*	*2*	*1.0*	26[A], 29[A]
		150 µg	*23*	*8*	*0.8*	29[A], 17[A]
		50.0 µg	*22*	*6*	*0.8*	26[A], 17[A]
		15.0 µg	*30*	*0*	*1.1*	30[A], 30[A]
		5.00 µg	*33*	*5*	*1.2*	29[A], 36[A]
		1.50 µg	*33*	*1*	*1.2*	32[A], 34[A]
	Water	100 µL	*28*	*1*		29[A], 27[A]
TA98	**2NF**	1.00 µg	*61*	*15*	*3.8*	50[A], 71[A]
TA100	**SA**	1.00 µg	*707*	*21*	*9.3*	721[A], 692[A]
TA1535	**SA**	1.00 µg	*721*	*37*	*80.1*	747[A], 695[A]
TA1537	**9AAD**	75.0 µg	*599*	*177*	*119.8*	724[A], 474[A]
WP2uvrA	**MMS**	1000 µg	*384*	*2*	*13.7*	385[A], 382[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PL.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PL
Date Plated: 6/1/2018
Evaluation Period: 6/4/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*16*	*4*	*1.1*	18[A], 13[A]
		1500 µg	*18*	*1*	*1.2*	17[A], 18[A]
		500 µg	*18*	*5*	*1.2*	21[A], 14[A]
		150 µg	*19*	*3*	*1.3*	17[A], 21[A]
		50.0 µg	*21*	*8*	*1.4*	26[A], 15[A]
		15.0 µg	*16*	*4*	*1.1*	13[A], 18[A]
		5.00 µg	*15*	*5*	*1.0*	11[A], 18[A]
		1.50 µg	*21*	*5*	*1.4*	17[A], 24[A]
	Water	100 µL	*15*	*0*		15[A], 15[A]
TA100	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*114*	*24*	*1.2*	131[A], 97[A]
		1500 µg	*118*	*3*	*1.3*	116[A], 120[A]
		500 µg	*98*	*7*	*1.1*	103[A], 93[A]
		150 µg	*116*	*1*	*1.3*	117[A], 115[A]
		50.0 µg	*109*	*14*	*1.2*	119[A], 99[A]
		15.0 µg	*108*	*6*	*1.2*	112[A], 103[A]
		5.00 µg	*82*	*15*	*0.9*	71[A], 92[A]
		1.50 µg	*100*	*2*	*1.1*	98[A], 101[A]
	Water	100 µL	*92*	*6*		96[A], 88[A]
TA1535	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*14*	*1*	*1.0*	13[A], 15[A]
		1500 µg	*12*	*2*	*0.9*	13[A], 10[A]
		500 µg	*12*	*2*	*0.9*	10[A], 13[A]
		150 µg	*12*	*4*	*0.9*	14[A], 9[A]
		50.0 µg	*13*	*2*	*0.9*	11[A], 14[A]
		15.0 µg	*7*	*1*	*0.5*	7[A], 6[A]
		5.00 µg	*10*	*1*	*0.7*	9[A], 11[A]
		1.50 µg	*14*	*1*	*1.0*	13[A], 14[A]
	Water	100 µL	*14*	*1*		13[A], 15[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PL.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PL
Date Plated: 6/1/2018
Evaluation Period: 6/4/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*11*	*3*	*1.4*	13^{A}, 9^{A}
		1500 µg	*10*	*5*	*1.3*	13^{A}, 6^{A}
		500 µg	*4*	*1*	*0.5*	5^{A}, 3^{A}
		150 µg	*9*	*0*	*1.1*	9^{A}, 9^{A}
		50.0 µg	*7*	*1*	*0.9*	6^{A}, 8^{A}
		15.0 µg	*8*	*4*	*1.0*	10^{A}, 5^{A}
		5.00 µg	*6*	*4*	*0.8*	3^{A}, 8^{A}
		1.50 µg	*9*	*3*	*1.1*	7^{A}, 11^{A}
	Water	100 µL	*8*	*3*		10^{A}, 6^{A}
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*34*	*9*	*1.0*	40^{A}, 27^{A}
		1500 µg	*32*	*0*	*1.0*	32^{A}, 32^{A}
		500 µg	*36*	*4*	*1.1*	33^{A}, 38^{A}
		150 µg	*28*	*6*	*0.8*	24^{A}, 32^{A}
		50.0 µg	*43*	*5*	*1.3*	46^{A}, 39^{A}
		15.0 µg	*30*	*16*	*0.9*	19^{A}, 41^{A}
		5.00 µg	*26*	*2*	*0.8*	24^{A}, 27^{A}
		1.50 µg	*29*	*4*	*0.9*	26^{A}, 32^{A}
	Water	100 µL	*33*	*8*		39^{A}, 27^{A}
TA98	**2AA**	1.00 µg	*232*	*20*	*15.5*	218^{A}, 246^{A}
TA100	**2AA**	2.00 µg	*1243*	*626*	*13.5*	1686^{A}, 800^{A}
TA1535	**2AA**	1.00 µg	*90*	*8*	*6.4*	96^{A}, 84^{A}
TA1537	**2AA**	2.00 µg	*39*	*0*	*4.9*	39^{A}, 39^{A}
WP2uvrA	**2AA**	15.0 µg	*247*	*35*	*7.5*	222^{A}, 272^{A}

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PL.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PL
Date Plated: 6/14/2018
Evaluation Period: 6/20/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*14*	*2*	*1.2*	11^{A}, 15^{A}, 15^{A}
		1500 µg	*14*	*3*	*1.2*	15^{A}, 17^{A}, 11^{A}
		500 µg	*13*	*2*	*1.1*	14^{A}, 13^{A}, 11^{A}
		150 µg	*15*	*0*	*1.3*	15^{A}, 15^{A}, 15^{A}
		50.0 µg	*15*	*2*	*1.3*	13^{A}, 17^{A}, 14^{A}
	Water	100 µL	*12*	*4*		16^{A}, 9^{A}, 11^{A}
TA100	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*98*	*5*	*1.0*	103^{A}, 93^{A}, 98^{A}
		1500 µg	*102*	*8*	*1.1*	104^{A}, 109^{A}, 93^{A}
		500 µg	*98*	*10*	*1.0*	89^{A}, 96^{A}, 108^{A}
		150 µg	*94*	*14*	*1.0*	86^{A}, 111^{A}, 86^{A}
		50.0 µg	*109*	*6*	*1.2*	109^{A}, 115^{A}, 104^{A}
	Water	100 µL	*94*	*12*		106^{A}, 83^{A}, 92^{A}
TA1535	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*14*	*1*	*1.2*	15^{A}, 13^{A}, 13^{A}
		1500 µg	*13*	*6*	*1.1*	13^{A}, 19^{A}, 8^{A}
		500 µg	*8*	*1*	*0.7*	8^{A}, 9^{A}, 8^{A}
		150 µg	*14*	*3*	*1.2*	16^{A}, 16^{A}, 10^{A}
		50.0 µg	*11*	*4*	*0.9*	9^{A}, 8^{A}, 15^{A}
	Water	100 µL	*12*	*4*		16^{A}, 10^{A}, 9^{A}
TA1537	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*6*	*2*	*0.8*	7^{A}, 3^{A}, 7^{A}
		1500 µg	*6*	*3*	*0.8*	8^{A}, 7^{A}, 3^{A}
		500 µg	*6*	*1*	*0.8*	5^{A}, 7^{A}, 6^{A}
		150 µg	*9*	*1*	*1.1*	8^{A}, 8^{A}, 10^{A}
		50.0 µg	*4*	*1*	*0.5*	3^{A}, 5^{A}, 5^{A}
	Water	100 µL	*8*	*2*		9^{A}, 5^{A}, 9^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PL.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PL
Date Plated: 6/14/2018
Evaluation Period: 6/20/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*33*	*3*	*1.1*	35^{A}, 29^{A}, 34^{A}
		1500 µg	*33*	*13*	*1.1*	31^{A}, 46^{A}, 21^{A}
		500 µg	*35*	*7*	*1.2*	43^{A}, 31^{A}, 31^{A}
		150 µg	*37*	*6*	*1.2*	31^{A}, 41^{A}, 40^{A}
		50.0 µg	*39*	*3*	*1.3*	40^{A}, 35^{A}, 41^{A}
	Water	100 µL	*30*	*5*		27^{A}, 36^{A}, 27^{A}
TA98	**2NF**	1.00 µg	*61*	*8*	*5.1*	57^{A}, 57^{A}, 70^{A}
TA100	**SA**	1.00 µg	*741*	*33*	*7.9*	705^{A}, 747^{A}, 771^{A}
TA1535	**SA**	1.00 µg	*718*	*29*	*59.8*	691^{A}, 749^{A}, 715^{A}
TA1537	**9AAD**	75.0 µg	*823*	*128*	*102.9*	677^{A}, 915^{A}, 878^{A}
WP2uvrA	**MMS**	1000 µg	*474*	*38*	*15.8*	431^{A}, 485^{A}, 505^{A}

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PL.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PL
Date Plated: 6/14/2018
Evaluation Period: 6/20/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*19*	*7*	*0.9*	27^{A}, 13^{A}, 17^{A}
		1500 µg	*19*	*4*	*0.9*	19^{A}, 16^{A}, 23^{A}
		500 µg	*22*	*8*	*1.0*	15^{A}, 19^{A}, 31^{A}
		150 µg	*19*	*3*	*0.9*	15^{A}, 21^{A}, 21^{A}
		50.0 µg	*23*	*7*	*1.0*	16^{A}, 23^{A}, 29^{A}
	Water	100 µL	*22*	*6*		16^{A}, 26^{A}, 25^{A}
TA100	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*120*	*4*	*1.3*	123^{A}, 116^{A}, 120^{A}
		1500 µg	*107*	*8*	*1.1*	114^{A}, 107^{A}, 99^{A}
		500 µg	*102*	*6*	*1.1*	107^{A}, 96^{A}, 104^{A}
		150 µg	*113*	*16*	*1.2*	128^{A}, 114^{A}, 97^{A}
		50.0 µg	*89*	*4*	*0.9*	93^{A}, 88^{A}, 86^{A}
	Water	100 µL	*96*	*10*		103^{A}, 101^{A}, 84^{A}
TA1535	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*13*	*6*	*1.2*	14^{A}, 6^{A}, 18^{A}
		1500 µg	*8*	*1*	*0.7*	9^{A}, 7^{A}, 7^{A}
		500 µg	*13*	*5*	*1.2*	17^{A}, 14^{A}, 8^{A}
		150 µg	*9*	*3*	*0.8*	6^{A}, 10^{A}, 11^{A}
		50.0 µg	*13*	*4*	*1.2*	8^{A}, 15^{A}, 16^{A}
	Water	100 µL	*11*	*3*		14^{A}, 8^{A}, 11^{A}
TA1537	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*7*	*3*	*0.8*	5^{A}, 6^{A}, 10^{A}
		1500 µg	*11*	*5*	*1.2*	8^{A}, 17^{A}, 9^{A}
		500 µg	*8*	*4*	*0.9*	3^{A}, 10^{A}, 10^{A}
		150 µg	*11*	*3*	*1.2*	8^{A}, 13^{A}, 13^{A}
		50.0 µg	*11*	*3*	*1.2*	14^{A}, 9^{A}, 10^{A}
	Water	100 µL	*9*	*3*		6^{A}, 11^{A}, 11^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 4 (CONT.)
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PL.503.BTL
Study Code: AF28PL
Experiment: B2
Date Plated: 6/14/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/20/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=3**	5000 µg	*36*	*4*	*1.0*	36[A], 40[A], 33[A]
		1500 µg	*37*	*4*	*1.0*	34[A], 42[A], 36[A]
		500 µg	*37*	*6*	*1.0*	43[A], 32[A], 35[A]
		150 µg	*34*	*6*	*0.9*	36[A], 27[A], 39[A]
		50.0 µg	*39*	*12*	*1.1*	48[A], 26[A], 44[A]
	Water	100 µL	*37*	*3*		39[A], 38[A], 34[A]
TA98	**2AA**	1.00 µg	*239*	*29*	*10.9*	245[A], 264[A], 207[A]
TA100	**2AA**	2.00 µg	*787*	*23*	*8.2*	766[A], 811[A], 783[A]
TA1535	**2AA**	1.00 µg	*66*	*13*	*6.0*	58[A], 60[A], 81[A]
TA1537	**2AA**	2.00 µg	*49*	*11*	*5.4*	56[A], 54[A], 36[A]
WP2uvrA	**2AA**	15.0 µg	*359*	*66*	*9.7*	287[A], 372[A], 418[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values 2016 revertants per plate											
Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	
SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control											

14. APPENDIX II: Study Protocol and Amendment

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PL.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 1500, 500, 150 and 50.0 µg per plate.

   Reason: To specify the dose levels to be used for the confirmatory mutagenicity assay based on the toxicity and precipitate profiles observed in the initial toxicity-mutation assay.

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PL.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, Ph.D., DABT
Sponsor Representative

13 June 2018
Date

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PL.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS
BioReliance Study Director

12 JUN 2018
Date

BioReliance Study Management

12-JUN-2018
Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF28PL.503.BTL

1. KEY PERSONNEL

Sponsor Information:

Sponsor	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor Number	C30049
Sponsor's Authorized Representative	Shawn Gannon, Ph.D., DABT The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899 Phone: 302-773-1376 Email: SHAWN.A.GANNON@chemours.com

Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	01-June-2018
Proposed Experimental Completion Date	28-June-2018
Proposed Report Date	13-July-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST SUBSTANCE INFORMATION

Identification	Sodium salt of Hydrolyzed TAF n=3
CAS No.	39492-90-5
Storage Conditions	Room Temperature Protect from light (Per BioReliance SOP)
Purity	99.4% (no correction factor will be used for dose formulations)
Molecular Weight	400.03 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused Test Substance will be returned to the sponsor prior to report finalization using the information below; unless the test substance is used on another study.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

Residual dose formulations will be discarded after use.

7. TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will he at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values
Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of

revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains

- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

Shawn Gannon, Ph.D., DABT
Sponsor Representative

8 May 2018
Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 30 MAY 2018

BioReliance Study Management — Date: 21-May-2018

15. APPENDIX III: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test for Induction of: Reverse mutation in bacterial cells

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

Metabolizing System: Aroclor-induced rat liver S9

Vehicle for Test Substance: Water

Treatment: Plate incorporation

Cytotoxic Effects: None

Genotoxic Effects: None

No. of Independent Assays: 2

No. of Replicate Cultures: 2 (B1) and 3 (B2)

Test Substance: Sodium salt of Hydrolyzed TAF n=3

Study No.: AF28PL.503.BTL

No. Cells Analyzed/Culture: 1.0 to 2.8 x 10^8 cells per plate

GLP Compliance: Yes

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Date(s) of Treatment: 01 June 2018 (B1) and 14 June 2018 (B2)

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B1: Initial Toxicity-Mutation Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	16 ± 3	76 ± 16	9 ± 2	5 ± 3	28 ± 1
	Sodium salt of Hydrolyzed TAF n=3	1.50	14 ± 1	69 ± 10	8 ± 3	5 ± 2	33 ± 1
		5.00	14 ± 0	78 ± 5	11 ± 1	8 ± 1	33 ± 5
		15.0	17 ± 9	83 ± 2	12 ± 1	5 ± 0	30 ± 0
		50.0	11 ± 1	80 ± 12	10 ± 2	7 ± 3	22 ± 6
		150	12 ± 7	76 ± 4	9 ± 1	5 ± 4	23 ± 8
		500	18 ± 1	88 ± 18	12 ± 2	7 ± 1	28 ± 2
		1500	18 ± 0	91 ± 4	13 ± 5	7 ± 1	33 ± 8
		5000	13 ± 6	86 ± 3	8 ± 1	6 ± 1	26 ± 4
	2NF	1.00	61 ± 15				
	SA	1.00		707 ± 21	721 ± 37		
	9AAD	75.0				599 ± 177	
	MMS	1000					384 ± 2
With Activation	Water	100 µL/plate	15 ± 0	92 ± 6	14 ± 1	8 ± 3	33 ± 8
	Sodium salt of Hydrolyzed TAF n=3	1.50	21 ± 5	100 ± 2	14 ± 1	9 ± 3	29 ± 4
		5.00	15 ± 5	82 ± 15	10 ± 1	6 ± 4	26 ± 2
		15.0	16 ± 4	108 ± 6	7 ± 1	8 ± 4	30 ± 16
		50.0	21 ± 8	109 ± 14	13 ± 2	7 ± 1	43 ± 5
		150	19 ± 3	116 ± 1	12 ± 4	9 ± 0	28 ± 6
		500	18 ± 5	98 ± 7	12 ± 2	4 ± 1	36 ± 4
		1500	18 ± 1	118 ± 3	12 ± 2	10 ± 5	32 ± 0
		5000	16 ± 4	114 ± 24	14 ± 1	11 ± 3	34 ± 9
	2AA	1.00	232 ± 20		90 ± 8		
	2AA	2.00		1243 ± 626		39 ± 0	
	2AA	15.0					247 ± 35

