Final Report

Study Title	*In Vivo* Mouse Bone Marrow Micronucleus Assay
Test Article	Perfluoroalkyl acrylate (Also called C6-SFA or 3,3,4,4,5,5,6,6,7,7,8,8,8-Tridecafluorooctyl acrylate)
Author	Yong Xu, MS, DABT
Sponsor	Daikin America, Inc. 2749 Hwy 20 Decatur, AL 35601
Test Facility	Covance Laboratories Inc. 9200 Leesburg Pike Vienna, Virginia 22182-1699
Covance Study Number	7958-102
Genetic Toxicology Assay Number	29737-0-455OECD
Report Issued	14 March 2008
Page Number	1 of 55

COMPLIANCE STATEMENT

In Vivo Mouse Bone Marrow Micronucleus Assay

I, the undersigned, hereby declare that the work was performed under my direction and that the findings provide a true and accurate record of the results obtained.

The study was conducted in accordance with the agreed protocol, unless otherwise stated, and the study objectives were achieved.

Except as noted below, all aspects of this study were in accordance with the Food and Drug Administration (FDA) Good Laboratory Practice Regulations, 21 CFR 58 and any applicable amendments.

Exceptions:

1) Documentation of the stability, purity, and/or characterization of the test article was conducted according to the Sponsor's Standard Operating Procedures (SOPs) but not under GLP conditions;

   Study Director Impact Statement: While the test article was tested in accordance with the accepted testing guidelines, the impact of having non-GLP documentation of the stability, purity and/or characterization of the test article cannot be fully evaluated at this time without additional information. This information may influence the evaluation of the results of this study.

2) The stability, homogeneity, and/or concentration of the dosing preparations were not analyzed.

   Study Director Impact Statement: While the test article was tested in accordance with the accepted testing guidelines, and the neat test article was appropriately characterized, the impact of not having verification of the stability, homogeneity, and/or concentration of the dosing formulations cannot be fully evaluated at this time without additional information. This information may influence the evaluation of the results of this study.

Yong Xu, MS, Diplomate, ABT
Study Director
Covance Laboratories Inc.

14 Mar. 2008
Date

QUALITY ASSURANCE STATEMENT

In Vivo Mouse Bone Marrow Micronucleus Assay

This report has been reviewed by the Quality Assurance Unit of Covance Laboratories Inc. and accurately reflects the raw data. The following study specific inspections were conducted and findings reported to the Study Director (SD) and associated management.

Inspection Dates		Phase	Date Reported to SD and SD Management
From	To		
28 Nov 2007	28 Nov 2007	Protocol Review	28 Nov 2007
20 Dec 2007	20 Dec 2007	Bone Marrow Harvest	20 Feb 2008
22 Feb 2008	25 Feb 2008	Draft Report and Data Review	25 Feb 2008
12 Mar 2008	12 Mar 2008	Protocol Amendment Review	12 Mar 2008
12 Mar 2008	13 Mar 2008	Draft to Final Report Review	13 Mar 2008

Jeannie Eberle
Representative
Quality Assurance Unit
Covance Laboratories Inc.

14 March 08
Date

KEY PERSONNEL

In Vivo Mouse Bone Marrow Micronucleus Assay

Study Monitor	Brad Hartong Daikin America, Inc.
Study Director	Yong Xu, MS, DABT Covance-Vienna
Study Toxicologist	Justin Butner, BS Covance-Vienna
Associate Director, Laboratory Animal Medicine	Stephen Curtis, DVM, DACLAM Covance-Vienna
Supervisor, Inlife	Karen Everett, BS Covance-Vienna
Supervisor, Postlife	Daksha N. Patel, BS, MT(ASCP), CLSp(CG) Covance-Vienna

CONTENTS

ABSTRACT

The objective of this study was to evaluate the test article, Perfluoroalkyl acrylate, for *in vivo* clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocytes (PCE) in CD-1®(ICR) BR mouse bone marrow.

In the dose range-finding study, Perfluoroalkyl acrylate was formulated in olive oil (see Protocol Deviation) and administered once by oral gavage to 3 males and 3 females per dose level. The animals were dosed at 500, 1000 and 2000 mg/kg and observed for up to 2 days after dosing for toxic signs and/or mortality. In a follow-up dose range-finding study, Perfluoroalkyl acrylate was formulated in olive oil with 0.5% Tween 80 and administered once by oral gavage to 2 or 3 males and 3 females per dose level (see Protocol Deviation). The animals were dosed at 250, 500, and 1000 mg/kg and observed for up to 2 days after dosing for toxic signs and/or mortality.

Based on the results of the dose range-finding study, the maximum tolerated dose was estimated to be 400 mg/kg. In the micronucleus assay, the test article was formulated in olive oil with 0.5% Tween 80 and administered once as follows.

Target Dose Level (mg/kg)	Stock Concentration (mg/mL)	Dosing Volume (mL/kg)	Route of Administration	Animals/Harvest Timepoint 24 Hour Male	48 Hour Male
Positive Control, 80	8	10	Oral Gavage	5	-
Vehicle Control, 0	0	20	Oral Gavage	5	5
100	5	20	Oral Gavage	5	-
200	10	20	Oral Gavage	5	-
400	20	20	Oral Gavage	5	5

Vehicle Control = olive oil with 0.5% Tween 80, Positive Control = Cyclophosphamide

Bone marrow was extracted and at least 2000 PCEs per animal were analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and normochromatic erythrocytes (NCEs) in at least the first 500 total erythrocytes for each animal.

The test article, Perfluoroalkyl acrylate, induced signs of clinical toxicity in the treated animals at 400 mg/kg, which included hypoactivity, squinted eyes, rough haircoat, hunched posture, wet haircoat-perineal, and/or body tremors. Perfluoroalkyl acrylate did not induce statistically significant increases in micronucleated PCEs at any test article dose examined (100, 200, and 400 mg/kg). In addition, Perfluoroalkyl acrylate was not cytotoxic to the bone marrow (i.e., no statistically significant decrease in the PCE:NCE ratios) at any dose of the test article. A statistically significant lower level of micronucleated PCEs and higher PCE:NCE ratio were found at 400 mg/kg without biological significance.

The test article, Perfluoroalkyl acrylate, was evaluated as negative in the mouse bone marrow micronucleus assay under the conditions of this assay.

STUDY CONDUCT

Purpose
This study was designed to evaluate the test article, Perfluoroalkyl acrylate, for *in vivo* clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocyte cells in CD-1®(ICR) BR mouse bone marrow.

Study Timetable
Study Initiation Date	16 November 2007
Experimental Start Date	29 November 2007
Experimental Completion Date	14 January 2008
Study Completion Date	14 March 2008

Protocol Adherence
This study was conducted in accordance with the protocol and amendment (Appendix 1), with the exception of the deviations noted in Appendix 1. The deviations did not affect the integrity or interpretability of the results of the study.