Key to Positive Controls

SA	sodium azide	2NF	2-nitrofluorene
2AA	2-aminoanthracene	MMS	methyl methanesulfonate
9AAD	9-Aminoacridine		

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B2: Confirmatory Mutagenicity Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	12 ± 4	94 ± 12	12 ± 4	8 ± 2	30 ± 5
	Sodium salt of Hydrolyzed TAF n=3	50.0	15 ± 2	109 ± 6	11 ± 4	4 ± 1	39 ± 3
		150	15 ± 0	94 ± 14	14 ± 3	9 ± 1	37 ± 6
		500	13 ± 2	98 ± 10	8 ± 1	6 ± 1	35 ± 7
		1500	14 ± 3	102 ± 8	13 ± 6	6 ± 3	33 ± 13
		5000	14 ± 2	98 ± 5	14 ± 1	6 ± 2	33 ± 3
	2NF	1.00	61 ± 8				
	SA	1.00		741 ± 33	718 ± 29		
	9AAD	75.0				823 ± 128	
	MMS	1000					474 ± 38
With Activation	Water	100 µL/plate	22 ± 6	96 ± 10	11 ± 3	9 ± 3	37 ± 3
	Sodium salt of Hydrolyzed TAF n=3	50.0	23 ± 7	89 ± 4	13 ± 4	11 ± 3	39 ± 12
		150	19 ± 3	113 ± 16	9 ± 3	11 ± 3	34 ± 6
		500	22 ± 8	102 ± 6	13 ± 5	8 ± 4	37 ± 6
		1500	19 ± 4	107 ± 8	8 ± 1	11 ± 5	37 ± 4
		5000	19 ± 7	120 ± 4	13 ± 6	7 ± 3	36 ± 4
	2AA	1.00	239 ± 29		66 ± 13		
	2AA	2.00		787 ± 23		49 ± 11	
	2AA	15.0					359 ± 66

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

Potassium salt of Hydrolyzed TAF n=2

Author

Emily Dakoulas, BS

Study Completion Date

30 August 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF28PM.503.BTL

Sponsor

The Chemours Company
1007 Market Street D-3008
Wilmington, DE 19899

Sponsor Number

C30049

1. STATEMENT OF COMPLIANCE

Study No. AF28PM.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

30 JAN 2018
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF28PM.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
19-Jun-2018	19-Jun-2018	Strain Characterization	19-Jun-2018	19-Jun-2018
19-Jun-2018	19-Jun-2018	Preparation of S9 Mixture	19-Jun-2018	19-Jun-2018
19-Jun-2018	25-Jun-2018	Protocol Review	25-Jun-2018	25-Jun-2018
13-Jul-2018	13-Jul-2018	Data/Draft Report	13-Jul-2018	13-Jul-2018
27-Aug-2018	27-Aug-2018	Final Report	27-Aug-2018	27-Aug-2018
27-Aug-2018	27-Aug-2018	Protocol Amendment Review	27-Aug-2018	27-Aug-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Lisa AnnMarie Fleshman 30-Aug-2018 12:29 pm GMT

Reason for signature: QA Approval

Printed by:Lisa AnnMarie Fleshman
Printed on:30-Aug-18

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4. STUDY INFORMATION

Study Conduct

Sponsor:	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor's Authorized Representative:	Shawn Gannon, Ph.D., DABT
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF28PM.503.BTL
Sponsor No.:	C30049

Test Substance

Identification:	Potassium salt of Hydrolyzed TAF n=2
CAS No.:	39492-89-2
Purity:	95% (per protocol)
Molecular Weight:	350.13 g/mol
Description:	White powder
Storage Conditions:	Room temperature, protected from light
Receipt Date:	02 May 2018

Study Dates

Study Initiation Date:	01 June 2018
Experimental Starting Date (first day of data collection):	01 June 2018
Experimental Start Date (first day test substance administered to test system):	05 June 2018
Experimental Completion Date:	26 June 2018

Key Personnel

Study Director:	Emily Dakoulas, BS

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, Potassium salt of Hydrolyzed TAF n=2, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Water was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Based upon these results, the maximum dose tested in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, the dose levels tested were 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate Potassium salt of Hydrolyzed TAF n=2 was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization using the information below.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

The vehicle used to deliver Potassium salt of Hydrolyzed TAF n=2 to the test system was water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Water	7732-18-5	Sigma-Aldrich	RNBF9658	Sterile-filtered	Mar 2019

To achieve a solution, the most concentrated dilution was sonicated at 28.0ºC for 5 minutes in the initial toxicity-mutation assay. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Water was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in water at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3925, Exp. Date: 21 Feb 2020; Lot No. 3961, Exp. Date: 15 May 2020) was purchased

commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and five dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	2.2	1.0	0.8	1.5	2.9
B2	1.2	1.1	1.5	1.9	2.8

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in water are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

Neither precipitate nor toxicity was observed.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 50.0, 150, 500, 1500 and 5000 µg per plate.

Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

A copy of the Common Technical Document Tables is included in Appendix III.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Potassium salt of Hydrolyzed TAF n=2 did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PM.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PM
Date Plated: 6/5/2018
Evaluation Period: 6/11/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*12*	*4*	*0.8*	15[A], 9[A]
		1500 µg	*13*	*5*	*0.8*	16[A], 9[A]
		500 µg	*16*	*1*	*1.0*	15[A], 17[A]
		150 µg	*14*	*0*	*0.9*	14[A], 14[A]
		50.0 µg	*11*	*5*	*0.7*	7[A], 14[A]
		15.0 µg	*12*	*5*	*0.8*	15[A], 8[A]
		5.00 µg	*14*	*6*	*0.9*	9[A], 18[A]
		1.50 µg	*14*	*1*	*0.9*	14[A], 13[A]
	Water	100 µL	*16*	*1*		15[A], 17[A]
TA100	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*94*	*4*	*1.0*	91[A], 97[A]
		1500 µg	*87*	*1*	*1.0*	87[A], 86[A]
		500 µg	*83*	*0*	*0.9*	83[A], 83[A]
		150 µg	*90*	*16*	*1.0*	79[A], 101[A]
		50.0 µg	*88*	*8*	*1.0*	93[A], 82[A]
		15.0 µg	*76*	*8*	*0.8*	70[A], 81[A]
		5.00 µg	*80*	*12*	*0.9*	88[A], 71[A]
		1.50 µg	*94*	*6*	*1.0*	89[A], 98[A]
	Water	100 µL	*90*	*1*		90[A], 89[A]
TA1535	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*12*	*1*	*0.9*	11[A], 13[A]
		1500 µg	*11*	*4*	*0.8*	14[A], 8[A]
		500 µg	*9*	*1*	*0.7*	8[A], 10[A]
		150 µg	*12*	*2*	*0.9*	13[A], 10[A]
		50.0 µg	*14*	*7*	*1.1*	19[A], 9[A]
		15.0 µg	*10*	*0*	*0.8*	10[A], 10[A]
		5.00 µg	*11*	*5*	*0.8*	14[A], 7[A]
		1.50 µg	*10*	*1*	*0.8*	9[A], 11[A]
	Water	100 µL	*13*	*0*		13[A], 13[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PM.503.BTL
Study Code: AF28PM
Experiment: B1
Date Plated: 6/5/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/11/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*7*	*2*	*0.9*	5[A], 8[A]
		1500 µg	*6*	*1*	*0.8*	6[A], 5[A]
		500 µg	*8*	*0*	*1.0*	8[A], 8[A]
		150 µg	*5*	*3*	*0.6*	3[A], 7[A]
		50.0 µg	*7*	*0*	*0.9*	7[A], 7[A]
		15.0 µg	*7*	*1*	*0.9*	6[A], 8[A]
		5.00 µg	*6*	*0*	*0.8*	6[A], 6[A]
		1.50 µg	*5*	*0*	*0.6*	5[A], 5[A]
	Water	100 µL	*8*	*1*		8[A], 7[A]
WP2uvrA	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*29*	*7*	*0.9*	34[A], 24[A]
		1500 µg	*37*	*8*	*1.2*	42[A], 31[A]
		500 µg	*36*	*4*	*1.1*	33[A], 39[A]
		150 µg	*32*	*8*	*1.0*	38[A], 26[A]
		50.0 µg	*36*	*3*	*1.1*	34[A], 38[A]
		15.0 µg	*45*	*13*	*1.4*	54[A], 35[A]
		5.00 µg	*30*	*1*	*0.9*	29[A], 31[A]
		1.50 µg	*32*	*4*	*1.0*	35[A], 29[A]
	Water	100 µL	*32*	*6*		36[A], 27[A]
TA98	**2NF**	1.00 µg	*71*	*28*	*4.4*	51[A], 90[A]
TA100	**SA**	1.00 µg	*642*	*30*	*7.1*	663[A], 620[A]
TA1535	**SA**	1.00 µg	*599*	*20*	*46.1*	613[A], 585[A]
TA1537	**9AAD**	75.0 µg	*1029*	*19*	*128.6*	1015[A], 1042[A]
WP2uvrA	**MMS**	1000 µg	*502*	*50*	*15.7*	466[A], 537[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PM.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PM
Date Plated: 6/5/2018
Evaluation Period: 6/11/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*16*	*1*	*0.8*	17[A], 15[A]
		1500 µg	*21*	*4*	*1.0*	24[A], 18[A]
		500 µg	*21*	*5*	*1.0*	17[A], 24[A]
		150 µg	*20*	*1*	*1.0*	19[A], 21[A]
		50.0 µg	*18*	*2*	*0.9*	16[A], 19[A]
		15.0 µg	*11*	*1*	*0.5*	11[A], 10[A]
		5.00 µg	*16*	*2*	*0.8*	14[A], 17[A]
		1.50 µg	*15*	*1*	*0.7*	14[A], 15[A]
	Water	100 µL	*21*	*0*		21[A], 21[A]
TA100	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*109*	*4*	*1.1*	106[A], 112[A]
		1500 µg	*114*	*14*	*1.2*	104[A], 124[A]
		500 µg	*98*	*8*	*1.0*	103[A], 92[A]
		150 µg	*87*	*8*	*0.9*	81[A], 92[A]
		50.0 µg	*104*	*19*	*1.1*	90[A], 117[A]
		15.0 µg	*109*	*3*	*1.1*	107[A], 111[A]
		5.00 µg	*107*	*1*	*1.1*	106[A], 108[A]
		1.50 µg	*91*	*1*	*0.9*	90[A], 91[A]
	Water	100 µL	*98*	*12*		89[A], 106[A]
TA1535	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*8*	*3*	*0.5*	6[A], 10[A]
		1500 µg	*17*	*2*	*1.0*	18[A], 15[A]
		500 µg	*17*	*4*	*1.0*	14[A], 19[A]
		150 µg	*16*	*1*	*0.9*	17[A], 15[A]
		50.0 µg	*15*	*3*	*0.9*	13[A], 17[A]
		15.0 µg	*12*	*2*	*0.7*	13[A], 10[A]
		5.00 µg	*10*	*4*	*0.6*	7[A], 13[A]
		1.50 µg	*10*	*0*	*0.6*	10[A], 10[A]
	Water	100 µL	*17*	*2*		15[A], 18[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PM.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PM
Date Plated: 6/5/2018
Evaluation Period: 6/11/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*5*	*2*	*0.5*	6[A], 3[A]
		1500 µg	*7*	*0*	*0.7*	7[A], 7[A]
		500 µg	*10*	*2*	*1.0*	8[A], 11[A]
		150 µg	*10*	*0*	*1.0*	10[A], 10[A]
		50.0 µg	*7*	*1*	*0.7*	6[A], 8[A]
		15.0 µg	*8*	*1*	*0.8*	7[A], 8[A]
		5.00 µg	*8*	*1*	*0.8*	7[A], 9[A]
		1.50 µg	*6*	*4*	*0.6*	3[A], 8[A]
	Water	100 µL	*10*	*4*		13[A], 7[A]
WP2uvrA	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*30*	*8*	*1.0*	36[A], 24[A]
		1500 µg	*42*	*1*	*1.4*	42[A], 41[A]
		500 µg	*34*	*2*	*1.1*	32[A], 35[A]
		150 µg	*29*	*4*	*1.0*	26[A], 31[A]
		50.0 µg	*28*	*1*	*0.9*	27[A], 29[A]
		15.0 µg	*26*	*1*	*0.9*	27[A], 25[A]
		5.00 µg	*38*	*6*	*1.3*	42[A], 33[A]
		1.50 µg	*36*	*5*	*1.2*	39[A], 32[A]
	Water	100 µL	*30*	*4*		27[A], 32[A]
TA98	**2AA**	1.00 µg	*267*	*6*	*12.7*	263[A], 271[A]
TA100	**2AA**	2.00 µg	*861*	*81*	*8.8*	803[A], 918[A]
TA1535	**2AA**	1.00 µg	*92*	*0*	*5.4*	92[A], 92[A]
TA1537	**2AA**	2.00 µg	*45*	*10*	*4.5*	38[A], 52[A]
WP2uvrA	**2AA**	15.0 µg	*327*	*46*	*10.9*	359[A], 294[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PM.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PM
Date Plated: 6/19/2018
Evaluation Period: 6/26/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*12*	*4*	*1.0*	9^{A}, 11^{A}, 16^{A}
		1500 µg	*11*	*4*	*0.9*	15^{A}, 11^{A}, 8^{A}
		500 µg	*12*	*4*	*1.0*	9^{A}, 10^{A}, 17^{A}
		150 µg	*12*	*5*	*1.0*	8^{A}, 18^{A}, 11^{A}
		50.0 µg	*12*	*5*	*1.0*	8^{A}, 10^{A}, 17^{A}
	Water	100 µL	*12*	*4*		16^{A}, 9^{A}, 10^{A}
TA100	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*98*	*6*	*1.2*	93^{A}, 104^{A}, 97^{A}
		1500 µg	*74*	*11*	*0.9*	67^{A}, 68^{A}, 86^{A}
		500 µg	*78*	*10*	*0.9*	89^{A}, 70^{A}, 75^{A}
		150 µg	*91*	*10*	*1.1*	79^{A}, 95^{A}, 98^{A}
		50.0 µg	*77*	*5*	*0.9*	76^{A}, 82^{A}, 73^{A}
	Water	100 µL	*85*	*15*		90^{A}, 68^{A}, 97^{A}
TA1535	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*11*	*4*	*1.0*	6^{A}, 14^{A}, 13^{A}
		1500 µg	*10*	*4*	*0.9*	7^{A}, 14^{A}, 8^{A}
		500 µg	*9*	*2*	*0.8*	11^{A}, 7^{A}, 10^{A}
		150 µg	*8*	*5*	*0.7*	3^{A}, 7^{A}, 13^{A}
		50.0 µg	*9*	*3*	*0.8*	7^{A}, 7^{A}, 13^{A}
	Water	100 µL	*11*	*2*		10^{A}, 9^{A}, 13^{A}
TA1537	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*5*	*2*	*0.8*	6^{A}, 6^{A}, 3^{A}
		1500 µg	*5*	*2*	*0.8*	7^{A}, 5^{A}, 3^{A}
		500 µg	*7*	*2*	*1.2*	8^{A}, 5^{A}, 8^{A}
		150 µg	*8*	*2*	*1.3*	7^{A}, 8^{A}, 10^{A}
		50.0 µg	*8*	*1*	*1.3*	9^{A}, 7^{A}, 7^{A}
	Water	100 µL	*6*	*1*		5^{A}, 6^{A}, 6^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PM.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PM
Date Plated: 6/19/2018
Evaluation Period: 6/26/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*37*	*5*	*1.2*	31[A], 40[A], 39[A]
		1500 µg	*33*	*4*	*1.1*	36[A], 33[A], 29[A]
		500 µg	*34*	*6*	*1.1*	29[A], 41[A], 32[A]
		150 µg	*38*	*5*	*1.2*	38[A], 43[A], 33[A]
		50.0 µg	*33*	*11*	*1.1*	32[A], 44[A], 22[A]
	Water	100 µL	*31*	*3*		33[A], 32[A], 27[A]
TA98	**2NF**	1.00 µg	*55*	*13*	*4.6*	50[A], 70[A], 46[A]
TA100	**SA**	1.00 µg	*761*	*27*	*9.0*	766[A], 785[A], 732[A]
TA1535	**SA**	1.00 µg	*519*	*38*	*47.2*	524[A], 479[A], 555[A]
TA1537	**9AAD**	75.0 µg	*684*	*2*	*114.0*	685[A], 684[A], 682[A]
WP2uvrA	**MMS**	1000 µg	*493*	*42*	*15.9*	448[A], 530[A], 502[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PM.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PM
Date Plated: 6/19/2018
Evaluation Period: 6/26/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*12*	*5*	*0.6*	7^{A}, 15^{A}, 15^{A}
		1500 µg	*19*	*2*	*1.0*	18^{A}, 21^{A}, 17^{A}
		500 µg	*19*	*2*	*1.0*	18^{A}, 18^{A}, 21^{A}
		150 µg	*16*	*6*	*0.8*	10^{A}, 17^{A}, 22^{A}
		50.0 µg	*19*	*3*	*1.0*	22^{A}, 16^{A}, 19^{A}
	Water	100 µL	*20*	*5*		23^{A}, 14^{A}, 22^{A}
TA100	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*110*	*2*	*1.3*	112^{A}, 111^{A}, 108^{A}
		1500 µg	*104*	*17*	*1.2*	122^{A}, 101^{A}, 88^{A}
		500 µg	*102*	*10*	*1.2*	109^{A}, 107^{A}, 91^{A}
		150 µg	*87*	*12*	*1.0*	78^{A}, 83^{A}, 100^{A}
		50.0 µg	*93*	*7*	*1.1*	101^{A}, 89^{A}, 89^{A}
	Water	100 µL	*86*	*3*		88^{A}, 82^{A}, 88^{A}
TA1535	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*7*	*4*	*0.6*	3^{A}, 7^{A}, 11^{A}
		1500 µg	*12*	*3*	*1.1*	9^{A}, 13^{A}, 14^{A}
		500 µg	*13*	*4*	*1.2*	16^{A}, 13^{A}, 9^{A}
		150 µg	*13*	*4*	*1.2*	17^{A}, 13^{A}, 10^{A}
		50.0 µg	*15*	*4*	*1.4*	11^{A}, 16^{A}, 18^{A}
	Water	100 µL	*11*	*4*		13^{A}, 6^{A}, 13^{A}
TA1537	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*7*	*3*	*1.2*	10^{A}, 5^{A}, 7^{A}
		1500 µg	*9*	*1*	*1.5*	10^{A}, 8^{A}, 9^{A}
		500 µg	*6*	*2*	*1.0*	3^{A}, 7^{A}, 7^{A}
		150 µg	*5*	*2*	*0.8*	5^{A}, 3^{A}, 6^{A}
		50.0 µg	*7*	*2*	*1.2*	5^{A}, 9^{A}, 6^{A}
	Water	100 µL	*6*	*3*		6^{A}, 9^{A}, 3^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 4 (CONT.)
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PM.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PM
Date Plated: 6/19/2018
Evaluation Period: 6/26/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Potassium salt of Hydrolyzed TAF n=2**	5000 µg	*34*	*2*	*1.0*	33[A], 36[A], 33[A]
		1500 µg	*32*	*1*	*1.0*	32[A], 31[A], 32[A]
		500 µg	*34*	*9*	*1.0*	41[A], 24[A], 38[A]
		150 µg	*34*	*4*	*1.0*	32[A], 39[A], 32[A]
		50.0 µg	*35*	*6*	*1.1*	30[A], 33[A], 42[A]
	Water	100 µL	*33*	*1*		32[A], 34[A], 34[A]
TA98	**2AA**	1.00 µg	*212*	*30*	*10.6*	204[A], 245[A], 186[A]
TA100	**2AA**	2.00 µg	*572*	*22*	*6.7*	568[A], 595[A], 552[A]
TA1535	**2AA**	1.00 µg	*74*	*1*	*6.7*	74[A], 75[A], 74[A]
TA1537	**2AA**	2.00 µg	*59*	*10*	*9.8*	48[A], 63[A], 67[A]
WP2uvrA	**2AA**	15.0 µg	*262*	*33*	*7.9*	262[A], 295[A], 229[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values 2016 revertants per plate											
Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	
SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control											