Regulatory Guidelines
The assay design was based on OECD Guideline 474, updated and adopted 21 July 1997.

Major Computer Systems
System[a]	Application Function
EMCS	Monitors and documents facility storage conditions (e.g., refrigerators, freezers, constant room temperatures and humidity levels)
EMCSDR	Transfers data from EMCS for reporting purposes
Cyscore	Randomizes animals and produces labels and forms
ANOVA/Program Trend	Statistical analysis
MTTS	Test article accessioning and dispensing

[a] All version numbers of the applications are maintained by Covance. Definitions for the acronyms can be found in the Glossary.

Record Retention
All raw data, documentation, records, the protocol and the final report generated as a result of this study will be stored in the Covance-Vienna archives for at least 1 year following finalization of the report. The Covance archives staff will contact the sponsor after at least 1 year following the report finalization to determine disposition of the archived materials (except for raw data on durable media, study correspondence, protocol, and final report which will be kept by Covance-Vienna). The Sponsor will authorize the transport of the materials to their site (or that of their designee), or authorize the transport of the materials to the archive facilities of EPL Archives, Inc., Sterling, VA (EPL). In the event the sponsor fails to indicate disposition, these materials will be transferred to the EPL storage facilities. Covance staff will have access to materials archived at EPL for continued research and/or regulatory audit.

TEST AND CONTROL ARTICLES

The test article was supplied by the Sponsor as a transparent, colorless liquid on 12 November 2007 and identified as follows.

Test article	CAS No.	Lot No.	Storage	Purity	Expiration Date
Perfluoroalkyl acrylate (C6-SFA)	17527-29-6	6X002	Room Temperature	99.7%	30 Oct 2008

The vehicle control article was olive oil with 0.5% Tween 80. The vehicle control article components were supplied as follows.

Control Article	CAS No.	Supplier	Lot/Batch No.	Storage	Purity	Expiration Date
Olive Oil	8001-25-0	Sigma Aldrich	057K6093	Room Temperature	NP	03 Dec 2010
Tween 80	9005-65-6	Sigma Aldrich	125K01921	Room Temperature	NP	30 Apr 2010

NP = Not Provided

The positive control article was cyclophosphamide. The positive control article components were supplied as follows.

Control Article	CAS No.	Supplier	Lot No.
Cyclophosphamide (CP)	6055-19-2	Sigma-Aldrich	C76K1050
Water for Cell Culture Applications	7732-18-5	Lonza (BioWhittaker®)	01116078

Information on synthesis methods, stability, purity, composition, or other characteristics that define the test article and control article components are on file with the Sponsor or the respective manufacturer(s). The Test Substance Data Sheet for the test article is presented in Appendix 2.

Test Article Disposition

Any remaining test article will be returned to the Sponsor after issuance of the audited draft report. The disposition of the remaining test article will be documented in the study file.

TEST SYSTEM

Test System Rationale

The micronucleus test can serve as a rapid screen for clastogenic agents and test articles which interfere with normal mitotic cell division (Schmid, 1975; Heddle *et al*., 1983; Heddle *et al*., 1991). Micronuclei are small chromatin bodies, consisting of entire

chromosomes and/or acentric chromosome fragments, which lag behind at mitotic anaphase. At telophase, these chromosomes and/or fragments are not segregated to either daughter nucleus and form single or multiple micronuclei in the cytoplasm. During maturation of hematopoietic cells from erythroblasts to erythrocytes, the nucleus is extruded. Micronuclei, if present, persist in the cytoplasm of these non-nucleated cells. Detection of micronuclei in non-nucleated cells eliminates the need to search for metaphase spreads in treated cell populations. Test articles affecting spindle-fiber function or formation can be detected through micronucleus induction (Schmid, 1975). In this study, enucleated immature red blood cells or polychromatic erythrocytes (PCEs) were analyzed for the presence of micronuclei.

Species and Justification

Dose Range-finding Study

Young adult male and female CD-1®(ICR) BR mice were received on 29 November 2007 from Harlan, Frederick, Maryland. This is an outbred strain that maximizes genetic heterogeneity and therefore tends to eliminate strain-specific response to test articles. The mouse has been routinely utilized as an animal model of choice for the mammalian bone marrow erythrocyte micronucleus assay.

Micronucleus Assay

Young adult male CD-1®(ICR) BR mice were received on 13 December 2007 from Harlan, Frederick, Maryland. This is an outbred strain that maximizes genetic heterogeneity and therefore tends to eliminate strain-specific response to test articles. The mouse has been routinely utilized as an animal model of choice for the mammalian bone marrow erythrocyte micronucleus assay.

Identification and Acclimation

Animals were randomized into groups according to Covance Standard Operating Procedures. Following randomization, each study animal was uniquely identified by ear tag. Animals were acclimated to laboratory conditions for at least 5 days, and released for study use by a staff examiner. Animals were considered acceptable for study use based upon data collected during acclimation.

Husbandry

Housing

The animals were housed in sanitary polycarbonate cages containing Sani-Chips® Hardwood Chip Laboratory bedding. The animals were housed up to five animals per cage during acclimation, and by full dose group/harvest timepoint after randomization. Each batch of wood chips was analyzed by the manufacturer for specific microorganisms and contaminants.

Environmental Conditions

Environmental controls were set to maintain the following animal room conditions: temperature range of 64 to 79°F, relative humidity range of 30 to 70%, 10 or greater air changes/hour, and a 12-hour light/12-hour dark cycle. The light/dark cycle was interrupted for non-study related activities. Actual temperature and humidity readings

were monitored continuously and averaged twice daily. Any variations to these conditions are maintained in the raw data and had no effect on the outcome of the study.

Diet, Water, and Contaminants

PMI Certified Rodent Diet® #5002 was available *ad libitum*. The manufacturer analyzed the diet for nutritional components and environmental contaminants. Tap water was available *ad libitum*. Water samples are routinely analyzed for specified microorganisms and environmental contaminants. Results of the diet and water analyses are reviewed for acceptability and are on file at Covance-Vienna. No contaminants were known to be present in the diet or water at levels that might interfere with this study.

EXPERIMENTAL DESIGN

Group Designations and Dose Levels

Dose Range-finding Study

The animals used in the dose range-finding study were dosed on 04 December 2007 (see Protocol Deviation) and on 12 December 2007. Animals were assigned to study groups as follows.