14. APPENDIX II: Study Protocol and Amendment

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PM.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 1500, 500, 150 and 50.0 µg per plate.

   Reason: To specify the dose levels to be used for the confirmatory mutagenicity assay based on the toxicity and precipitate profiles observed in the initial toxicity-mutation assay.

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PM.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, Ph.D., DABT
Sponsor Representative

19 June 2018
Date

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PM.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS
BioReliance Study Director

18 JUN 2018
Date

BioReliance Study Management

18 Jun 2018
Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF28PM.503.BTL

1. KEY PERSONNEL

Sponsor Information:

Sponsor	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor Number	C30049
Sponsor's Authorized Representative	Shawn Gannon, Ph.D., DABT The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899 Phone: 302-773-1376 Email: SHAWN.A.GANNON@chemours.com

Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	05-June-2018
Proposed Experimental Completion Date	03-July-2018
Proposed Report Date	18-July-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST SUBSTANCE INFORMATION

Identification	Potassium salt of Hydrolyzed TAF n=2
CAS No.	39492-89-2
Storage Conditions	Room Temperature Protect from light (Per BioReliance SOP)
Purity	95% (no correction factor will be used for dose formulations)
Molecular Weight	350.13 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused Test Substance will be returned to the sponsor prior to report finalization using the information below; unless the test substance is used on another study.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

Residual dose formulations will be discarded after use.

7. **TEST SYSTEM**
The tester strains will include the *S. typhimurium* histidine auxotropbs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values
Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of

revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains

- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

Shawn Gannon, Ph.D., DABT
Sponsor Representative

8 May 2018
Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 01 JUN 2018

BioReliance Study Management — Date: 01-JUN-18

15. APPENDIX III: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test for Induction of: Reverse mutation in bacterial cells

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

Metabolizing System: Aroclor-induced rat liver S9

Vehicle for Test Substance: Water

Treatment: Plate incorporation

Cytotoxic Effects: None

Genotoxic Effects: None

No. of Independent Assays: 2

No. of Replicate Cultures: 2 (B1) and 3 (B2)

Test Substance: Potassium salt of Hydrolyzed TAF n=2

Study No.: AF28PM.503.BTL

No. Cells Analyzed/Culture: 0.8 to 2.9 x 10^8 cells per plate

GLP Compliance: Yes

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Date(s) of Treatment: 05 June 2018 (B1) and 19 June 2018 (B2)

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B1: Initial Toxicity-Mutation Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	16 ± 1	90 ± 1	13 ± 0	8 ± 1	32 ± 6
	Potassium salt of Hydrolyzed TAF n=2	1.50	14 ± 1	94 ± 6	10 ± 1	5 ± 0	32 ± 4
		5.00	14 ± 6	80 ± 12	11 ± 5	6 ± 0	30 ± 1
		15.0	12 ± 5	76 ± 8	10 ± 0	7 ± 1	45 ± 13
		50.0	11 ± 5	88 ± 8	14 ± 7	7 ± 0	36 ± 3
		150	14 ± 0	90 ± 16	12 ± 2	5 ± 3	32 ± 8
		500	16 ± 1	83 ± 0	9 ± 1	8 ± 0	36 ± 4
		1500	13 ± 5	87 ± 1	11 ± 4	6 ± 1	37 ± 8
		5000	12 ± 4	94 ± 4	12 ± 1	7 ± 2	29 ± 7
	2NF	1.00	71 ± 28				
	SA	1.00		642 ± 30	599 ± 20		
	9AAD	75.0				1029 ± 19	
	MMS	1000					502 ± 50
With Activation	Water	100 µL/plate	21 ± 0	98 ± 12	17 ± 2	10 ± 4	30 ± 4
	Potassium salt of Hydrolyzed TAF n=2	1.50	15 ± 1	91 ± 1	10 ± 0	6 ± 4	36 ± 5
		5.00	16 ± 2	107 ± 1	10 ± 4	8 ± 1	38 ± 6
		15.0	11 ± 1	109 ± 3	12 ± 2	8 ± 1	26 ± 1
		50.0	18 ± 2	104 ± 19	15 ± 3	7 ± 1	28 ± 1
		150	20 ± 1	87 ± 8	16 ± 1	10 ± 0	29 ± 4
		500	21 ± 5	98 ± 8	17 ± 4	10 ± 2	34 ± 2
		1500	21 ± 4	114 ± 14	17 ± 2	7 ± 0	42 ± 1
		5000	16 ± 1	109 ± 4	8 ± 3	5 ± 2	30 ± 8
	2AA	1.00	267 ± 6		92 ± 0		
	2AA	2.00		861 ± 81		45 ± 10	
	2AA	15.0					327 ± 46

Key to Positive Controls

SA	sodium azide	2NF	2-nitrofluorene
2AA	2-aminoanthracene	MMS	methyl methanesulfonate
9AAD	9-Aminoacridine		

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B2: Confirmatory Mutagenicity Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	12 ± 4	85 ± 15	11 ± 2	6 ± 1	31 ± 3
	Potassium salt of Hydrolyzed TAF n=2	50.0	12 ± 5	77 ± 5	9 ± 3	8 ± 1	33 ± 11
		150	12 ± 5	91 ± 10	8 ± 5	8 ± 2	38 ± 5
		500	12 ± 4	78 ± 10	9 ± 2	7 ± 2	34 ± 6
		1500	11 ± 4	74 ± 11	10 ± 4	5 ± 2	33 ± 4
		5000	12 ± 4	98 ± 6	11 ± 4	5 ± 2	37 ± 5
	2NF	1.00	55 ± 13				
	SA	1.00		761 ± 27	519 ± 38		
	9AAD	75.0				684 ± 2	
	MMS	1000					493 ± 42
With Activation	Water	100 µL/plate	20 ± 5	86 ± 3	11 ± 4	6 ± 3	33 ± 1
	Potassium salt of Hydrolyzed TAF n=2	50.0	19 ± 3	93 ± 7	15 ± 4	7 ± 2	35 ± 6
		150	16 ± 6	87 ± 12	13 ± 4	5 ± 2	34 ± 4
		500	19 ± 2	102 ± 10	13 ± 4	6 ± 2	34 ± 9
		1500	19 ± 2	104 ± 17	12 ± 3	9 ± 1	32 ± 1
		5000	12 ± 5	110 ± 2	7 ± 4	7 ± 3	34 ± 2
	2AA	1.00	212 ± 30		74 ± 1		
	2AA	2.00		572 ± 22		59 ± 10	
	2AA	15.0					262 ± 33

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

Sodium salt of Hydrolyzed TAF n=1

Author

Emily Dakoulas, BS

Study Completion Date

27 August 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF28PN.503.BTL

Sponsor

The Chemours Company
1007 Market Street D-3008
Wilmington, DE 19899

Sponsor Number

C30049

1. STATEMENT OF COMPLIANCE

Study No. AF28PN.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

27 Nov 2018
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF28PN.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
14-Jun-2018	15-Jun-2018	Protocol Review	15-Jun-2018	15-Jun-2018
19-Jun-2018	19-Jun-2018	Dilution of the test article and/or positive control	20-Jun-2018	20-Jun-2018
13-Jul-2018	13-Jul-2018	Data/Draft Report	13-Jul-2018	13-Jul-2018
23-Aug-2018	23-Aug-2018	Final Report	23-Aug-2018	23-Aug-2018
23-Aug-2018	23-Aug-2018	Protocol Amendment Review	23-Aug-2018	23-Aug-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Carlos Bonilla 27-Aug-2018 6:30 pm GMT

Reason for signature: QA Approval

Printed by:Carlos Bonilla

Printed on:27-Aug-18

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4. STUDY INFORMATION

Study Conduct

Sponsor:	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor's Authorized Representative:	Shawn Gannon, Ph.D., DABT
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF28PN.503.BTL
Sponsor No.:	C30049

Test Substance

Identification:	Sodium salt of Hydrolyzed TAF n=1
CAS No.:	39492-88-1
Purity:	99.9% (per protocol)
Molecular Weight:	268.03 g/mol
Description:	White powder
Storage Conditions:	Room temperature, protected from light
Receipt Date:	02 May 2018

Study Dates

Study Initiation Date:	01 June 2018
Experimental Starting Date (first day of data collection):	01 June 2018
Experimental Start Date (first day test substance administered to test system):	05 June 2018
Experimental Completion Date:	26 June 2018

Key Personnel

Study Director:	Emily Dakoulas, BS

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, Sodium salt of Hydrolyzed TAF n=1, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Water was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Based upon these results, the maximum dose tested in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, the dose levels tested were 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate Sodium salt of Hydrolyzed TAF n=1 was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization using the information below.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

The vehicle used to deliver Sodium salt of Hydrolyzed TAF n=1 to the test system was water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Water	7732-18-5	Sigma-Aldrich	RNBF9658	Sterile-filtered	Mar 2019
			RNBG4913		Dec 2019

To achieve a solution, the most concentrated dilution was sonicated at 21.4ºC for 1 minute in the initial toxicity-mutation assay. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Water was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in water at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3925, Exp. Date: 21 Feb 2020; Lot No. 3961, Exp. Date: 15 May 2020) was purchased

commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and five dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	2.2	1.0	0.8	1.5	2.9
B2	1.2	1.1	1.5	1.9	2.8

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in water are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

Neither precipitate nor toxicity was observed.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 50.0, 150, 500, 1500 and 5000 µg per plate.

Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

A copy of the Common Technical Document Tables is included in Appendix III.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Sodium salt of Hydrolyzed TAF n=1 did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PN.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PN
Date Plated: 6/5/2018
Evaluation Period: 6/11/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*11*	*4*	*0.8*	8[A], 14[A]
		1500 µg	*19*	*6*	*1.4*	15[A], 23[A]
		500 µg	*14*	*1*	*1.0*	15[A], 13[A]
		150 µg	*11*	*5*	*0.8*	7[A], 14[A]
		50.0 µg	*14*	*6*	*1.0*	10[A], 18[A]
		15.0 µg	*12*	*3*	*0.9*	10[A], 14[A]
		5.00 µg	*9*	*0*	*0.6*	9[A], 9[A]
		1.50 µg	*10*	*1*	*0.7*	9[A], 10[A]
	Water	100 µL	*14*	*4*		16[A], 11[A]
TA100	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*90*	*8*	*1.1*	84[A], 95[A]
		1500 µg	*78*	*3*	*1.0*	80[A], 76[A]
		500 µg	*79*	*4*	*1.0*	76[A], 81[A]
		150 µg	*88*	*1*	*1.1*	87[A], 89[A]
		50.0 µg	*88*	*11*	*1.1*	96[A], 80[A]
		15.0 µg	*75*	*11*	*0.9*	67[A], 83[A]
		5.00 µg	*85*	*2*	*1.1*	83[A], 86[A]
		1.50 µg	*80*	*21*	*1.0*	95[A], 65[A]
	Water	100 µL	*79*	*11*		71[A], 86[A]
TA1535	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*10*	*6*	*0.8*	6[A], 14[A]
		1500 µg	*10*	*0*	*0.8*	10[A], 10[A]
		500 µg	*13*	*0*	*1.0*	13[A], 13[A]
		150 µg	*9*	*1*	*0.7*	8[A], 10[A]
		50.0 µg	*13*	*2*	*1.0*	14[A], 11[A]
		15.0 µg	*12*	*1*	*0.9*	13[A], 11[A]
		5.00 µg	*7*	*0*	*0.5*	7[A], 7[A]
		1.50 µg	*11*	*8*	*0.8*	16[A], 5[A]
	Water	100 µL	*13*	*2*		11[A], 14[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PN.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PN
Date Plated: 6/5/2018
Evaluation Period: 6/11/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*6*	*0*	*1.0*	6^{A}, 6^{A}
		1500 µg	*5*	*3*	*0.8*	7^{A}, 3^{A}
		500 µg	*7*	*0*	*1.2*	7^{A}, 7^{A}
		150 µg	*5*	*2*	*0.8*	3^{A}, 6^{A}
		50.0 µg	*6*	*1*	*1.0*	6^{A}, 5^{A}
		15.0 µg	*7*	*1*	*1.2*	6^{A}, 8^{A}
		5.00 µg	*7*	*0*	*1.2*	7^{A}, 7^{A}
		1.50 µg	*7*	*1*	*1.2*	6^{A}, 7^{A}
	Water	100 µL	*6*	*4*		3^{A}, 9^{A}
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*35*	*0*	*1.0*	35^{A}, 35^{A}
		1500 µg	*36*	*6*	*1.1*	31^{A}, 40^{A}
		500 µg	*31*	*5*	*0.9*	34^{A}, 27^{A}
		150 µg	*34*	*7*	*1.0*	39^{A}, 29^{A}
		50.0 µg	*30*	*4*	*0.9*	32^{A}, 27^{A}
		15.0 µg	*38*	*12*	*1.1*	46^{A}, 29^{A}
		5.00 µg	*35*	*13*	*1.0*	26^{A}, 44^{A}
		1.50 µg	*36*	*15*	*1.1*	25^{A}, 46^{A}
	Water	100 µL	*34*	*1*		33^{A}, 34^{A}
TA98	**2NF**	1.00 µg	*69*	*21*	*4.9*	83^{A}, 54^{A}
TA100	**SA**	1.00 µg	*600*	*35*	*7.6*	575^{A}, 625^{A}
TA1535	**SA**	1.00 µg	*564*	*21*	*43.4*	549^{A}, 579^{A}
TA1537	**9AAD**	75.0 µg	*858*	*120*	*143.0*	773^{A}, 943^{A}
WP2uvrA	**MMS**	1000 µg	*513*	*25*	*15.1*	531^{A}, 495^{A}