Vehicle Article Used	Target Dose Level (mg/kg)	Dose Concentration (mg/mL)	Dosing Volume (mL/kg)	Route of Administration	Number of Animals Male	Female
Olive oil [a]	500	25	20	Oral Gavage	3	3
Olive oil [a]	1000	50	20	Oral Gavage	3	3
Olive oil [a]	2000	100	20	Oral Gavage	3	3
0.5% Tween 80 in olive oil	250	12.5	20	Oral Gavage	3	3
0.5% Tween 80 in olive oil	500	25	20	Oral Gavage	3	3
0.5% Tween 80 in olive oil	1000	50	20	Oral Gavage	3	3

[a] See Protocol Deviations

At initiation of treatment, the animals dosed on 04 December 2007 were approximately 8 weeks old, and their body weights ranged from 33.4 to 37.9 g and 25.5 to 30.2 g, for the males and females, respectively. At initiation of treatment, the animals dosed on 12 December 2007 were approximately 10 weeks old, and their body weights ranged from 34.8 to 42.1 g (see Protocol Deviation) and 24.3 to 29.1 g, for the males and females, respectively.

Since no appropriate toxicity data were available (e.g., the same species, strain, same route, etc.), a dose range-finding study was performed using the same treatment regimen used in the micronucleus assay. Both males and females were dosed. The dose range-finding study was designed on the basis of information provided by the Sponsor. The daily observations of toxic signs and/or mortality data were used to estimate the highest appropriate dose level (maximum tolerated dose) for the micronucleus assay.

Micronucleus Assay
The animals used in the micronucleus assay were dosed on 19 December 2007. Animals were assigned to study groups as follows.

Target Dose Level (mg/kg)	Stock Concentration (mg/mL)	Dosing Volume (mL/kg)	Route of Administration	Animals/Harvest Timepoint 24 Hour Male	48 Hour Male
Positive Control, 80	8	10	Oral Gavage	5	-
Vehicle Control, 0	0	20	Oral Gavage	5	5
100	5	20	Oral Gavage	5	-
200	10	20	Oral Gavage	5	-
400	20	20	Oral Gavage	5	5

Vehicle Control = olive oil with 0.5% Tween 80, Positive Control = Cyclophosphamide

Since no relevant differences in toxicity between the sexes were observed in the dose range-finding study, only males were used in the micronucleus assay. The high dose, unless non-toxic, should have produced some indication of toxicity, e.g., toxic signs, death, or depression of the ratio of PCEs to NCEs. The use of a high dose, as defined above, increased the likelihood that a weak clastogen could be detected.

At initiation of treatment, the animals were approximately 8 weeks old, and their body weights ranged from 33.2 to 43.7 g (see Protocol Deviations). Animals not used on study were euthanized and discarded.

Rationale for Dosage Design and Route of Administration
The treatment regimen was a single oral gavage dose administration The oral route was selected because this is a relevant route of administration and is routinely used for this assay.

PROCEDURES

Dose Preparation
Prior to dosing, the top stock of the test article was prepared by adding the appropriate volume of the vehicle, olive oil with 0.5% Tween 80, to a pre-weighed quantity of the test article and mixed, forming a homogeneous suspension. Lower concentrations were obtained by dilution with the vehicle. The formulations were held at room temperature prior to dosing and stirred during the dosing procedure.

Dose Analyses
The Sponsor was responsible for the determination and documentation of the identity, strength, purity, stability and uniformity of the test article and the determination of stability, homogeneity and concentration of the dosing preparations. The Test Substance Data Sheet is presented in Appendix 2.

Duplicate samples of 1.0 mL were collected from the high, middle, and low dosing formulations and the vehicle and stored in amber glass vials under refrigerated conditions

(2 to 8°C). One set of samples was shipped to the Sponsor at the following address for possible analysis (which was not conducted):

Brad Hartong
Daikin America, Inc.
2749 Hwy 20
Decatur, AL 35601

The remaining set was stored as backup. Backup samples were disposed of upon approval by the Sponsor at the end of the study and documented in the study file.

Observation of Animals

Clinical Observations

All animals were examined immediately after dosing, approximately 1 hour after dosing, and at least daily for the duration of this assay for signs of clinical toxicity and/or mortality.

Disposition of Animals

Animals that were found dead or sacrificed *in extremis* (euthanized by CO_2 inhalation followed by incision of the diaphragm) were discarded without necropsy (see Protocol Deviation). All surviving animals were euthanized by CO_2 inhalation followed by incision of the diaphragm, and discarded without necropsy.

Micronucleus Assay

Extraction of Bone Marrow

The hind limb bones (tibias) were removed for marrow extraction from five surviving animals in the positive control, low and mid dose groups, and from ten surviving animals in the control and high dose groups. For each animal, the marrow flushed from the bones was combined in an individual centrifuge tube containing 3 to 5 mL fetal bovine serum (one tube per animal).

Preparation of Slides

Following centrifugation to pellet the marrow, the supernatant was removed by aspiration and portions of the pellet were spread on slides and air-dried. The slides were fixed in methanol, stained in May-Grünwald solution and Giemsa, and protected by mounting with coverslips. For control of bias, all slides were coded prior to analysis.

Slide Analysis

Slides prepared from the bone marrow collected from five animals per group at the designated harvest timepoints were scored for micronuclei and the PCE to NCE cell ratio. The micronucleus frequency (expressed as percent micronucleated cells) was determined by analyzing the number of micronucleated PCEs from at least 2000 PCEs per animal. The PCE:NCE ratio was determined by scoring the number of PCEs and NCEs observed while scoring at least 500 erythrocytes per animal.

The criteria for the identification of micronuclei were those of Schmid (1976). Micronuclei were darkly stained and generally round, although almond- and ring-shaped micronuclei occasionally occurred. Micronuclei were sharp bordered and generally between one-twentieth and one-fifth the size of the PCEs. The unit of scoring was the micronucleated cell, not the micronucleus; thus, the occasional cell with more than one micronucleus was counted as one micronucleated PCE, not two (or more) micronuclei.

The staining procedure permitted the differentiation by color of PCEs and NCEs (bluish-gray and red, respectively).

The historical background frequency of micronucleated cells was expressed as percentage micronucleated cells based on the number of PCEs analyzed. The historical background frequency of micronuclei in the mouse strains at this laboratory is about 0.0 to 0.4%, which is within the range of the published data (Salamone and Mavournin, 1994).

Statistical Evaluation
The following statistical methods were used to analyze the micronucleus data.

- Assay data analysis was performed using an analysis of variance (Winer, 1971) on untransformed proportions of cells with micronuclei per animal and on untransformed PCE:NCE ratios when the variances were homogeneous. Ranked proportions were used for heterogeneous variances.
- If the analysis of variance was statistically significant ($p \leq 0.05$), Dunnett's t-test (Dunnett, 1955; 1964) was used to determine which dose groups, if any, were statistically significantly different from the vehicle control. Analyses were performed separately for each sampling time.

The 100, 200, and 400 mg/kg dose groups, as well as the positive control group, were compared with the vehicle control group at the 5% probability level.

Statistical significance is designated throughout the text of this report by the term *significant*. Statistical analysis programs are referenced accordingly in the appropriate section of this report.

Data Presentation
Individual animal data and data are summarized by dose group for the different timepoints and are presented in the Tables section of the report.

Assay Acceptance Criteria
Acceptable Controls
The vehicle control group mean must lie within the historical control range and will usually be less than 0.4% micronucleated PCEs.

There must be a statistically significant elevation of the mean of the positive control group relative to the vehicle control group, and the positive control response must be consistent with historical positive control data.

Acceptable High Dose
Generally the high dose should reach the limit dose or produce some indication of toxicity, e.g., toxic signs and/or mortality in the test article dosed animals and/or a reduction in the PCE:NCE ratio. If there are solubility constraints, the highest dose tested will be the solubility limit or higher doses if a well-dispersed suspension is obtained that does not settle out rapidly.

Assay Evaluation Criteria
The criteria for a positive response is the detection of a statistically significant increase in micronucleated PCEs for at least one dose level, and a statistically significant dose-related response. A test article that does not induce both of these responses is considered negative. Statistical significance is not the only determinant of a positive response; the Study Director also considers the biological relevance of the results in the final evaluation.

RESULTS AND DISCUSSION

Dose Range-finding Study
Survival and Clinical Observations
The mortality data for Perfluoroalkyl acrylate in Olive oil (see Protocol Deviation) are summarized in Table 1. Clinical observations are presented in Table 2.

Mortality was observed in 3 out of 3 males at 2000 mg/kg and 2 out of 3 males at 1000 mg/kg. Mortality was observed in one of three females at 500, 1000, and 2000 mg/kg.

Clinical signs of toxicity observed at 2000 mg/kg included squinted eyes and slight hypoactivity in males and squinted eyes, hypoactivity, irregular respiration, and hunched posture in females. Clinical signs of toxicity observed in animals at 1000 mg/kg included squinted eyes, slight hypoactivity, wet haircoat – ventral cervical, yellow genital discharge, hypoactivity, irregular respiration, and hunched posture. Clinical signs of toxicity observed in animals 500 mg/kg included squinted eyes, slight hypoactivity, hypoactivity, irregular respiration, hunched posture, and body tremors.

The mortality data for Perfluoroalkyl acrylate in 0.5% Tween 80 in Olive oil are summarized in Table 3. Clinical observations are presented in Table 4.

Mortality was observed in 1 out of 2 males at 1000 mg/kg, and 1 out of 3 males at 500 mg/kg. One out of 2 males at 1000 mg/kg was humanely sacrificed due to excessive toxicity. Mortality was observed in one of three females at 1000 mg/kg.

Clinical signs of toxicity observed in animals at 1000 mg/kg included squinted eyes, slight hypoactivity, hypoactivity, irregular respiration, hunched posture, rough haircoat, and coldness to the touch. Clinical signs of toxicity observed in animals at 500 mg/kg

included hunched posture and rough haircoat. Clinical signs of toxicity observed in animals 250 mg/kg included hunched posture and rough haircoat.

Conclusion
Based on these results, the maximum tolerated dose was estimated to be 400 mg/kg. Since no relevant differences in toxicity between the sexes were observed in the dose range-finding study, only males were used in the micronucleus assay.

Micronucleus Assay
Survival and Clinical Observations
The mortality data for this assay are summarized in Table 5. Clinical observations are presented in Table 6.

The test article, Perfluoroalkyl acrylate, did not induce mortality in any of the animals dosed. All animals in the 100 mg/kg and 200 mg/kg dose groups appeared normal immediately after dosing and remained healthy until the appropriate harvest timepoint. All animals in the vehicle and positive control groups appeared normal after dosing and remained healthy until the appropriate harvest timepoint.

Clinical signs of toxicity observed in the remaining animals in the 400 mg/kg dose group included hypoactivity and squinted eyes in 3 of 10 animals, rough haircoat and hunched posture in 10 of 10 animals, wet haircoat-perineal in 1 of 10 animals, and body tremors in 1 of 10 animals.

Results and Interpretation
Data are summarized by dose group for the different timepoints and are presented in Table 7. Individual animal data are presented in Tables 8 and 9.

The test article, Perfluoroalkyl acrylate, induced signs of clinical toxicity in the treated animals at 400 mg/kg, which included hypoactivity, squinted eyes, rough haircoat, hunched posture, wet haircoat-perineal, and/or body tremors. Perfluoroalkyl acrylate did not induce statistically significant increases in micronucleated PCEs at any test article dose examined (100, 200, and 400 mg/kg). In addition, Perfluoroalkyl acrylate was not cytotoxic to the bone marrow (i.e., no statistically significant decrease in the PCE:NCE ratios) at any dose of the test article. A statistically significant lower level of micronucleated PCEs and higher PCE:NCE ratio were found at 400 mg/kg without biological significance.

The vehicle control group had approximately ≤0.04% micronucleated PCEs and the group mean was within the historical control range (Appendix 3). The positive control, cyclophosphamide, induced a statistically significant increase in micronucleated PCEs as compared to that of the vehicle control, with a mean and standard deviation of 2.22 ± 0.73%.

CONCLUSION

The test article, Perfluoroalkyl acrylate, was evaluated as negative in the mouse bone marrow micronucleus assay under the conditions of this assay.

TABLES

Table 1:
Mortality Summary –
Dose Range-finding Study

Target Dose Level[a] (mg/kg)	Number of Males (Died/Total Dosed)	Number of Females (Died/Total Dosed)
500	0/3	1/3
1000	2/3	1/3
2000	3/3	1/3

[a] Vehicle article: Olive oil

Table 2:
Clinical Observations –
Dose Range-finding Study

Target Dose Level[a] (mg/kg)	Sex	Animal ID	Time After Dosing			
			IPD	1 hour PD	1 day	2 days
500	M	8267	0	0	0	0
		8268	0	1,2	0	0
		8272	0	0	2	1,6,7
	F	8273	0	0	0	4
		8280	0	0	0	1,2,7,8
		8282	0	0	0	9
1000	M	8261	0	0	5	0
		8269	0	0	4	-
		8270	0	0	4	-
	F	8276	0	0	0	1,6,7,8
		8277	0	3	0	1,6,7,8
		8278	0	0	1,2	4
2000	M	8262	0	1,2	4	-
		8266	0	1,2	4	-
		8271	0	1,2	4	-
	F	8275	0	0	0	4
		8279	0	0	0	1,6,7,8
		8283	0	0	0	1,6,7,8

Key: 0 = Normal, 1 = squinted eyes, 2 = slightly hypoactive, 3 = wet haircoat-ventral cervical, 4 = found dead, 5 = yellow genital discharge, 6 = hypoactive, 7 = irregular respiration, 8 = hunched posture, 9 = body tremors, 10 = rough haircoat, 11 = cold to touch
IPD = Immediately post dosing
PD = Post dosing
[a] Vehicle article: Olive oil

Table 3:
Mortality Summary –
Dose Range-finding Study

Target Dose Level[a] (mg/kg)	Number of Males (Died/Total Dosed)	Number of Females (Died/Total Dosed)
250	0/3	0/3
500	1/3	0/3
1000	1/2[b]	1/3

[a] Vehicle article: Olive oil with 0.5% Tween 80
[b] One animal was humanely euthanized on Day 1 due to excessive toxicity.