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PN.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PN
Date Plated: 6/5/2018
Evaluation Period: 6/11/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*16*	*1*	*0.8*	15[A], 17[A]
		1500 µg	*29*	*4*	*1.4*	26[A], 32[A]
		500 µg	*23*	*1*	*1.1*	22[A], 24[A]
		150 µg	*16*	*3*	*0.8*	18[A], 14[A]
		50.0 µg	*17*	*0*	*0.8*	17[A], 17[A]
		15.0 µg	*22*	*5*	*1.0*	25[A], 18[A]
		5.00 µg	*17*	*8*	*0.8*	22[A], 11[A]
		1.50 µg	*18*	*2*	*0.9*	19[A], 16[A]
	Water	100 µL	*21*	*8*		15[A], 26[A]
TA100	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*108*	*7*	*1.1*	103[A], 113[A]
		1500 µg	*104*	*15*	*1.0*	93[A], 114[A]
		500 µg	*98*	*1*	*1.0*	97[A], 99[A]
		150 µg	*125*	*23*	*1.2*	108[A], 141[A]
		50.0 µg	*106*	*1*	*1.0*	107[A], 105[A]
		15.0 µg	*101*	*4*	*1.0*	103[A], 98[A]
		5.00 µg	*102*	*6*	*1.0*	106[A], 98[A]
		1.50 µg	*98*	*4*	*1.0*	100[A], 95[A]
	Water	100 µL	*101*	*7*		106[A], 96[A]
TA1535	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*18*	*6*	*1.3*	13[A], 22[A]
		1500 µg	*12*	*2*	*0.9*	13[A], 10[A]
		500 µg	*12*	*3*	*0.9*	10[A], 14[A]
		150 µg	*13*	*6*	*0.9*	9[A], 17[A]
		50.0 µg	*13*	*4*	*0.9*	16[A], 10[A]
		15.0 µg	*12*	*4*	*0.9*	9[A], 14[A]
		5.00 µg	*11*	*4*	*0.8*	8[A], 13[A]
		1.50 µg	*7*	*1*	*0.5*	6[A], 8[A]
	Water	100 µL	*14*	*5*		10[A], 17[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PN.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PN
Date Plated: 6/5/2018
Evaluation Period: 6/11/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*5*	*2*	*0.8*	6[A], 3[A]
		1500 µg	*8*	*1*	*1.3*	9[A], 7[A]
		500 µg	*4*	*3*	*0.7*	2[A], 6[A]
		150 µg	*7*	*2*	*1.2*	5[A], 8[A]
		50.0 µg	*4*	*2*	*0.7*	5[A], 2[A]
		15.0 µg	*4*	*1*	*0.7*	5[A], 3[A]
		5.00 µg	*6*	*1*	*1.0*	6[A], 5[A]
		1.50 µg	*5*	*2*	*0.8*	6[A], 3[A]
	Water	100 µL	*6*	*1*		6[A], 5[A]
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*35*	*1*	*1.2*	34[A], 36[A]
		1500 µg	*37*	*8*	*1.2*	31[A], 42[A]
		500 µg	*32*	*1*	*1.1*	32[A], 31[A]
		150 µg	*30*	*1*	*1.0*	29[A], 31[A]
		50.0 µg	*29*	*4*	*1.0*	31[A], 26[A]
		15.0 µg	*29*	*6*	*1.0*	33[A], 24[A]
		5.00 µg	*31*	*1*	*1.0*	31[A], 30[A]
		1.50 µg	*33*	*11*	*1.1*	25[A], 41[A]
	Water	100 µL	*30*	*4*		27[A], 32[A]
TA98	**2AA**	1.00 µg	*239*	*19*	*11.4*	225[A], 252[A]
TA100	**2AA**	2.00 µg	*547*	*7*	*5.4*	552[A], 542[A]
TA1535	**2AA**	1.00 µg	*83*	*6*	*5.9*	87[A], 79[A]
TA1537	**2AA**	2.00 µg	*70*	*26*	*11.7*	88[A], 51[A]
WP2uvrA	**2AA**	15.0 µg	*247*	*16*	*8.2*	235[A], 258[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PN.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PN
Date Plated: 6/19/2018
Evaluation Period: 6/26/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*13*	*3*	*1.0*	16^{A}, 13^{A}, 11^{A}
		1500 µg	*13*	*5*	*1.0*	18^{A}, 8^{A}, 13^{A}
		500 µg	*11*	*3*	*0.8*	8^{A}, 13^{A}, 13^{A}
		150 µg	*13*	*3*	*1.0*	11^{A}, 16^{A}, 13^{A}
		50.0 µg	*13*	*4*	*1.0*	10^{A}, 17^{A}, 13^{A}
	Water	100 µL	*13*	*2*		14^{A}, 14^{A}, 11^{A}
TA100	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*73*	*22*	*0.9*	68^{A}, 55^{A}, 97^{A}
		1500 µg	*89*	*9*	*1.2*	89^{A}, 81^{A}, 98^{A}
		500 µg	*92*	*6*	*1.2*	92^{A}, 86^{A}, 98^{A}
		150 µg	*83*	*3*	*1.1*	80^{A}, 82^{A}, 86^{A}
		50.0 µg	*83*	*8*	*1.1*	76^{A}, 83^{A}, 91^{A}
	Water	100 µL	*77*	*9*		87^{A}, 72^{A}, 71^{A}
TA1535	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*12*	*4*	*1.0*	8^{A}, 15^{A}, 14^{A}
		1500 µg	*10*	*4*	*0.8*	13^{A}, 11^{A}, 6^{A}
		500 µg	*9*	*2*	*0.8*	9^{A}, 11^{A}, 7^{A}
		150 µg	*16*	*1*	*1.3*	16^{A}, 17^{A}, 16^{A}
		50.0 µg	*10*	*5*	*0.8*	6^{A}, 15^{A}, 10^{A}
	Water	100 µL	*12*	*3*		13^{A}, 9^{A}, 14^{A}
TA1537	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*4*	*2*	*0.8*	3^{A}, 3^{A}, 6^{A}
		1500 µg	*5*	*0*	*1.0*	5^{A}, 5^{A}, 5^{A}
		500 µg	*7*	*2*	*1.4*	7^{A}, 5^{A}, 9^{A}
		150 µg	*7*	*4*	*1.4*	11^{A}, 3^{A}, 6^{A}
		50.0 µg	*6*	*3*	*1.2*	6^{A}, 3^{A}, 9^{A}
	Water	100 µL	*5*	*2*		3^{A}, 6^{A}, 6^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PN.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PN
Date Plated: 6/19/2018
Evaluation Period: 6/26/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*35*	*1*	*1.1*	36[A], 35[A], 35[A]
		1500 µg	*37*	*4*	*1.1*	42[A], 34[A], 36[A]
		500 µg	*34*	*6*	*1.0*	31[A], 41[A], 31[A]
		150 µg	*40*	*9*	*1.2*	41[A], 48[A], 31[A]
		50.0 µg	*24*	*9*	*0.7*	15[A], 24[A], 32[A]
	Water	100 µL	*33*	*3*		35[A], 30[A], 34[A]
TA98	**2NF**	1.00 µg	*52*	*14*	*4.0*	40[A], 48[A], 67[A]
TA100	**SA**	1.00 µg	*653*	*24*	*8.5*	627[A], 657[A], 675[A]
TA1535	**SA**	1.00 µg	*590*	*30*	*49.2*	611[A], 603[A], 556[A]
TA1537	**9AAD**	75.0 µg	*521*	*129*	*104.2*	388[A], 529[A], 645[A]
WP2uvrA	**MMS**	1000 µg	*462*	*38*	*14.0*	487[A], 418[A], 480[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PN.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PN
Date Plated: 6/19/2018
Evaluation Period: 6/26/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*14*	*1*	*1.0*	15^{A}, 14^{A}, 14^{A}
		1500 µg	*15*	*2*	*1.1*	17^{A}, 13^{A}, 14^{A}
		500 µg	*14*	*4*	*1.0*	17^{A}, 15^{A}, 9^{A}
		150 µg	*17*	*4*	*1.2*	16^{A}, 22^{A}, 14^{A}
		50.0 µg	*17*	*5*	*1.2*	22^{A}, 13^{A}, 17^{A}
	Water	100 µL	*14*	*2*		16^{A}, 13^{A}, 14^{A}
TA100	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*94*	*5*	*0.9*	88^{A}, 98^{A}, 96^{A}
		1500 µg	*92*	*10*	*0.9*	98^{A}, 81^{A}, 97^{A}
		500 µg	*94*	*10*	*0.9*	98^{A}, 101^{A}, 83^{A}
		150 µg	*101*	*4*	*1.0*	105^{A}, 98^{A}, 101^{A}
		50.0 µg	*89*	*2*	*0.9*	91^{A}, 88^{A}, 87^{A}
	Water	100 µL	*100*	*7*		96^{A}, 108^{A}, 97^{A}
TA1535	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*13*	*5*	*1.3*	18^{A}, 10^{A}, 10^{A}
		1500 µg	*12*	*1*	*1.2*	11^{A}, 13^{A}, 11^{A}
		500 µg	*8*	*2*	*0.8*	9^{A}, 9^{A}, 5^{A}
		150 µg	*10*	*4*	*1.0*	8^{A}, 14^{A}, 7^{A}
		50.0 µg	*8*	*2*	*0.8*	8^{A}, 7^{A}, 10^{A}
	Water	100 µL	*10*	*2*		11^{A}, 11^{A}, 8^{A}
TA1537	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*8*	*3*	*1.3*	11^{A}, 6^{A}, 7^{A}
		1500 µg	*5*	*2*	*0.8*	6^{A}, 2^{A}, 6^{A}
		500 µg	*6*	*1*	*1.0*	7^{A}, 5^{A}, 5^{A}
		150 µg	*5*	*3*	*0.8*	9^{A}, 3^{A}, 3^{A}
		50.0 µg	*7*	*2*	*1.2*	5^{A}, 6^{A}, 9^{A}
	Water	100 µL	*6*	*1*		5^{A}, 7^{A}, 5^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 4 (CONT.)
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PN.503.BTL
Study Code: AF28PN
Experiment: B2
Date Plated: 6/19/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/26/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Sodium salt of Hydrolyzed TAF n=1**	5000 µg	*31*	*2*	*1.1*	30^{A}, 30^{A}, 34^{A}
		1500 µg	*31*	*3*	*1.1*	33^{A}, 33^{A}, 27^{A}
		500 µg	*34*	*6*	*1.2*	39^{A}, 36^{A}, 27^{A}
		150 µg	*36*	*9*	*1.2*	46^{A}, 31^{A}, 30^{A}
		50.0 µg	*31*	*3*	*1.1*	29^{A}, 30^{A}, 35^{A}
	Water	100 µL	*29*	*5*		32^{A}, 23^{A}, 32^{A}
TA98	**2AA**	1.00 µg	*217*	*15*	*15.5*	218^{A}, 231^{A}, 202^{A}
TA100	**2AA**	2.00 µg	*778*	*19*	*7.8*	772^{A}, 763^{A}, 800^{A}
TA1535	**2AA**	1.00 µg	*74*	*16*	*7.4*	60^{A}, 91^{A}, 72^{A}
TA1537	**2AA**	2.00 µg	*40*	*6*	*6.7*	35^{A}, 47^{A}, 38^{A}
WP2uvrA	**2AA**	15.0 µg	*289*	*1*	*10.0*	290^{A}, 290^{A}, 288^{A}

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values 2016 revertants per plate											
Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	
SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control											

14. APPENDIX II: Study Protocol and Amendment

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PN.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 1500, 500, 150 and 50.0 µg per plate.

   Reason: To specify the dose levels to be used for the confirmatory assay based on the toxicity and precipitate profiles observed in the initial toxicity-mutation assay.

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PN.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, Ph.D., DABT
Sponsor Representative

14 June 2018
Date

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PN.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS
BioReliance Study Director

18 Jun 2018
Date

BioReliance Study Management

18 Jun 2018
Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF28PN.503.BTL

1. KEY PERSONNEL

Sponsor Information:

Sponsor	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor Number	C30049
Sponsor's Authorized Representative	Shawn Gannon, Ph.D., DABT The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899 Phone: 302-773-1376 Email: SHAWN.A.GANNON@chemours.com

Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	06-June-2018
Proposed Experimental Completion Date	03-July-2018
Proposed Report Date	18-July-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST SUBSTANCE INFORMATION

Identification	Sodium salt of Hydrolyzed TAF n=1
CAS No.	39492-88-1
Storage Conditions	Room Temperature Protect from light (Per BioReliance SOP)
Purity	99.9% (no correction factor will be used for dose formulations)
Molecular Weight	268.03 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused Test Substance will be returned to the sponsor prior to report finalization using the information below; unless the test substance is used on another study.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

Residual dose formulations will be discarded after use.

7. **TEST SYSTEM**
The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/ml, for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values
Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of

revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains

- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test substance, its stability and the stability and strengtb of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or tbe cbaracterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may bc issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp$^-$ reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

Shawn Gannon, Ph.D., DABT
Sponsor Representative

8 May 2018
Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 01 JUN 2018

BioReliance Study Management — Date: 01-Jun-18

15. APPENDIX III: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test for Induction of: Reverse mutation in bacterial cells

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

Metabolizing System: Aroclor-induced rat liver S9

Vehicle for Test Substance: Water

Treatment: Plate incorporation

Cytotoxic Effects: None

Genotoxic Effects: None

No. of Independent Assays: 2

No. of Replicate Cultures: 2 (B1) and 3 (B2)

Test Substance: Sodium salt of Hydrolyzed TAF n=1

Study No.: AF28PN.503.BTL

No. Cells Analyzed/Culture: 0.8 to 2.9 x 10^8 cells per plate

GLP Compliance: Yes

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Date(s) of Treatment: 05 June 2018 (B1) and 19 June 2018 (B2)

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B1: Initial Toxicity-Mutation Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	14 ± 4	79 ± 11	13 ± 2	6 ± 4	34 ± 1
	Sodium salt of Hydrolyzed TAF n=1	1.50	10 ± 1	80 ± 21	11 ± 8	7 ± 1	36 ± 15
		5.00	9 ± 0	85 ± 2	7 ± 0	7 ± 0	35 ± 13
		15.0	12 ± 3	75 ± 11	12 ± 1	7 ± 1	38 ± 12
		50.0	14 ± 6	88 ± 11	13 ± 2	6 ± 1	30 ± 4
		150	11 ± 5	88 ± 1	9 ± 1	5 ± 2	34 ± 7
		500	14 ± 1	79 ± 4	13 ± 0	7 ± 0	31 ± 5
		1500	19 ± 6	78 ± 3	10 ± 0	5 ± 3	36 ± 6
		5000	11 ± 4	90 ± 8	10 ± 6	6 ± 0	35 ± 0
	2NF	1.00	69 ± 21				
	SA	1.00		600 ± 35	564 ± 21		
	9AAD	75.0				858 ± 120	
	MMS	1000					513 ± 25
With Activation	Water	100 µL/plate	21 ± 8	101 ± 7	14 ± 5	6 ± 1	30 ± 4
	Sodium salt of Hydrolyzed TAF n=1	1.50	18 ± 2	98 ± 4	7 ± 1	5 ± 2	33 ± 11
		5.00	17 ± 8	102 ± 6	11 ± 4	6 ± 1	31 ± 1
		15.0	22 ± 5	101 ± 4	12 ± 4	4 ± 1	29 ± 6
		50.0	17 ± 0	106 ± 1	13 ± 4	4 ± 2	29 ± 4
		150	16 ± 3	125 ± 23	13 ± 6	7 ± 2	30 ± 1
		500	23 ± 1	98 ± 1	12 ± 3	4 ± 3	32 ± 1
		1500	29 ± 4	104 ± 15	12 ± 2	8 ± 1	37 ± 8
		5000	16 ± 1	108 ± 7	18 ± 6	5 ± 2	35 ± 1
	2AA	1.00	239 ± 19		83 ± 6		
	2AA	2.00		547 ± 7		70 ± 26	
	2AA	15.0					247 ± 16

Key to Positive Controls

SA	sodium azide	2NF	2-nitrofluorene
2AA	2-aminoanthracene	MMS	methyl methanesulfonate
9AAD	9-Aminoacridine		

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B2: Confirmatory Mutagenicity Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	13 ± 2	77 ± 9	12 ± 3	5 ± 2	33 ± 3
	Sodium salt of Hydrolyzed TAF n=1	50.0	13 ± 4	83 ± 8	10 ± 5	6 ± 3	24 ± 9
		150	13 ± 3	83 ± 3	16 ± 1	7 ± 4	40 ± 9
		500	11 ± 3	92 ± 6	9 ± 2	7 ± 2	34 ± 6
		1500	13 ± 5	89 ± 9	10 ± 4	5 ± 0	37 ± 4
		5000	13 ± 3	73 ± 22	12 ± 4	4 ± 2	35 ± 1
	2NF	1.00	52 ± 14				
	SA	1.00		653 ± 24	590 ± 30		
	9AAD	75.0				521 ± 129	
	MMS	1000					462 ± 38
With Activation	Water	100 µL/plate	14 ± 2	100 ± 7	10 ± 2	6 ± 1	29 ± 5
	Sodium salt of Hydrolyzed TAF n=1	50.0	17 ± 5	89 ± 2	8 ± 2	7 ± 2	31 ± 3
		150	17 ± 4	101 ± 4	10 ± 4	5 ± 3	36 ± 9
		500	14 ± 4	94 ± 10	8 ± 2	6 ± 1	34 ± 6
		1500	15 ± 2	92 ± 10	12 ± 1	5 ± 2	31 ± 3
		5000	14 ± 1	94 ± 5	13 ± 5	8 ± 3	31 ± 2
	2AA	1.00	217 ± 15		74 ± 16		
	2AA	2.00		778 ± 19		40 ± 6	
	2AA	15.0					289 ± 1

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

Sodium salt of PSEPVE Acid

Author

Emily Dakoulas, BS

Study Completion Date

30 August 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF28PP.503.BTL

Sponsor

The Chemours Company
1007 Market Street D-3008
Wilmington, DE 19899

Sponsor Number

C30049

1. STATEMENT OF COMPLIANCE

Study No. AF28PP.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

30 NOV 2018
Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF28PP.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
05-Jun-2018	05-Jun-2018	Protocol Review	05-Jun-2018	05-Jun-2018
05-Jun-2018	05-Jun-2018	Preparation of S9 Mixture	05-Jun-2018	05-Jun-2018
17-Jul-2018	17-Jul-2018	Data/Draft Report	17-Jul-2018	17-Jul-2018
17-Jul-2018	17-Jul-2018	Protocol Amendment Review	17-Jul-2018	17-Jul-2018
27-Aug-2018	27-Aug-2018	Final Report	27-Aug-2018	27-Aug-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Lisa AnnMarie Fleshman 30-Aug-2018 12:26 pm GMT

Reason for signature: QA Approval

Printed by:Lisa AnnMarie Fleshman
Printed on:30-Aug-18

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4. STUDY INFORMATION

Study Conduct

Sponsor:	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor's Authorized Representative:	Shawn Gannon, Ph.D., DABT
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF28PP.503.BTL
Sponsor No.:	C30049

Test Substance

Identification:	Sodium salt of PSEPVE Acid
CAS No.:	65086-48-8
Purity:	96.6% (per protocol)
Molecular Weight:	466.11 g/mol
Description:	White powder
Storage Conditions:	Room temperature, protected from light
Receipt Date:	02 May 2018

Study Dates

Study Initiation Date:	01 June 2018
Experimental Starting Date (first day of data collection):	01 June 2018
Experimental Start Date (first day test substance administered to test system):	05 June 2018
Experimental Completion Date:	27 June 2018

Key Personnel

Study Director:	Emily Dakoulas, BS

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, Sodium salt of PSEPVE Acid, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Water was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Based upon these results, the maximum dose tested in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, the dose levels tested were 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate Sodium salt of PSEPVE Acid was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization using the information below.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

The vehicle used to deliver Sodium salt of PSEPVE Acid to the test system was water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Water	7732-18-5	Sigma-Aldrich	RNBF9658	Sterile-filtered	Mar 2019
			RNBG4913		Dec 2019

To achieve a solution, the most concentrated dilution was sonicated at 28.3ºC for 1 minute in the initial toxicity-mutation assay. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Water was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in water at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3925, Exp. Date: 21 Feb 2020; Lot No. 3961, Exp. Date: 15 May 2020) was purchased

commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and five dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	2.2	1.0	0.8	1.5	2.9
B2	2.0	1.2	1.7	1.7	4.0

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in water are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

Neither precipitate nor toxicity was observed.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 50.0, 150, 500, 1500 and 5000 µg per plate.

Neither precipitate nor toxicity was observed.

A copy of the Common Technical Document Tables is included in Appendix III.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Sodium salt of PSEPVE Acid did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PP.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PP
Date Plated: 6/5/2018
Evaluation Period: 6/12/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of PSEPVE Acid**	5000 µg	*17*	*1*	*1.1*	18^{A}, 16^{A}
		1500 µg	*16*	*4*	*1.1*	18^{A}, 13^{A}
		500 µg	*15*	*5*	*1.0*	18^{A}, 11^{A}
		150 µg	*15*	*0*	*1.0*	15^{A}, 15^{A}
		50.0 µg	*21*	*2*	*1.4*	19^{A}, 22^{A}
		15.0 µg	*17*	*8*	*1.1*	11^{A}, 22^{A}
		5.00 µg	*20*	*2*	*1.3*	21^{A}, 18^{A}
		1.50 µg	*14*	*1*	*0.9*	15^{A}, 13^{A}
	Water	100 µL	*15*	*6*		19^{A}, 11^{A}
TA100	**Sodium salt of PSEPVE Acid**	5000 µg	*105*	*37*	*1.0*	131^{A}, 79^{A}
		1500 µg	*101*	*14*	*1.0*	111^{A}, 91^{A}
		500 µg	*94*	*8*	*0.9*	88^{A}, 100^{A}
		150 µg	*90*	*20*	*0.9*	104^{A}, 76^{A}
		50.0 µg	*101*	*12*	*1.0*	109^{A}, 92^{A}
		15.0 µg	*98*	*16*	*1.0*	87^{A}, 109^{A}
		5.00 µg	*96*	*5*	*0.9*	92^{A}, 99^{A}
		1.50 µg	*112*	*6*	*1.1*	107^{A}, 116^{A}
	Water	100 µL	*103*	*18*		90^{A}, 115^{A}
TA1535	**Sodium salt of PSEPVE Acid**	5000 µg	*14*	*1*	*1.4*	13^{A}, 14^{A}
		1500 µg	*12*	*1*	*1.2*	11^{A}, 13^{A}
		500 µg	*13*	*3*	*1.3*	11^{A}, 15^{A}
		150 µg	*16*	*4*	*1.6*	18^{A}, 13^{A}
		50.0 µg	*13*	*4*	*1.3*	10^{A}, 15^{A}
		15.0 µg	*11*	*6*	*1.1*	7^{A}, 15^{A}
		5.00 µg	*13*	*2*	*1.3*	11^{A}, 14^{A}
		1.50 µg	*13*	*4*	*1.3*	10^{A}, 15^{A}
	Water	100 µL	*10*	*1*		10^{A}, 9^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PP.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PP
Date Plated: 6/5/2018
Evaluation Period: 6/12/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Sodium salt of PSEPVE Acid**	5000 µg	*10*	*1*	*1.1*	10^{A}, 9^{A}
		1500 µg	*8*	*4*	*0.9*	5^{A}, 10^{A}
		500 µg	*9*	*2*	*1.0*	10^{A}, 7^{A}
		150 µg	*11*	*6*	*1.2*	6^{A}, 15^{A}
		50.0 µg	*11*	*7*	*1.2*	16^{A}, 6^{A}
		15.0 µg	*14*	*1*	*1.6*	14^{A}, 13^{A}
		5.00 µg	*7*	*1*	*0.8*	8^{A}, 6^{A}
		1.50 µg	*10*	*0*	*1.1*	10^{A}, 10^{A}
	Water	100 µL	*9*	*1*		8^{A}, 10^{A}
WP2uvrA	**Sodium salt of PSEPVE Acid**	5000 µg	*31*	*1*	*1.0*	32^{A}, 30^{A}
		1500 µg	*33*	*5*	*1.1*	36^{A}, 29^{A}
		500 µg	*37*	*8*	*1.2*	42^{A}, 31^{A}
		150 µg	*33*	*0*	*1.1*	33^{A}, 33^{A}
		50.0 µg	*43*	*2*	*1.4*	44^{A}, 41^{A}
		15.0 µg	*40*	*9*	*1.3*	46^{A}, 33^{A}
		5.00 µg	*21*	*5*	*0.7*	24^{A}, 17^{A}
		1.50 µg	*30*	*5*	*1.0*	33^{A}, 26^{A}
	Water	100 µL	*30*	*12*		21^{A}, 38^{A}
TA98	**2NF**	1.00 µg	*68*	*11*	*4.5*	60^{A}, 75^{A}
TA100	**SA**	1.00 µg	*754*	*27*	*7.3*	773^{A}, 735^{A}
TA1535	**SA**	1.00 µg	*648*	*49*	*64.8*	613^{A}, 683^{A}
TA1537	**9AAD**	75.0 µg	*514*	*34*	*57.1*	490^{A}, 538^{A}
WP2uvrA	**MMS**	1000 µg	*367*	*43*	*12.2*	336^{A}, 397^{A}