Table 4:
Clinical Observations –
Dose Range-finding Study

Target Dose Level[a] (mg/kg)	Sex	Animal ID	Time After Dosing			
			IPD	1 hour PD	1 day	2 days
250	M	8263	0	0	8,10	0
		8264	0	0	8,10	0
		8265	0	0	8,10	0
	F	8274	0	0	8,10	0
		8281	0	0	8,10	0
		8284	0	0	8,10	0
500	M	8334	0	0	4	-
		8339	0	0	8,10	0
		8340	0	0	8,10	0
	F	8344	0	0	8	0
		8345	0	0	8	0
		8346	0	0	8	0
1000	M	8335	0	1,2,7,10	4	-
		8336	0	1,2,7,10	1,6,7,8,10,11→12	-
	F	8343	0	2,8,10	1,2,7,8,10	1,6,10
		8347	0	10	8,10	1,6,10
		8350	0	2,7,8,10	4	-

Key: 0 = Normal, 1 = squinted eyes, 2 = slightly hypoactive, 3 = wet haircoat-ventral cervical, 4 = found dead, 5 = yellow genital discharge, 6 = hypoactive, 7 = irregular respiration, 8 = hunched posture, 9 = body tremors, 10 = rough haircoat, 11 = cold to touch, 12 = animal humanely euthanized due to excessive toxicity
IPD = Immediately post dosing
PD = Post dosing
→ = followed by
[a] Vehicle article: Olive oil with 0.5% Tween 80

Table 5:
Mortality Summary –
Micronucleus Assay

Target Dose Level[a] (mg/kg)	Number of Males (Died/Total Dosed)
100	0/5
200	0/5
400	0/10

Table 6:
Clinical Observations –
Micronucleus Assay

Target Dose Level (mg/kg)	Harvest Timepoint	Animal ID	Time After Dosing			
			IPD	1 hour PD	1 day	2 days
Vehicle Control 0	24	All	0	0	0	NA
	48	All	0	0	0→0	0
Positive Control 80	24	All	0[a]	0	0	NA
100	24	All	0	0	0	NA
200	24	All	0	0	0	NA
400	24	8410	0	0	1,2,3,5	NA
		8426	0	0	3,5	NA
		8432	0	0	3.5	NA
		8433	0	0	1,2,3,4,5	NA
		8440	0	0	1,2,3,5,6	NA
	48	8412	0	0	3,5→3,5	3,5
		8417	0	0	3,5→3,5	3,5
		8424	0	0	3,5→3,5	3,5
		8430	0	0	3,5→3,5	3,5
		8436	0	0	3,5→3,5	3,5

Key: 0 = Normal, 1 = hypoactive, 2 = squinted eyes, 3 = rough haircoat, 4 = wet haircoat-perineal, 5 = hunched posture, 6 = body tremors
NA = not applicable, animal harvested at the 24-hour harvest timepoint.
IPD = Immediately post dosing
PD = Post dosing
→ = followed by
[a] Immediate post dose observation time recorded for animal 8428 was prior to dosing.

Table 7: Micronucleus Assay – Summary Table

Assay No.: 29737-0-455OECD
Test Article: Perfluoroalkyl acrylate
Initiation of Dosing: 19 December 2007

Treatment	Dose	Harvest Time	% Micronucleated PCEs Mean of 2000 per Animal ± S.D. Males	Ratio PCE:NCE Mean ± S.D. Males
Controls				
Vehicle	VC 20 mL/kg	24 hr	0.08 ± 0.03	0.25 ± 0.10
		48 hr	0.04 ± 0.04	0.40 ± 0.15
Positive	CP 80 mg/kg	24 hr	2.22 ± 0.73*	0.45 ± 0.16**
Test Article	100 mg/kg	24 hr	0.04 ± 0.04	0.48 ± 0.19
	200 mg/kg	24 hr	0.06 ± 0.02	0.49 ± 0.10
	400 mg/kg	24 hr	0.01 ± 0.02***	0.54 ± 0.17*
		48 hr	0.05 ± 0.06	0.30 ± 0.08

* Significantly greater than the corresponding vehicle control, $p \leq 0.01$.
** Significantly greater than the corresponding vehicle control, $p \leq 0.05$.
*** Significantly less than the corresponding vehicle control, $p \leq 0.05$.
CP = Cyclophosphamide
PCE = Polychromatic erythrocyte
NCE = Normochromatic erythrocyte
VC = Olive oil with 0.5% Tween 80

Table 8: Micronucleus Assay – 24-Hour Male Individual Animal Data

Assay No.: 29737-0-455OECD
Test Article: Perfluoroalkyl acrylate
Initiation of Dosing: 19 December 2007

Treatment	Dose	Animal Number	# MN PCE/ 2000 PCE	Ratio PCE:NCE
Vehicle Control	VC 20 mL/kg	8411	2	0.38
		8418	1	0.33
		8420	1	0.19
		8427	2	0.24
		8443	2	0.13
Positive Control	CP 80 mg/kg	6706	30	0.70
		8416	49	0.39
		8428	60	0.47
		8434	55	0.41
		8444	28	0.28
Test Article	100 mg/kg	8415	1	0.45
		8425	2	0.27
		8439	0	0.70
		8441	1	0.32
		8442	0	0.66
	200 mg/kg	6701	2	0.45
		6702	1	0.41
		6704	1	0.40
		6705	1	0.61
		8413	1	0.59
	400 mg/kg	8410	0	0.75
		8426	0	0.59
		8432	0	0.38
		8433	1	0.37
		8440	0	0.62

CP = Cyclophosphamide
PCE = Polychromatic erythrocyte
MN PCE = Micronucleated PCE
NCE = Normochromatic erythrocyte
VC = Olive oil with 0.5% Tween 80

Table 9: Micronucleus Assay – 48-Hour Male Individual Animal Data

Assay No.: 29737-0-455OECD
Test Article: Perfluoroalkyl acrylate
Initiation of Dosing: 19 December 2007

Treatment	Dose	Animal Number	# MN PCE/ 2000 PCE	Ratio PCE:NCE
Vehicle Control	VC 20 mL/kg	8419	1	0.24
		8422	0	0.48
		8423	0	0.42
		8429	1	0.60
		8435	2	0.28
Test Article	400 mg/kg	8412	0	0.38
		8417	3	0.21
		8424	0	0.32
		8430	1	0.23
		8436	1	0.38

PCE = Polychromatic erythrocyte
MN PCE = Micronucleated PCE
NCE = Normochromatic erythrocyte
VC = Olive oil with 0.5% Tween 80