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PP.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PP
Date Plated: 6/5/2018
Evaluation Period: 6/12/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of PSEPVE Acid**	5000 µg	*20*	*1*	*0.7*	19[A], 21[A]
		1500 µg	*17*	*3*	*0.6*	15[A], 19[A]
		500 µg	*21*	*3*	*0.7*	19[A], 23[A]
		150 µg	*23*	*5*	*0.8*	19[A], 26[A]
		50.0 µg	*32*	*3*	*1.1*	34[A], 30[A]
		15.0 µg	*27*	*6*	*0.9*	22[A], 31[A]
		5.00 µg	*22*	*0*	*0.7*	22[A], 22[A]
		1.50 µg	*22*	*1*	*0.7*	23[A], 21[A]
	Water	100 µL	*30*	*8*		36[A], 24[A]
TA100	**Sodium salt of PSEPVE Acid**	5000 µg	*120*	*4*	*1.1*	117[A], 122[A]
		1500 µg	*109*	*8*	*1.0*	103[A], 114[A]
		500 µg	*113*	*14*	*1.0*	123[A], 103[A]
		150 µg	*100*	*12*	*0.9*	108[A], 91[A]
		50.0 µg	*114*	*14*	*1.0*	104[A], 124[A]
		15.0 µg	*116*	*10*	*1.0*	123[A], 109[A]
		5.00 µg	*106*	*7*	*1.0*	111[A], 101[A]
		1.50 µg	*103*	*6*	*0.9*	99[A], 107[A]
	Water	100 µL	*111*	*5*		114[A], 107[A]
TA1535	**Sodium salt of PSEPVE Acid**	5000 µg	*9*	*1*	*0.5*	10[A], 8[A]
		1500 µg	*10*	*0*	*0.6*	10[A], 10[A]
		500 µg	*15*	*3*	*0.9*	17[A], 13[A]
		150 µg	*11*	*3*	*0.6*	13[A], 9[A]
		50.0 µg	*11*	*0*	*0.6*	11[A], 11[A]
		15.0 µg	*18*	*2*	*1.1*	19[A], 16[A]
		5.00 µg	*11*	*3*	*0.6*	9[A], 13[A]
		1.50 µg	*15*	*6*	*0.9*	11[A], 19[A]
	Water	100 µL	*17*	*6*		13[A], 21[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PP.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PP
Date Plated: 6/5/2018
Evaluation Period: 6/12/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Sodium salt of PSEPVE Acid**	5000 µg	*9*	*3*	*1.3*	7[A], 11[A]
		1500 µg	*7*	*1*	*1.0*	6[A], 8[A]
		500 µg	*8*	*1*	*1.1*	7[A], 8[A]
		150 µg	*11*	*3*	*1.6*	13[A], 9[A]
		50.0 µg	*11*	*3*	*1.6*	9[A], 13[A]
		15.0 µg	*12*	*4*	*1.7*	14[A], 9[A]
		5.00 µg	*7*	*6*	*1.0*	11[A], 3[A]
		1.50 µg	*9*	*0*	*1.3*	9[A], 9[A]
	Water	100 µL	*7*	*0*		7[A], 7[A]
WP2uvrA	**Sodium salt of PSEPVE Acid**	5000 µg	*36*	*3*	*1.0*	34[A], 38[A]
		1500 µg	*39*	*1*	*1.1*	38[A], 39[A]
		500 µg	*36*	*5*	*1.0*	39[A], 32[A]
		150 µg	*34*	*4*	*1.0*	36[A], 31[A]
		50.0 µg	*37*	*14*	*1.1*	47[A], 27[A]
		15.0 µg	*35*	*6*	*1.0*	39[A], 31[A]
		5.00 µg	*28*	*6*	*0.8*	24[A], 32[A]
		1.50 µg	*45*	*6*	*1.3*	41[A], 49[A]
	Water	100 µL	*35*	*7*		40[A], 30[A]
TA98	**2AA**	1.00 µg	*245*	*10*	*8.2*	252[A], 238[A]
TA100	**2AA**	2.00 µg	*718*	*37*	*6.5*	692[A], 744[A]
TA1535	**2AA**	1.00 µg	*78*	*6*	*4.6*	74[A], 82[A]
TA1537	**2AA**	2.00 µg	*51*	*2*	*7.3*	49[A], 52[A]
WP2uvrA	**2AA**	15.0 µg	*328*	*30*	*9.4*	306[A], 349[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PP.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PP
Date Plated: 6/20/2018
Evaluation Period: 6/27/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of PSEPVE Acid**	5000 µg	*15*	*6*	*0.8*	12^{A}, 12^{A}, 22^{A}
		1500 µg	*15*	*4*	*0.8*	15^{A}, 19^{A}, 12^{A}
		500 µg	*17*	*6*	*0.9*	12^{A}, 16^{A}, 24^{A}
		150 µg	*16*	*9*	*0.8*	6^{A}, 24^{A}, 17^{A}
		50.0 µg	*17*	*3*	*0.9*	17^{A}, 15^{A}, 20^{A}
	Water	100 µL	*19*	*4*		21^{A}, 22^{A}, 15^{A}
TA100	**Sodium salt of PSEPVE Acid**	5000 µg	*92*	*7*	*0.9*	84^{A}, 94^{A}, 98^{A}
		1500 µg	*93*	*10*	*0.9*	94^{A}, 102^{A}, 83^{A}
		500 µg	*100*	*2*	*1.0*	100^{A}, 98^{A}, 102^{A}
		150 µg	*100*	*12*	*1.0*	114^{A}, 97^{A}, 90^{A}
		50.0 µg	*93*	*6*	*0.9*	89^{A}, 99^{A}, 90^{A}
	Water	100 µL	*98*	*3*		94^{A}, 99^{A}, 100^{A}
TA1535	**Sodium salt of PSEPVE Acid**	5000 µg	*14*	*2*	*0.9*	14^{A}, 16^{A}, 12^{A}
		1500 µg	*15*	*2*	*0.9*	14^{A}, 15^{A}, 17^{A}
		500 µg	*15*	*3*	*0.9*	11^{A}, 17^{A}, 17^{A}
		150 µg	*18*	*2*	*1.1*	16^{A}, 20^{A}, 17^{A}
		50.0 µg	*15*	*1*	*0.9*	14^{A}, 15^{A}, 15^{A}
	Water	100 µL	*16*	*3*		15^{A}, 19^{A}, 14^{A}
TA1537	**Sodium salt of PSEPVE Acid**	5000 µg	*7*	*1*	*0.9*	7^{A}, 6^{A}, 7^{A}
		1500 µg	*8*	*3*	*1.0*	11^{A}, 6^{A}, 7^{A}
		500 µg	*9*	*3*	*1.1*	6^{A}, 11^{A}, 9^{A}
		150 µg	*7*	*1*	*0.9*	6^{A}, 7^{A}, 7^{A}
		50.0 µg	*7*	*2*	*0.9*	5^{A}, 7^{A}, 9^{A}
	Water	100 µL	*8*	*4*		4^{A}, 11^{A}, 9^{A}
WP2uvrA	**Sodium salt of PSEPVE Acid**	5000 µg	*27*	*2*	*1.0*	28^{A}, 24^{A}, 28^{A}
		1500 µg	*24*	*4*	*0.9*	24^{A}, 20^{A}, 28^{A}
		500 µg	*26*	*5*	*1.0*	21^{A}, 30^{A}, 27^{A}
		150 µg	*29*	*4*	*1.1*	33^{A}, 26^{A}, 27^{A}
		50.0 µg	*27*	*2*	*1.0*	28^{A}, 27^{A}, 25^{A}
	Water	100 µL	*26*	*6*		27^{A}, 31^{A}, 20^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PP.503.BTL
Study Code: AF28PP
Experiment: B2
Date Plated: 6/20/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/27/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**2NF**	1.00 µg	*67*	*4*	*3.5*	64[A], 71[A], 66[A]
TA100	**SA**	1.00 µg	*715*	*33*	*7.3*	682[A], 748[A], 716[A]
TA1535	**SA**	1.00 µg	*762*	*47*	*47.6*	812[A], 718[A], 755[A]
TA1537	**9AAD**	75.0 µg	*768*	*49*	*96.0*	781[A], 810[A], 714[A]
WP2uvrA	**MMS**	1000 µg	*461*	*47*	*17.7*	416[A], 510[A], 457[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PP.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PP
Date Plated: 6/20/2018
Evaluation Period: 6/27/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Sodium salt of PSEPVE Acid**	5000 µg	*20*	*2*	*1.1*	22^{A}, 19^{A}, 19^{A}
		1500 µg	*20*	*2*	*1.1*	22^{A}, 20^{A}, 19^{A}
		500 µg	*19*	*4*	*1.0*	15^{A}, 22^{A}, 21^{A}
		150 µg	*20*	*3*	*1.1*	22^{A}, 20^{A}, 17^{A}
		50.0 µg	*19*	*3*	*1.0*	20^{A}, 16^{A}, 21^{A}
	Water	100 µL	*19*	*2*		19^{A}, 17^{A}, 21^{A}
TA100	**Sodium salt of PSEPVE Acid**	5000 µg	*127*	*13*	*1.2*	123^{A}, 116^{A}, 141^{A}
		1500 µg	*126*	*8*	*1.2*	125^{A}, 135^{A}, 119^{A}
		500 µg	*107*	*12*	*1.0*	120^{A}, 105^{A}, 97^{A}
		150 µg	*105*	*3*	*1.0*	103^{A}, 109^{A}, 104^{A}
		50.0 µg	*102*	*4*	*0.9*	106^{A}, 98^{A}, 102^{A}
	Water	100 µL	*109*	*1*		109^{A}, 108^{A}, 110^{A}
TA1535	**Sodium salt of PSEPVE Acid**	5000 µg	*13*	*2*	*0.9*	15^{A}, 11^{A}, 12^{A}
		1500 µg	*16*	*2*	*1.1*	17^{A}, 14^{A}, 16^{A}
		500 µg	*12*	*3*	*0.9*	12^{A}, 10^{A}, 15^{A}
		150 µg	*13*	*4*	*0.9*	11^{A}, 17^{A}, 10^{A}
		50.0 µg	*11*	*1*	*0.8*	12^{A}, 11^{A}, 10^{A}
	Water	100 µL	*14*	*2*		12^{A}, 16^{A}, 14^{A}
TA1537	**Sodium salt of PSEPVE Acid**	5000 µg	*10*	*1*	*1.7*	9^{A}, 9^{A}, 11^{A}
		1500 µg	*7*	*2*	*1.2*	6^{A}, 9^{A}, 5^{A}
		500 µg	*7*	*2*	*1.2*	6^{A}, 9^{A}, 5^{A}
		150 µg	*8*	*2*	*1.3*	7^{A}, 10^{A}, 7^{A}
		50.0 µg	*7*	*2*	*1.2*	6^{A}, 9^{A}, 7^{A}
	Water	100 µL	*6*	*1*		6^{A}, 7^{A}, 6^{A}
WP2uvrA	**Sodium salt of PSEPVE Acid**	5000 µg	*32*	*5*	*1.0*	33^{A}, 27^{A}, 37^{A}
		1500 µg	*31*	*1*	*0.9*	30^{A}, 31^{A}, 31^{A}
		500 µg	*36*	*1*	*1.1*	37^{A}, 35^{A}, 36^{A}
		150 µg	*33*	*3*	*1.0*	31^{A}, 37^{A}, 31^{A}
		50.0 µg	*35*	*3*	*1.1*	33^{A}, 38^{A}, 35^{A}
	Water	100 µL	*33*	*5*		28^{A}, 33^{A}, 37^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 4 (CONT.)
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PP.503.BTL
Study Code: AF28PP
Experiment: B2
Date Plated: 6/20/2018
Exposure Method: Plate incorporation assay
Evaluation Period: 6/27/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**2AA**	1.00 µg	*218*	*27*	*11.5*	203[A], 249[A], 203[A]
TA100	**2AA**	2.00 µg	*750*	*34*	*6.9*	742[A], 788[A], 721[A]
TA1535	**2AA**	1.00 µg	*83*	*13*	*5.9*	82[A], 97[A], 71[A]
TA1537	**2AA**	2.00 µg	*43*	*9*	*7.2*	33[A], 50[A], 45[A]
WP2uvrA	**2AA**	15.0 µg	*300*	*14*	*9.1*	312[A], 302[A], 285[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values 2016 revertants per plate											
Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	
SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control											

14. APPENDIX II: Study Protocol and Amendment

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PP.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 1500, 500, 150 and 50.0 µg per plate.

   Reason: To specify the dose levels to be used for the confirmatory mutagenicity assay based on the toxicity and precipitate profiles observed in the initial toxicity-mutation assay.

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PP.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, Ph.D., DABT
Sponsor Representative

19 June 2018
Date

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PP.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS
BioReliance Study Director

19 JUN 2018
Date

BioReliance Study Management

19 June 2018
Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF28PP.503.BTL

1. KEY PERSONNEL

Sponsor Information:

Sponsor	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor Number	C30049
Sponsor's Authorized Representative	Shawn Gannon, Ph.D., DABT The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899 Phone: 302-773-1376 Email: SHAWN.A.GANNON@chemours.com

Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	06-June-2018
Proposed Experimental Completion Date	06-July-2018
Proposed Report Date	20-July-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices. and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST SUBSTANCE INFORMATION

Identification	Sodium salt of PSEPVE Acid
CAS No.	65086-48-8
Storage Conditions	Room Temperature Protect from light (Per BioReliance SOP)
Purity	96.6% (no correction factor will be used for dose formulations)
Molecular Weight	466.11 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused Test Substance will be returned to the sponsor prior to report finalization using the information below; unless the test substance is used on another study.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

Residual dose formulations will be discarded after use.

7. **TEST SYSTEM**
The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test suhstance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values
Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of

revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains

- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

Shawn Gannon, Ph.D., DABT
Sponsor Representative

8 May 2018
Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 01 JUN 2018

BioReliance Study Management — Date: 01-JUN-18

15. APPENDIX III: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test for Induction of: Reverse mutation in bacterial cells

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

Metabolizing System: Aroclor-induced rat liver S9

Vehicle for Test Substance: Water

Treatment: Plate incorporation

Cytotoxic Effects: None

Genotoxic Effects: None

No. of Independent Assays: 2

No. of Replicate Cultures: 2 (B1) and 3 (B2)

Test Substance: Sodium salt of PSEPVE Acid

Study No.: AF28PP.503.BTL

No. Cells Analyzed/Culture: 0.8 to 4.0 x 10^8 cells per plate

GLP Compliance: Yes

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Date(s) of Treatment: 05 June 2018 (B1) and 20 June 2018 (B2)

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B1: Initial Toxicity-Mutation Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	15 ± 6	103 ± 18	10 ± 1	9 ± 1	30 ± 12
	Sodium salt of PSEPVE Acid	1.50	14 ± 1	112 ± 6	13 ± 4	10 ± 0	30 ± 5
		5.00	20 ± 2	96 ± 5	13 ± 2	7 ± 1	21 ± 5
		15.0	17 ± 8	98 ± 16	11 ± 6	14 ± 1	40 ± 9
		50.0	21 ± 2	101 ± 12	13 ± 4	11 ± 7	43 ± 2
		150	15 ± 0	90 ± 20	16 ± 4	11 ± 6	33 ± 0
		500	15 ± 5	94 ± 8	13 ± 3	9 ± 2	37 ± 8
		1500	16 ± 4	101 ± 14	12 ± 1	8 ± 4	33 ± 5
		5000	17 ± 1	105 ± 37	14 ± 1	10 ± 1	31 ± 1
	2NF	1.00	68 ± 11				
	SA	1.00		754 ± 27	648 ± 49		
	9AAD	75.0				514 ± 34	
	MMS	1000					367 ± 43
With Activation	Water	100 µL/plate	30 ± 8	111 ± 5	17 ± 6	7 ± 0	35 ± 7
	Sodium salt of PSEPVE Acid	1.50	22 ± 1	103 ± 6	15 ± 6	9 ± 0	45 ± 6
		5.00	22 ± 0	106 ± 7	11 ± 3	7 ± 6	28 ± 6
		15.0	27 ± 6	116 ± 10	18 ± 2	12 ± 4	35 ± 6
		50.0	32 ± 3	114 ± 14	11 ± 0	11 ± 3	37 ± 14
		150	23 ± 5	100 ± 12	11 ± 3	11 ± 3	34 ± 4
		500	21 ± 3	113 ± 14	15 ± 3	8 ± 1	36 ± 5
		1500	17 ± 3	109 ± 8	10 ± 0	7 ± 1	39 ± 1
		5000	20 ± 1	120 ± 4	9 ± 1	9 ± 3	36 ± 3
	2AA	1.00	245 ± 10		78 ± 6		
	2AA	2.00		718 ± 37		51 ± 2	
	2AA	15.0					328 ± 30

Key to Positive Controls

SA	sodium azide	2NF	2-nitrofluorene
2AA	2-aminoanthracene	MMS	methyl methanesulfonate
9AAD	9-Aminoacridine		

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD) (B2: Confirmatory Mutagenicity Assay)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	19 ± 4	98 ± 3	16 ± 3	8 ± 4	26 ± 6
	Sodium salt of PSEPVE Acid	50.0	17 ± 3	93 ± 6	15 ± 1	7 ± 2	27 ± 2
		150	16 ± 9	100 ± 12	18 ± 2	7 ± 1	29 ± 4
		500	17 ± 6	100 ± 2	15 ± 3	9 ± 3	26 ± 5
		1500	15 ± 4	93 ± 10	15 ± 2	8 ± 3	24 ± 4
		5000	15 ± 6	92 ± 7	14 ± 2	7 ± 1	27 ± 2
	2NF	1.00	67 ± 4				
	SA	1.00		715 ± 33	762 ± 47		
	9AAD	75.0				768 ± 49	
	MMS	1000					461 ± 47
With Activation	Water	100 µL/plate	19 ± 2	109 ± 1	14 ± 2	6 ± 1	33 ± 5
	Sodium salt of PSEPVE Acid	50.0	19 ± 3	102 ± 4	11 ± 1	7 ± 2	35 ± 3
		150	20 ± 3	105 ± 3	13 ± 4	8 ± 2	33 ± 3
		500	19 ± 4	107 ± 12	12 ± 3	7 ± 2	36 ± 1
		1500	20 ± 2	126 ± 8	16 ± 2	7 ± 2	31 ± 1
		5000	20 ± 2	127 ± 13	13 ± 2	10 ± 1	32 ± 5
	2AA	1.00	218 ± 27		83 ± 13		
	2AA	2.00		750 ± 34		43 ± 9	
	2AA	15.0					300 ± 14

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005)

Test Substance

Potassium salt of Hydro PSEPVE Acid

Author

Emily Dakoulas, BS

Study Completion Date

29 August 2018

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AF28PR.503.BTL

Sponsor

The Chemours Company
1007 Market Street D-3008
Wilmington, DE 19899

Sponsor Number

C30049

1. STATEMENT OF COMPLIANCE

Study No. AF28PR.503.BTL was conducted in compliance with the following regulation: US EPA GLP Standards 40 CFR 792 (TSCA). This regulation is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries. The following exceptions were noted:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily Dakoulas, BS
Study Director

Date

2. QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AF28PR.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
13-Jun-2018	13-Jun-2018	Plating	13-Jun-2018	13-Jun-2018
13-Jun-2018	13-Jun-2018	Strain Characterization	14-Jun-2018	14-Jun-2018
05-Jul-2018	05-Jul-2018	Protocol Amendment Review	05-Jul-2018	05-Jul-2018
05-Jul-2018	05-Jul-2018	Protocol Review	05-Jul-2018	05-Jul-2018
06-Jul-2018	06-Jul-2018	Data/Draft Report	06-Jul-2018	06-Jul-2018
20-Aug-2018	21-Aug-2018	Final Report	21-Aug-2018	21-Aug-2018

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Luleayenwa Aberra-Degu 28-Aug-2018 8:14 pm GMT

Reason for signature: QA Approval

Printed by:Luleayenwa Aberra-Degu
Printed on:28-Aug-18

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4. STUDY INFORMATION

Study Conduct

Sponsor:	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor's Authorized Representative:	Shawn Gannon, Ph.D., DABT
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AF28PR.503.BTL
Sponsor No.:	C30049

Test Substance

Identification:	Potassium salt of Hydro PSEPVE Acid
CAS No.:	259140-44-8
Purity:	97.7% (per protocol)
Molecular Weight:	502.22 g/mol
Description:	White powder
Storage Conditions:	Room temperature, protected from light
Receipt Date:	02 May 2018

Study Dates

Study Initiation Date:	24 May 2018
Experimental Starting Date (first day of data collection):	25 May 2018
Experimental Start Date (first day test substance administered to test system):	31 May 2018
Experimental Completion Date:	25 June 2018

Key Personnel

Study Director:	Emily Dakoulas, BS

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Ankit Patel, BS
Report Writer:	Gayathri Jayakumar, MPS

5. SUMMARY

The test substance, Potassium salt of Hydro PSEPVE Acid, was tested to evaluate its mutagenic potential by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Water was used as the vehicle.

In the initial toxicity-mutation assay, the dose levels tested were 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Based upon these results, the maximum dose tested in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, the dose levels tested were 50.0, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

These results indicate Potassium salt of Hydro PSEPVE Acid was negative for the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

6. PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system.

Historical control data are found in Appendix I. Copies of the study protocol and amendment are included in Appendix II.

7. CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The identity, strength, purity, stability and composition or other characteristics to define the test substance have not been determined.

All unused Test Substance was returned to the sponsor prior to report finalization using the information below.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

The vehicle used to deliver Potassium salt of Hydro PSEPVE Acid to the test system was water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Water	7732-18-5	Sigma-Aldrich	RNBF9658	Sterile-filtered	Mar 2019

Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light. To achieve a solution, the most concentrated dilution was sonicated at 31.7ºC for 22 minutes in the confirmatory mutagenicity assay.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 30-Nov-2019 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2019 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBT8080V Exp. Date Jan-2020 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. BCBK1177V Exp. Date 31-Mar-2019 CAS No. 52417-22-8 Purity 99.5%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBX5165V Exp. Date 31-Oct-2020 CAS No. 66-27-3 Purity 99.5%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

8. MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Salmonella tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Solubility Determination

Water was the vehicle of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in water at a concentration of approximately 50 mg/mL in the solubility test conducted at BioReliance.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3925, Exp. Date: 21 Feb 2020; Lot No. 3961, Exp. Date: 15 May 2020) was purchased

commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and eight dose levels of the test substance, in duplicate, in the presence and absence of Aroclor-induced rat liver S9. Dose levels for the confirmatory mutagenicity assay were based upon lack of post-treatment toxicity.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. TA98, TA100, TA1535, TA1537 and WP2 *uvr*A were exposed to the vehicle alone, positive controls and five dose levels of the test substance, in triplicate, in the presence and absence of Aroclor-induced rat liver S9.