APPENDICES

Appendix 1
Protocol, Protocol Amendment, and Protocol Deviations

COVANCE
THE DEVELOPMENT SERVICES COMPANY

COVANCE STUDY NO. 7958-102
GENETIC TOXICOLOGY ASSAY NO. 29737-0-455OECD
PROTOCOL 455OECD EDITION 4

IN VIVO MOUSE BONE MARROW MICRONUCLEUS ASSAY

PART 1. SPONSOR INFORMATION AND APPROVALS

1.0 SPONSOR IDENTIFICATION

Company Name: Daikin America, Inc.
2749 Hwy 20
Address: Decatur, AL 35601

2.0 TEST ARTICLE IDENTIFICATION

Perfluoroalkyl acrylate (Also called C6-SFA or 3,3,4,4,5,5,6,6,7,7,8,8,8-Tridecafluorooctyl acrylate)

3.0 TEST ARTICLE ANALYSIS

Determination and documentation of the identity, strength, purity, stability and uniformity of the test article, as defined in the Good Laboratory Practice (GLP) regulations, is the responsibility of the Sponsor. The Sponsor should provide these test article characterization data (a Certificate of Analysis or equivalent) for review by the Study Director and inclusion in the final report. Determination of the stability, homogeneity and concentration of the dosing preparations used on this study is the responsibility of the Sponsor. Dose formulation samples will be saved as directed in Part 2, section 4.2 of this protocol. The GLP compliance statement in the final report will note any exceptions if these test article characterization data and/or dosing solution analyses (along with an accompanying GLP compliance statement) are not provided to the Study Director prior to the issuance of the final report.

4.0 REGULATORY COMPLIANCE

The study will be conducted by Covance Laboratories Inc. at 9200 Leesburg Pike, Vienna, Virginia 22182 (Covance-Vienna). All aspects of the study performed by Covance will be in compliance with the following GLP regulations:

☐ OECD ☒ FDA ☐ EPA-TSCA ☐ EPA-FIFRA

☐ MAFF ☐ MHLW

5.0 QUALITY ASSURANCE

The protocol, at least one critical phase of the work in progress, and the final report will be audited by Covance Quality Assurance Unit (QAU). Portions of this study not conducted by Covance will be audited by the Sponsor's QAU or designee. Inspection reports and a Quality Assurance Statement should be

provided to the Study Director for all study activities, including those not performed by Covance.

6.0 STUDY DATES

Proposed Experimental Start Date:	4 December 2007
Proposed Experimental Termination Date:	22 January 2008
Proposed Audited Draft Report Date:	15 February 2008

7.0 APPROVAL OF STUDY PROTOCOL

The final version of the protocol was approved by the sponsor for study director signature on 16 November 2007.

Study Director:

Date 16 NOV 2007

Yong Xu, MS. DABT
Genetic and Molecular Toxicology
Covance-Vienna
yong.xu@covance.com
703 245 2200 ext. 5056

Testing Facility Management:

Date 16 Nov 2007

Timothy E. Lawlor, MA
Associate Director
Genetic and Molecular Toxicology
Covance-Vienna

Sponsor's Authorized Representative:

Date 12/6/07

Brad Hartong
Daikin America, Inc.
2749 Hwy 20
Decatur, AL 35601
Phone: 256-260-6329
Fax: 256-260-6303
E-mail: hartong@daikin-america.com

PART 2. STUDY PROTOCOL

1.0 OBJECTIVE

The objective of this study is to evaluate a test article for *in vivo* clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocyte cells in mouse bone marrow. The assay design is based on OECD Guideline 474, updated and adopted 21 July 1997.

2.0 TEST SYSTEM RATIONALE

The micronucleus test can serve as a rapid screen for clastogenic agents and test articles which interfere with normal mitotic cell division (Schmid, 1975; Heddle *et al.*, 1983; Heddle *et al.*, 1991). Micronuclei are small chromatin bodies, consisting of entire chromosomes and/or acentric chromosome fragments, which lag behind at mitotic anaphase. At telophase, these chromosomes and/or fragments are not segregated to either daughter nucleus, and form single or multiple micronuclei in the cytoplasm. During maturation of hematopoietic cells from erythroblasts to erythrocytes the nucleus is extruded. Micronuclei, if present, persist in the cytoplasm of these non-nucleated cells. Detection of micronuclei in non-nucleated cells eliminates the need to search for metaphase spreads in treated cell populations. Test articles affecting spindle-fiber function or formation as well as clastogenic agents can be detected through micronucleus induction (Schmid, 1975). In this study, enucleated immature red blood cells or polychromatic erythrocytes (PCEs), will be analyzed for the presence of micronuclei.

3.0 MATERIALS

3.1 Animals and Animal Husbandry

Young adult male and/or female mice of the CD-1® (ICR) BR strain, 8-10 weeks old at the time of dosing, will be purchased from Charles River Laboratories or Harlan. The CD-1® mouse is an outbred strain that maximizes genetic heterogeneity and therefore tends to eliminate strain-specific response to test articles.

Animals will be isolated by sex and housed at ≤5/polycarbonate cage containing Sani-Chips® Hardwood Chip Laboratory bedding. Each batch of wood chips is analyzed for specific microorganisms and contaminants. Environmental controls will be set to maintain temperatures of 64°F - 79°F with a relative humidity of 30% to 70%. The lighting controls will be set to maintain a 12-hour light/12-hour dark cycle which may be interrupted as needed for study or non-study related activities. The air handling controls will be set for at least 10 air changes per hour in the study room. A commercial diet (PMI® Certified Rodent Diet® #5002) and

tap water will be available *ad libitum*. The feed is analyzed by the manufacturer for concentrations of specified heavy metals, aflatoxin, chlorinated hydrocarbons, organophosphates, and specified nutrients. The water is analyzed biannually on a retrospective basis for specified microorganisms, pesticides, heavy metals, alkalinity, and halogens. Animals will be acclimated for at least 5 days before being used in this assay.

Animals will be randomly assigned to study groups according to Covance Standard Operating Procedures. Animals will be weighed prior to dosing and will be dosed based upon the individual animal weights. The body weights of the individual mice will be in the range of 20 to 40 grams. The weight variation of animals should be minimal and will not exceed ±20% of the mean weight of each sex. Animals will be uniquely identified by ear tag. Treatment groups will be identified by cage label/card.

Sanitary cages will be used. Personnel handling animals or working within the animal facilities will be required to wear suitable protective garments and equipment.

3.2 Justification of Species

The mouse has been routinely utilized as an animal model of choice for the mammalian bone marrow erythrocyte micronucleus assay.

3.3 Test Article

Solid or liquid test articles are suitable for this study. The test article is identified in Part 1 of this protocol. Storage conditions will be specified by the Sponsor. Residual test article will be returned to the Sponsor after the completion of dosing.