Treatment of Test System

Media used in the treatment of the test system were as indicated below.

Component	Medium			
	Minimal top agar	Minimal bottom agar	Nutrient bottom agar	Nutrient broth
	Concentration in Medium			
BBL Select agar (W/V)	0.8% (W/V)	--	--	--
Vogel-Bonner minimal medium E	--	1.5% (W/V)	1.5% (W/V)	--
Sodium chloride	0.5% (W/V)	--	--	--
L-histidine, D-biotin and L-tryptophan solution	50 mM each	--	--	--
Sterile water	25 mL/100 mL agar (when agar not used with S9 or Sham mix)	--	--	--
Oxoid Nutrient Broth No. 2 (dry powder)	--	--	2.5% (W/V)	2.5% (W/V)
Vogel-Bonner salt solution	--	--	--	Supplied at 20 mL/L

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50.0 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid:

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene.

Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive).

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL.

The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control and exceed the corresponding acceptable vehicle control range cited above.

A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance at JK Records as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

9. RESULTS AND DISCUSSION

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	1.3	1.4	1.1	1.4	3.1
B2	1.3	1.1	1.5	1.8	2.9

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay conducted at dose levels of 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate in water are presented in Tables 1 and 2. The maximum dose of 5000 µg per plate was achieved using a concentration of 50.0 mg/mL and a 100 µL plating aliquot.

Neither precipitate nor toxicity was observed.

No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. Based upon the results of the initial toxicity-mutation assay, the dose levels selected for the confirmatory mutagenicity assay were 50.0, 150, 500, 1500 and 5000 µg per plate.

Neither precipitate nor toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

A copy of the Common Technical Document Tables is included in Appendix III.

10. CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Potassium salt of Hydro PSEPVE Acid did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

11. REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

12. DATA TABLES

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PR.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PR
Date Plated: 5/31/2018
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*20*	*3*	*1.3*	22[A], 18[A]
		1500 µg	*18*	*2*	*1.2*	19[A], 16[A]
		500 µg	*15*	*5*	*1.0*	11[A], 18[A]
		150 µg	*20*	*1*	*1.3*	21[A], 19[A]
		50.0 µg	*16*	*3*	*1.1*	14[A], 18[A]
		15.0 µg	*17*	*3*	*1.1*	15[A], 19[A]
		5.00 µg	*10*	*0*	*0.7*	10[A], 10[A]
		1.50 µg	*19*	*6*	*1.3*	14[A], 23[A]
	Water	100 µL	*15*	*0*		15[A], 15[A]
TA100	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*82*	*6*	*1.0*	86[A], 78[A]
		1500 µg	*100*	*17*	*1.2*	88[A], 112[A]
		500 µg	*93*	*4*	*1.1*	95[A], 90[A]
		150 µg	*104*	*5*	*1.3*	100[A], 107[A]
		50.0 µg	*85*	*9*	*1.0*	91[A], 78[A]
		15.0 µg	*85*	*4*	*1.0*	82[A], 88[A]
		5.00 µg	*77*	*5*	*0.9*	80[A], 73[A]
		1.50 µg	*96*	*13*	*1.2*	86[A], 105[A]
	Water	100 µL	*83*	*0*		83[A], 83[A]
TA1535	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*11*	*1*	*0.9*	10[A], 11[A]
		1500 µg	*14*	*8*	*1.2*	19[A], 8[A]
		500 µg	*11*	*0*	*0.9*	11[A], 11[A]
		150 µg	*8*	*1*	*0.7*	9[A], 7[A]
		50.0 µg	*12*	*2*	*1.0*	13[A], 10[A]
		15.0 µg	*10*	*9*	*0.8*	16[A], 3[A]
		5.00 µg	*12*	*2*	*1.0*	10[A], 13[A]
		1.50 µg	*10*	*1*	*0.8*	9[A], 10[A]
	Water	100 µL	*12*	*1*		11[A], 13[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 1 (CONT.)
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AF28PR.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PR
Date Plated: 5/31/2018
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*8*	*4*	*0.9*	5[A], 11[A]
		1500 µg	*8*	*4*	*0.9*	5[A], 10[A]
		500 µg	*9*	*3*	*1.0*	7[A], 11[A]
		150 µg	*10*	*2*	*1.1*	8[A], 11[A]
		50.0 µg	*11*	*3*	*1.2*	13[A], 9[A]
		15.0 µg	*13*	*4*	*1.4*	10[A], 15[A]
		5.00 µg	*9*	*3*	*1.0*	7[A], 11[A]
		1.50 µg	*5*	*3*	*0.6*	3[A], 7[A]
	Water	100 µL	*9*	*1*		8[A], 10[A]
WP2uvrA	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*25*	*3*	*0.8*	27[A], 23[A]
		1500 µg	*33*	*1*	*1.1*	33[A], 32[A]
		500 µg	*30*	*8*	*1.0*	36[A], 24[A]
		150 µg	*35*	*5*	*1.2*	31[A], 38[A]
		50.0 µg	*32*	*4*	*1.1*	29[A], 35[A]
		15.0 µg	*43*	*6*	*1.4*	38[A], 47[A]
		5.00 µg	*25*	*1*	*0.8*	24[A], 25[A]
		1.50 µg	*34*	*3*	*1.1*	32[A], 36[A]
	Water	100 µL	*30*	*6*		34[A], 25[A]
TA98	**2NF**	1.00 µg	*66*	*1*	*4.4*	65[A], 66[A]
TA100	**SA**	1.00 µg	*722*	*62*	*8.7*	766[A], 678[A]
TA1535	**SA**	1.00 µg	*742*	*93*	*61.8*	676[A], 808[A]
TA1537	**9AAD**	75.0 µg	*826*	*94*	*91.8*	759[A], 892[A]
WP2uvrA	**MMS**	1000 µg	*368*	*12*	*12.3*	376[A], 359[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PR.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay
Study Code: AF28PR
Date Plated: 5/31/2018
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*23*	*10*	*1.0*	30[A], 16[A]
		1500 µg	*20*	*4*	*0.9*	17[A], 22[A]
		500 µg	*25*	*6*	*1.1*	29[A], 21[A]
		150 µg	*24*	*3*	*1.1*	26[A], 22[A]
		50.0 µg	*21*	*2*	*1.0*	22[A], 19[A]
		15.0 µg	*27*	*8*	*1.2*	21[A], 32[A]
		5.00 µg	*27*	*5*	*1.2*	23[A], 30[A]
		1.50 µg	*24*	*1*	*1.1*	23[A], 25[A]
	Water	100 µL	*22*	*7*		27[A], 17[A]
TA100	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*106*	*4*	*1.0*	103[A], 109[A]
		1500 µg	*121*	*3*	*1.1*	119[A], 123[A]
		500 µg	*109*	*17*	*1.0*	97[A], 121[A]
		150 µg	*124*	*10*	*1.2*	117[A], 131[A]
		50.0 µg	*107*	*6*	*1.0*	111[A], 103[A]
		15.0 µg	*113*	*13*	*1.1*	103[A], 122[A]
		5.00 µg	*89*	*12*	*0.8*	80[A], 97[A]
		1.50 µg	*100*	*2*	*0.9*	98[A], 101[A]
	Water	100 µL	*107*	*4*		104[A], 109[A]
TA1535	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*14*	*1*	*1.0*	14[A], 13[A]
		1500 µg	*13*	*4*	*0.9*	10[A], 15[A]
		500 µg	*13*	*3*	*0.9*	15[A], 11[A]
		150 µg	*14*	*4*	*1.0*	11[A], 16[A]
		50.0 µg	*17*	*1*	*1.2*	16[A], 17[A]
		15.0 µg	*14*	*4*	*1.0*	16[A], 11[A]
		5.00 µg	*11*	*1*	*0.8*	10[A], 11[A]
		1.50 µg	*15*	*5*	*1.1*	11[A], 18[A]
	Water	100 µL	*14*	*5*		17[A], 10[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 2 (CONT.)
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AF28PR.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AF28PR
Date Plated: 5/31/2018
Evaluation Period: 6/5/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*12*	*5*	*1.2*	8[A], 15[A]
		1500 µg	*12*	*1*	*1.2*	11[A], 13[A]
		500 µg	*11*	*5*	*1.1*	7[A], 14[A]
		150 µg	*15*	*1*	*1.5*	16[A], 14[A]
		50.0 µg	*17*	*6*	*1.7*	13[A], 21[A]
		15.0 µg	*8*	*1*	*0.8*	7[A], 9[A]
		5.00 µg	*14*	*4*	*1.4*	11[A], 17[A]
		1.50 µg	*10*	*2*	*1.0*	11[A], 8[A]
	Water	100 µL	*10*	*4*		7[A], 13[A]
WP2uvrA	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*45*	*4*	*1.2*	42[A], 47[A]
		1500 µg	*51*	*12*	*1.3*	42[A], 59[A]
		500 µg	*46*	*5*	*1.2*	49[A], 42[A]
		150 µg	*42*	*6*	*1.1*	38[A], 46[A]
		50.0 µg	*45*	*5*	*1.2*	48[A], 41[A]
		15.0 µg	*42*	*1*	*1.1*	41[A], 42[A]
		5.00 µg	*37*	*8*	*1.0*	43[A], 31[A]
		1.50 µg	*41*	*8*	*1.1*	35[A], 46[A]
	Water	100 µL	*38*	*9*		44[A], 31[A]
TA98	**2AA**	1.00 µg	*198*	*13*	*9.0*	207[A], 188[A]
TA100	**2AA**	2.00 µg	*635*	*11*	*5.9*	627[A], 642[A]
TA1535	**2AA**	1.00 µg	*94*	*19*	*6.7*	80[A], 107[A]
TA1537	**2AA**	2.00 µg	*45*	*16*	*4.5*	56[A], 33[A]
WP2uvrA	**2AA**	15.0 µg	*286*	*2*	*7.5*	284[A], 287[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

[A]: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PR.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PR
Date Plated: 6/13/2018
Evaluation Period: 6/25/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*14*	*4*	*0.8*	18^{A}, 10^{A}, 13^{A}
		1500 µg	*18*	*2*	*1.0*	21^{A}, 17^{A}, 17^{A}
		500 µg	*22*	*2*	*1.2*	24^{A}, 22^{A}, 21^{A}
		150 µg	*20*	*1*	*1.1*	21^{A}, 19^{A}, 21^{A}
		50.0 µg	*21*	*4*	*1.2*	17^{A}, 22^{A}, 24^{A}
	Water	100 µL	*18*	*1*		17^{A}, 18^{A}, 18^{A}
TA100	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*106*	*1*	*1.1*	106^{A}, 106^{A}, 107^{A}
		1500 µg	*106*	*12*	*1.1*	92^{A}, 111^{A}, 114^{A}
		500 µg	*94*	*9*	*1.0*	99^{A}, 99^{A}, 83^{A}
		150 µg	*96*	*15*	*1.0*	88^{A}, 86^{A}, 113^{A}
		50.0 µg	*98*	*3*	*1.0*	101^{A}, 95^{A}, 98^{A}
	Water	100 µL	*97*	*16*		79^{A}, 108^{A}, 104^{A}
TA1535	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*16*	*4*	*1.3*	21^{A}, 14^{A}, 14^{A}
		1500 µg	*15*	*5*	*1.3*	17^{A}, 18^{A}, 9^{A}
		500 µg	*14*	*6*	*1.2*	9^{A}, 21^{A}, 11^{A}
		150 µg	*17*	*1*	*1.4*	17^{A}, 18^{A}, 16^{A}
		50.0 µg	*10*	*1*	*0.8*	11^{A}, 10^{A}, 9^{A}
	Water	100 µL	*12*	*4*		11^{A}, 9^{A}, 17^{A}
TA1537	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*10*	*5*	*1.1*	11^{A}, 14^{A}, 5^{A}
		1500 µg	*12*	*3*	*1.3*	10^{A}, 10^{A}, 16^{A}
		500 µg	*12*	*5*	*1.3*	14^{A}, 7^{A}, 16^{A}
		150 µg	*11*	*1*	*1.2*	10^{A}, 11^{A}, 11^{A}
		50.0 µg	*9*	*1*	*1.0*	9^{A}, 9^{A}, 8^{A}
	Water	100 µL	*9*	*3*		11^{A}, 5^{A}, 11^{A}

Key to Automatic Count Flags

^{A}: Automatic count

TABLE 3 (CONT.)
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AF28PR.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PR
Date Plated: 6/13/2018
Evaluation Period: 6/25/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*28*	*2*	*0.9*	29[A], 26[A], 29[A]
		1500 µg	*30*	*4*	*1.0*	27[A], 34[A], 30[A]
		500 µg	*34*	*7*	*1.1*	34[A], 40[A], 27[A]
		150 µg	*39*	*7*	*1.3*	40[A], 46[A], 32[A]
		50.0 µg	*29*	*3*	*0.9*	32[A], 27[A], 29[A]
	Water	100 µL	*31*	*2*		29[A], 33[A], 31[A]
TA98	**2NF**	1.00 µg	*65*	*9*	*3.6*	57[A], 65[A], 74[A]
TA100	**SA**	1.00 µg	*741*	*48*	*7.6*	790[A], 740[A], 694[A]
TA1535	**SA**	1.00 µg	*759*	*16*	*63.3*	757[A], 744[A], 776[A]
TA1537	**9AAD**	75.0 µg	*630*	*26*	*70.0*	645[A], 600[A], 645[A]
WP2uvrA	**MMS**	1000 µg	*497*	*5*	*16.0*	491[A], 499[A], 500[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PR.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay
Study Code: AF28PR
Date Plated: 6/13/2018
Evaluation Period: 6/25/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*24*	*4*	*1.0*	29[A], 22[A], 21[A]
		1500 µg	*21*	*5*	*0.9*	18[A], 27[A], 19[A]
		500 µg	*26*	*5*	*1.1*	22[A], 31[A], 24[A]
		150 µg	*24*	*3*	*1.0*	22[A], 24[A], 27[A]
		50.0 µg	*29*	*4*	*1.2*	31[A], 24[A], 32[A]
	Water	100 µL	*24*	*2*		26[A], 23[A], 22[A]
TA100	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*116*	*9*	*1.1*	127[A], 111[A], 111[A]
		1500 µg	*100*	*15*	*1.0*	103[A], 113[A], 83[A]
		500 µg	*108*	*13*	*1.1*	122[A], 105[A], 97[A]
		150 µg	*105*	*8*	*1.0*	114[A], 99[A], 103[A]
		50.0 µg	*115*	*21*	*1.1*	91[A], 124[A], 129[A]
	Water	100 µL	*102*	*19*		123[A], 92[A], 90[A]
TA1535	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*11*	*7*	*0.7*	7[A], 19[A], 8[A]
		1500 µg	*16*	*4*	*1.1*	21[A], 13[A], 14[A]
		500 µg	*18*	*2*	*1.2*	19[A], 19[A], 15[A]
		150 µg	*16*	*4*	*1.1*	17[A], 11[A], 19[A]
		50.0 µg	*11*	*3*	*0.7*	13[A], 7[A], 13[A]
	Water	100 µL	*15*	*6*		15[A], 9[A], 21[A]
TA1537	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*11*	*4*	*1.2*	8[A], 16[A], 9[A]
		1500 µg	*10*	*4*	*1.1*	13[A], 5[A], 11[A]
		500 µg	*13*	*1*	*1.4*	14[A], 13[A], 13[A]
		150 µg	*11*	*4*	*1.2*	15[A], 11[A], 7[A]
		50.0 µg	*12*	*2*	*1.3*	11[A], 15[A], 11[A]
	Water	100 µL	*9*	*1*		10[A], 8[A], 9[A]

Key to Automatic Count Flags

[A]: Automatic count

TABLE 4 (CONT.)
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AF28PR.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AF28PR
Date Plated: 6/13/2018
Evaluation Period: 6/25/2018

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2uvrA	**Potassium salt of Hydro PSEPVE Acid**	5000 µg	*32*	*3*	*0.9*	31^{A}, 36^{A}, 30^{A}
		1500 µg	*32*	*9*	*0.9*	36^{A}, 38^{A}, 21^{A}
		500 µg	*35*	*6*	*1.0*	41^{A}, 32^{A}, 31^{A}
		150 µg	*32*	*3*	*0.9*	33^{A}, 34^{A}, 29^{A}
		50.0 µg	*33*	*2*	*1.0*	31^{A}, 35^{A}, 34^{A}
	Water	100 µL	*34*	*2*		32^{A}, 35^{A}, 34^{A}
TA98	**2AA**	1.00 µg	*179*	*77*	*7.5*	226^{A}, 90^{A}, 220^{A}
TA100	**2AA**	2.00 µg	*609*	*66*	*6.0*	534^{A}, 653^{A}, 641^{A}
TA1535	**2AA**	1.00 µg	*101*	*15*	*6.7*	101^{A}, 116^{A}, 86^{A}
TA1537	**2AA**	2.00 µg	*71*	*8*	*7.9*	63^{A}, 79^{A}, 71^{A}
WP2uvrA	**2AA**	15.0 µg	*312*	*24*	*9.2*	287^{A}, 335^{A}, 314^{A}

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic Count Flags

^{A}: Automatic count

13. APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values
2016
revertants per plate

Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	15	5	6	34	5-25	22	6	8	42	10-34
	Pos	198	174	36	1826		287	159	47	1916	
TA100	Neg	90	12	60	146	66-114	94	14	63	181	66-122
	Pos	629	159	186	1383		620	294	192	3483	
TA1535	Neg	12	4	3	31	4-20	12	4	3	26	4-20
	Pos	541	164	34	1082		150	122	27	1114	
TA1537	Neg	8	3	1	21	2-14	9	3	2	23	3-15
	Pos	368	227	21	1791		91	90	17	951	
WP2 *uvr*A	Neg	24	7	7	44	10-38	27	7	8	51	13-41
	Pos	336	119	25	876		300	111	41	1059	

SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control

14. APPENDIX II: Study Protocol and Amendment

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PR.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

1. Page 7, Section 8, Experimental Design and Methodology – Confirmatory Mutagenicity Assay

   Effective: Date of Study Director signature on this amendment

   Add:
   The doses will be 5000, 1500, 500, 150 and 50.0 µg per plate.