3.4 Control Articles

3.4.1 Vehicle Control Article

The vehicle control article will be olive oil with 0.5% Tween 80. The vehicle control animals will be dosed with the vehicle by the same route as, and concurrently with, the test article and in amounts equal to the maximum volumes administered to the experimental animals.

The dosing volume usually will not exceed 20 mL/kg for oral gavage and intraperitoneal administrations; the use of higher volumes/kg must be justified.

*3.4.2 **Positive Control Article***

Cyclophosphamide (CP; CAS #6055-19-2) will be used as the positive control article. This control article will be dissolved in deionized water at ~8 mg/mL and administered by oral gavage at ~10 mL/kg to achieve a dose of ~80 mg/kg. The positive control animals are dosed once with CP by oral gavage with a single bone marrow sample time of approximately 24 hours after dosing.

4.0 EXPERIMENTAL DESIGN

4.1 Test Article Handling

If test article solubility information is not provided by the Sponsor, a preliminary solubility test will be performed. The vehicles generally used in the study are deionized water, 0.9% saline (CAS No. 7647-14-5), corn oil (CAS No. 8001-30-7), or 0.5% aqueous carboxymethyl cellulose solution (CAS No. 9004-32-4). The vehicle selected will be the one which gives the best solubility or in which an evenly dispersed suspension can be prepared.

4.2 Test Article Analysis

Samples will be taken of the dosing formulations used in definitive micronucleus assay. If the dosing preparations are suspensions, duplicate samples of 1.0 mL each will be taken from the top, middle, and bottom of the low and high dose formulations and duplicate samples of 1.0 mL each will be taken from the middle of all remaining dosing formulations, including the vehicle control. If the dosing preparations are solutions, duplicate samples of 1.0 mL will be taken from the high, middle, and low dose concentrations, including the vehicle.

The samples will be stored refrigerated (set to maintain 2 to 8°C). One set of samples will be shipped under refrigerated conditions (ice packs) to the following address for storage and possible analysis. The remaining set will be stored as backup at Covance-Vienna.

Brad Hartong
Daikin America, Inc.
2749 Hwy 20
Decatur, AL 35601
Phone: 256-260-6329
E-mail: hartong@daikin-america.com

Any backup samples not analyzed will be disposed of after issuance of the audited draft report and upon the request of the Study Director.

4.3 Dose Range-finding Study

A dose range-finding study will be performed using both sexes and the same treatment regimen to be used in the micronucleus assay. The dose range-finding study will be designed on the basis of information provided by the Sponsor wherever possible. The actual number of groups will be determined on a case by case basis, but each group will consist of 1 to 3 animals of the specified sex(es).

Animals will be treated with the test article once. The standard method of treatment using a single administration of the test article will be used. Animals will be dosed by oral gavage (PO).

If no toxicity information is available, then the highest dose tested in the dose range-finding study will be 2000 mg/kg, or a dose limit approach will be used. The objective is to use a minimum number of animals in locating an appropriate high dose for test articles having little or no toxicity data. For a dose limit approach, 1 to 3 animals of the specified sex(es) will be exposed to a dose expected to cause toxic signs. Depending on the toxic signs obtained, other groups of 1 to 3 animals will be dosed with lower or higher doses, as appropriate. This procedure will continue until a dose causing toxic signs without lethality can be estimated. The highest applied dose for test articles causing little or no toxicity will be 2000 mg/kg. The doses evaluated in the dose range-finding study will be documented in the raw data and presented in the report.

Dosing formulations will be prepared either on the day prior to or on the day of dosing and held at room temperature. The dosing volume usually will not exceed 20 mL/kg for PO and IP administrations.

The animals will be observed immediately after dosing, approximately 1 hour after dosing, and daily (or more often, as necessary) for toxic signs and mortality for the duration of the study. The observation period will be up to 2 days after the last administration. Unscheduled deaths will be discarded without necropsy. All animals will be euthanized by CO_2 inhalation followed by incision of the diaphragm and then disposed of without necropsy. The daily observations of toxic signs and/or mortality data will be used to estimate the highest appropriate dose (maximum tolerated dose) for the micronucleus assay.

4.4 Micronucleus Assay

4.4.1 Dose Selection

If the dose range-finding study shows no difference in toxicity between the sexes, only males will be used in the micronucleus assay. When differences in toxicity are observed, both sexes will be used, with the dose selection process being performed independently for each sex. Only females will be used if the test article exposure to humans is intended specifically for females.

The high dose will be determined from the dose range-finding study or will be the limit dose of 2000 mg/kg for test articles having no toxicity. The high dose for toxic test articles should produce some indication of toxicity, e.g., toxic signs, death, or depression of the ratio of PCEs to normochromatic erythrocytes (NCEs).

One-half and one-quarter of this high dose normally will be used as the intermediate and low doses, respectively, but the low dose should produce little or no toxicity. The use of a high dose, as defined above, increases the likelihood that a weak clastogen will be detected and is therefore recommended. The doses selected will be listed in an amendment to the protocol.

4.4.2 Dosing Regimen and Sampling Times

The experimental parameters for dosing will be the same as used for the dose range-finding study, unless specified otherwise by amendment to the protocol. A minimum of five animals of the appropriate sex(es) will be used for each dose group, positive and vehicle control, for each sampling time used. An additional group consisting of 3-5 animals of appropriate sex(es) may be dosed as a secondary dose group for the high dose group. This group will be added if lethality is likely to occur, and these animals will be used only as replacements for animals lost in the high dose group prior to the scheduled sampling times. Any secondary dose group animals not used as replacements will not have bone marrow collected and will be euthanized at the completion of the assay and discarded without necropsy. The inclusion of the secondary dose group will be determined by the Study Director and will be specified in an amendment to the protocol.

For the acute dosing regimen (single administration), the sampling times will be approximately 24 and 48 hours after administration of the test article. For the multiple dosing regimen, one sample

time at approximately 24 hours after the last administration will be used. These two procedures therefore use different numbers of animals, as summarized in the following table. The acute dosing regimen will be used unless the Sponsor specifies the use of multiple treatments.

Allocation of Animals [a] for the Micronucleus Assay

Group Number	Treatment	Acute Treatment Sampling Times		OR	Multiple Treatments
		24 Hours	48 Hours		
1	Positive Control	5	-		5
2	Vehicle Control	5	5		5
3	Low Dose	5	-		5
4	Mid Dose	5	-		5
5	High Dose	5	5		5
		Total	35[a]	OR	25[a]

a The animal numbers are for one sex only; the total numbers are increased 2-fold for assays using both sexes

Dosing formulations will be prepared either on the day prior to or on the day of dosing and held at room temperature. Dosing volumes usually will not exceed 20 mL/kg for PO or IP administrations. The animals will be observed immediately after dosing, approximately 1 hour after dosing, and daily (or more often, as necessary) for toxic signs and mortality for the duration of the study. All animals will be euthanized by CO_2 inhalation followed by the incision of the diaphragm.