   Reason: To specify the dose levels to be used for the confirmatory mutagenicity assay based on the toxicity and precipitate profiles observed in the initial toxicity-mutation assay.

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PR.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Sponsor Approval:

Shawn Gannon, Ph.D., DABT
Sponsor Representative

11 June 2018
Date

PROTOCOL AMENDMENT 1

Sponsor: The Chemours Company

BioReliance Study No.: AF28PR.503.BTL; **Sponsor No.:** C30049

Title: Bacterial Reverse Mutation Assay

Study Director and Test Facility Management Approvals:

Emily Dakoulas, BS
BioReliance Study Director

08 JUN 2018
Date

BioReliance Study Management

08-JUN-18
Date

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study Number	AF28PR.503.BTL

1. KEY PERSONNEL

Sponsor Information:

Sponsor	The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899
Sponsor Number	C30049
Sponsor's Authorized Representative	Shawn Gannon, Ph.D., DABT The Chemours Company 1007 Market Street D-3008 Wilmington, DE 19899 Phone: 302-773-1376 Email: SHAWN.A.GANNON@chemours.com

Test Facility Information:

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@sial.com
BioReliance Quality Assurance Representative	Luleayenwa (Lula) Aberra-Degu, RQAP-GLP BioReliance Corporation Phone: 301-610-2667 Email: Luleayenwa.aberra-degu@sial.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	07-June-2018
Proposed Experimental Completion Date	03-July-2018
Proposed Report Date	18-July-2018

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

The regulation listed is compatible to non-US regulations, OECD Principles of Good Laboratory Practice (C(97)186/Final); Japanese Ministry of Health, Labor and Welfare Good Laboratory Practices (Ordinance Nos. 21 and 114, if applicable); Japanese Ministry of Agriculture, Forestry and Fisheries Good Laboratory Practices (No. 11 Nousan-6283); Japanese Ministry of Economy, Trade and Industry Good Laboratory Practices, and allows submission of the report under the Mutual Acceptance of Data (MAD) agreement with applicable OECD member countries.

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report. If no regulatory compliance statement to any GLP regulations is made by the Test Site(s), a GLP exception will be added to the compliance page of the final report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

At a minimum, Test Site QA is responsible for auditing the raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. Additional audits are conducted as directed by Test Site QA SOPS. Email Testing Facility Management at RCK-Tox-TFM@bioreliance.com. A signed QA Statement documenting the type of audits performed, the dates performed, and the dates in which the audit results were reported to the Study Director, Principal Investigator and their respective management must be submitted by the Test Site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997 and ISO/IEC 17025:2005 (ISO/IEC, 2005).

6. TEST SUBSTANCE INFORMATION

Identification	Potassium salt of Hydro PSEPVE Acid
CAS No.	259140-44-8
Storage Conditions	Room Temperature Protect from light (Per BioReliance SOP)
Purity	97.7% (no correction factor will be used for dose formulations)
Molecular Weight	502.22 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused Test Substance will be returned to the sponsor prior to report finalization using the information below; unless the test substance is used on another study.

Alex Petlick
The Chemours Company
200 Powder Mill Rd
Experimental Station
E402/5317
Wilmington, DE 19803
Phone: +1 (302) 353-5444
Email: Alexandra.Petlick@chemours.com

Residual dose formulations will be discarded after use.

7. **TEST SYSTEM**
The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System

Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate

Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix

100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control articles or the positive control dose formulations. The neat positive control articles and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these articles will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50.0, 15.0, 5.00 and 1.50 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50.0 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50.0 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data (95% control limits), all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate with the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive). Untreated controls, when part of the design, must also be within the ranges cited below.

95% Control Limits (99% Upper Limit)					
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
-S9	5-25 (30)	66-114 (126)	4-20 (24)	2-14 (17)	10-38 (45)
+S9	10-34 (40)	66-122 (136)	4-20 (24)	3-15 (18)	13-41 (48)
With Study Director justification, values including the 99% control limit and above are acceptable.					

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values
Each mean positive control value must exhibit at least a 3.0-fold increase over the respective mean vehicle control value for each tester strain and exceed the corresponding acceptable vehicle control range cited above.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of

revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value and above the corresponding acceptable vehicle control range.

An equivocal response is an increase in a revertant count that is greater than the acceptable vehicle control range but lacks a dose response or does not achieve the respective fold increase threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains

- Test conditions
- Results
- Discussion of results
- Conclusion
- Historical Control Data (vehicle and positive controls with ranges, means and standard deviations)
- Copy of the protocol and any amendment
- Contributing reports (if applicable)
- Information about the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations, if provided by the Sponsor
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the original signed protocol, amendment(s) (if applicable), and the original signed final report will be archived by BioReliance as directed by the applicable SOP. A copy of the draft report, including Study Director and Sponsor comments, if applicable, will be archived electronically by BioReliance. Following the SOP retention period, the Sponsor will be contacted by BioReliance for disposition instructions or return of materials. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database.

BioReliance reserves the right to retain true copies (i.e. photocopies, scans, microfilm, or other accurate reproductions of the original records) for at least the minimum retention period specified by the relevant regulations.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

Shawn Gannon, Ph.D., DABT
Sponsor Representative

8 May 2018
Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 24 MAY 2018

BioReliance Study Management — Date: 24-MAY-18

15. APPENDIX III: Common Technical Document Tables

2.6.7.8 Genotoxicity: In Vitro

Report Title: Bacterial Reverse Mutation Assay

Test for Induction of: Reverse mutation in bacterial cells

Species/Strain: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvr*A

Metabolizing System: Aroclor-induced rat liver S9

Vehicle for Test Substance: Water

Treatment: Plate incorporation

Cytotoxic Effects: None

Genotoxic Effects: None

No. of Independent Assays: 2

No. of Replicate Cultures: 2 (B1) and 3 (B2)

Test Substance: Potassium salt of Hydro PSEPVE Acid

Study No.: AF28PR.503.BTL

No. Cells Analyzed/Culture: 1.1 to 3.1 x 10^8 cells per plate

GLP Compliance: Yes

Vehicle for Positive Controls: DMSO, except sterile water for sodium azide

Date(s) of Treatment: 31 May 2018 (B1) and 13 June 2018 (B2)

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	15 ± 0	83 ± 0	12 ± 1	9 ± 1	30 ± 6
	Potassium salt of Hydro PSEPVE Acid	1.50	19 ± 6	96 ± 13	10 ± 1	5 ± 3	34 ± 3
		5.00	10 ± 0	77 ± 5	12 ± 2	9 ± 3	25 ± 1
		15.0	17 ± 3	85 ± 4	10 ± 9	13 ± 4	43 ± 6
		50.0	16 ± 3	85 ± 9	12 ± 2	11 ± 3	32 ± 4
		150	20 ± 1	104 ± 5	8 ± 1	10 ± 2	35 ± 5
		500	15 ± 5	93 ± 4	11 ± 0	9 ± 3	30 ± 8
		1500	18 ± 2	100 ± 17	14 ± 8	8 ± 4	33 ± 1
		5000	20 ± 3	82 ± 6	11 ± 1	8 ± 4	25 ± 3
	2NF	1.00	66 ± 1				
	SA	1.00		722 ± 62	742 ± 93		
	9AAD	75.0				826 ± 94	
	MMS	1000					368 ± 12
With Activation	Water	100 µL/plate	22 ± 7	107 ± 4	14 ± 5	10 ± 4	38 ± 9
	Potassium salt of Hydro PSEPVE Acid	1.50	24 ± 1	100 ± 2	15 ± 5	10 ± 2	41 ± 8
		5.00	27 ± 5	89 ± 12	11 ± 1	14 ± 4	37 ± 8
		15.0	27 ± 8	113 ± 13	14 ± 4	8 ± 1	42 ± 1
		50.0	21 ± 2	107 ± 6	17 ± 1	17 ± 6	45 ± 5
		150	24 ± 3	124 ± 10	14 ± 4	15 ± 1	42 ± 6
		500	25 ± 6	109 ± 17	13 ± 3	11 ± 5	46 ± 5
		1500	20 ± 4	121 ± 3	13 ± 4	12 ± 1	51 ± 12
		5000	23 ± 10	106 ± 4	14 ± 1	12 ± 5	45 ± 4
	2AA	1.00	198 ± 13		94 ± 19		
	2AA	2.00		635 ± 11		45 ± 16	
	2AA	15.0					286 ± 2

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)				
			TA98	TA100	TA1535	TA1537	WP2uvrA
Without Activation	Water	100 µL/plate	18 ± 1	97 ± 16	12 ± 4	9 ± 3	31 ± 2
	Potassium salt of Hydro PSEPVE Acid	50.0	21 ± 4	98 ± 3	10 ± 1	9 ± 1	29 ± 3
		150	20 ± 1	96 ± 15	17 ± 1	11 ± 1	39 ± 7
		500	22 ± 2	94 ± 9	14 ± 6	12 ± 5	34 ± 7
		1500	18 ± 2	106 ± 12	15 ± 5	12 ± 3	30 ± 4
		5000	14 ± 4	106 ± 1	16 ± 4	10 ± 5	28 ± 2
	2NF	1.00	65 ± 9				
	SA	1.00		741 ± 48	759 ± 16		
	9AAD	75.0				630 ± 26	
	MMS	1000 g					497 ± 5
With Activation	Water	100 µL/plate	24 ± 2	102 ± 19	15 ± 6	9 ± 1	34 ± 2
	Potassium salt of Hydro PSEPVE Acid	50.0	29 ± 4	115 ± 21	11 ± 3	12 ± 2	33 ± 2
		150	24 ± 3	105 ± 8	16 ± 4	11 ± 4	32 ± 3
		500	26 ± 5	108 ± 13	18 ± 2	13 ± 1	35 ± 6
		1500	21 ± 5	100 ± 15	16 ± 4	10 ± 4	32 ± 9
		5000	24 ± 4	116 ± 9	11 ± 7	11 ± 4	32 ± 3
	2AA	1.00	179 ± 77		101 ± 15		
	2AA	2.00		609 ± 66		71 ± 8	
	2AA	15.0					312 ± 24

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

PUBLIC COPY

September 25, 2018

Via Federal Express

Document Processing Center (Mail Code 7407M)
Room 6428
Attention: 8(e) Coordinator – FYI Letter
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
1201 Constitution Ave., NW
Washington, DC 20004

Dear 8(e) Coordinator:

Test Number	Test Substance Abbreviation*	Test Substance	CAS RN (if available)
1	Sodium Salt of Hydrolyzed TAF n = 0	Acetic acid, 2,2-difluoro-2-(trifluoromethoxy)-, sodium salt (1:1)	21837-98-9
2	Sodium Salt of Hydrolyzed TAF n = 1	Acetic acid, 2-[difluoro(trifluoromethoxy)methoxy]-2,2-difluoro-, sodium salt (1:1)	No CAS RN
3	Potassium Salt of Hydrolyzed TAF n = 2	Acetic acid, 2-[[difluoro(trifluoromethoxy)methoxy]difluoromethoxy]-2,2-difluoro-, potassium salt (1:1)	No CAS RN
4	Sodium Salt Hydrolyzed TAF n = 3	3,5,7,9-Tetraoxadecanoic acid, 2,2,4,4,6,6,8,8,10,10,10-undecafluoro-, sodium salt (1:1)	1035377-21-9
5	Sodium Salt of Hydrolyzed TAF n = 4	3,5,7,9,11-Pentaoxadodecanoic acid, 2,2,4,4,6,6,8,8,10,10,12,12,12-tridecafluoro-, sodium salt (1:1)	No CAS RN
6	Potassium Salt of Hydro PSEPVE Acid	Ethanesulfonic acid, 2-[1-[difluoro(1,2,2,2-tetrafluoroethoxy)methyl]-1,2,2,2-tetrafluoroethoxy]-1,1,2,2-tetrafluoro-, potassium salt (1:1)	259140-44-8
7	Sodium Salt of PSEPVE Acid	Ethanesulfonic acid, 2-[1-[difluoro[(1,2,2-trifluoroethenyl)oxy]methyl]-1,2,2,2-tetrafluoroethoxy]-1,1,2,2-tetrafluoro-, sodium salt (1:1)	No CAS RN
8	PFECA A	Butanoic acid, 2,2,3,3,4,4-hexafluoro-4-(trifluoromethoxy)-	863090-89-5
9	PFECA F	Propanoic acid, 2,2,3,3-tetrafluoro-3-(trifluoromethoxy)-	377-73-1
10	PFECA G	Butanoic acid, 2,2,3,3,4,4-hexafluoro-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethoxy]-	801212-59-9

* TAF = telomeric acid fluoride; PSEPVE = perfluorosulfonyl ethyl vinyl ether; PFECA = perfluoroalkyl ether carboxylic acid

This letter is to inform you of the results of Ames studies with the above-referenced test substances. Results indicate that “each test substance” was negative for the ability to induce reverse mutations at selected loci of several strains of Salmonella typhimurium and at the tryptophan locus of Escherichia coli strain WP2 uvrA in the presence and absence of an exogenous metabolic activation system.

This information is provided to the TSCA 8(e) Office for information in view of the Agency's continued interest in perfluorinated substances and as a precautionary measure.

I hereby certify to the best of my knowledge and belief that all information entered on this form is complete and accurate.

I further certify that, pursuant to 15 U.S.C. § 2613(c), for all claims for confidentiality made with this submission, all information submitted to substantiate such claims is true and correct, and that it is true and correct that

(i) My company has taken reasonable measures to protect the confidentiality of the information;

(ii) I have determined that the information is not required to be disclosed or otherwise made available to the public under any other Federal law;

(iii) I have a reasonable basis to conclude that disclosure of the information is likely to cause substantial harm to the competitive position of my company; and

(iv) I have a reasonable basis to believe that the information is not readily discoverable through reverse engineering.

Any knowing and willful misrepresentation is subject to criminal penalty pursuant to 18 U.S.C. § 1001.

Substantiation of our claim of confidentiality is included herewith as **Attachment 1**. Please contact me if you have any questions about this submission or need further clarification.

Sincerely,

Attachment 1