4.4.3 Extraction of Bone Marrow

The hind limb bones (tibias or femurs) will be removed from all surviving animals for marrow extraction. The marrow will be flushed from the bone and transferred to centrifuge tubes containing 3-5 mL fetal bovine serum (one tube per animal).

4.4.4 Preparation of Slides

Following centrifugation to pellet the marrow, the supernatant will be removed by aspiration and portions of the pellet will be spread on slides and air-dried. The slides will then be fixed in methanol, stained in May-Grünwald solution and Giemsa, and protected by mounting with coverslips. For control of bias, all slides will be coded prior to analysis.

4.4.5 Slide Analysis

All surviving animals in each treatment and control group will be analyzed. At least 2000 PCEs per animal will be scored when possible. Data from those individual experimental animals with either fewer than 500 PCEs or with a PCE to NCE ratio less than 20% of the vehicle control value will not be used for calculation of group means or for statistical evaluation unless directed by the Study Director. The historical background frequency of micronucleated cells will be expressed as percent micronucleated cells based on the number of PCEs analyzed. The historical background frequency of micronucleated cells will be expressed as percent micronucleated cells based on the number of PCEs analyzed. The historical background frequency of micronuclei in the CD-1® mouse strain in this laboratory is around 0.0-0.4%, which is within the range of the published data (Salamone and Mavournin, 1994).

The frequency of PCEs versus mature erythrocytes (NCEs) will be determined by scoring the number of PCEs and NCEs observed in the optic fields while scoring at least the first 500 erythrocytes on the slide.

The criteria for the identification of micronuclei are those of Schmid (1976). Micronuclei are darkly stained and generally round, although almond and ring-shaped micronuclei occasionally occur. Micronuclei have sharp borders and are generally between 1/20 and 1/5 the size of the PCE. The unit of scoring is the micronucleated cell, not the micronucleus; thus, the occasional cell with more than one micronucleus is counted as one micronucleated PCE, not two (or more) micronuclei.

The staining procedure permits the differentiation by color of polychromatic and normochromatic erythrocytes (bluish-grey and red, respectively).

5.0 DATA

5.1 Data Presentation

The data reported will include the number of PCEs scored, the number of micronucleated PCEs, and the ratio of polychromatic to normochromatic erythrocytes for each experimental animal. For each treatment group, the mean percent micronucleated PCEs and the mean PCE:NCE ratio will be calculated, as well as the standard deviation of the mean for each parameter.

5.2 Study Acceptance Criteria

5.2.1 Acceptable Controls

The vehicle control group must lie within the historical control range and will usually be less than 0.4% micronucleated PCEs.

There must be a statistically significant elevation of the mean of the positive control group relative to the vehicle control group, and the positive control response must be consistent with historical positive control data.

5.2.2 Acceptable High Dose

Generally the high dose should reach the limit dose or produce some indication of toxicity, e.g., toxic signs and/or mortality in the test article dosed animals and/or a reduction in the PCE:NCE ratio. If there are solubility constraints, the highest dose tested will be the solubility limit or higher doses if a well-dispersed suspension is obtained that does not settle out rapidly.

5.3 Study Evaluation Criteria

Assay data analysis will be performed using an analysis of variance (Winer, 1971) on untransformed proportions of cells with micronuclei per animal when the variances are homogeneous. Ranked proportions will be used for heterogeneous variances. If the analysis of variance is significant ($p \leq 0.05$), a Dunnett's t-test (Dunnett, 1955; 1964) will be used to determine which dose groups, if any, are significantly different from the vehicle control. Analyses will be performed separately for each sampling time (and sex, if more than one sex). Additionally, parametric or nonparametric tests for trend (e.g. Cochrane-Armitage) may be employed to identify any dose-related response.

The criteria for a positive response is the detection of a statistically significant positive response for at least one dose group and/or a statistically significant dose-related response. A test article that does not induce both of these responses will be considered negative. Equivocal results may require further investigation (at additional cost). Statistical significance will not be the only determinant of a positive response, and the study director will consider the biological relevance of the results in the final evaluation.

6.0 REFERENCES

Dunnett, C. W., "A Multiple Comparisons Procedure for Comparing Several Treatments with a Control," *Journal of the American Statistical Association, 50*:1096-1121 (1955).

Dunnett, C. W., "New Tables for Multiple Comparisons with a Control," *Biometrics, 20*:482-491 (1964).

Heddle, J. A., Hite, M., Kirkhart, B., Mavournin, K. H., MacGregor, J. T., Newell, G. W. and Salamone, M. F., "The Induction of Micronuclei as a Measure of Genotoxicity," *Mutation Research, 123*:61-118 (1983).

Heddle, J. A., Cimino, M. C., Hayashi, M., Romagna, F., Shelby, M. D., Tucker, J. D., Vanparys, Ph., and MacGregor, J. T., "Micronuclei as an Index of Cytogenetic Damage: Past, Present, and Future," *Environmental and Molecular Mutagenesis, 18*:277-291 (1991).

OECD, "Mammalian Erythrocyte Micronucleus Test," *OECD Guidelines for Testing of Chemicals*, Section 4, Guideline 474, updated and adopted 21 July 1997.

Salamone, M. F. and Mavournin, K. H., "Bone Marrow Micronucleus Assay: a Review of the Mouse Stocks Used and Their Published Mean Spontaneous Micronucleus Frequencies," *Environmental and Molecular Mutagenesis, 23*:239-273 (1994).

Schmid, W., "The Micronucleus Test," *Mutation Research, 31*:9-15 (1975).

Schmid, W., "The Micronucleus Test for Cytogenetic Analysis," *Chemical Mutagens, Principles and Methods for Their Detection*, (ed.) Hollaender, A., Vol. 4, pp. 31-53, Plenum Press (1976).

Winer, B. J., *Statistical Principles in Experimental Design*, Second Ed., McGraw-Hill: New York (1971).

7.0 REPORT FORMAT

Covance employs a standard report format for each study design. The final report will provide the following information:

- Sponsor identification.
- Quality Assurance statement.
- Statement of GLP compliance.

- Study Director signature.
- Test article identification, a physical description of the test article, CAS number (if known), and date of receipt.
- Type of study, protocol number, and Covance Study Number.
- Dates of study initiation and completion.
- Identification of Study Director and laboratory supervisor.
- Method information, per OECD guideline 474.
- Doses at which the test article was tested.
- Assay evaluation and acceptance criteria.
- Clinical signs observed in the dose range-finding study (if applicable) and in the definitive assay.
- Interpretation of results.
- Conclusions.
- References.
- Test results presented in tabular form.
- Historical control data summarized in tabular form.

8.0 CHANGES AND REVISIONS

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

9.0 ANIMAL CARE AND USE STATEMENT

All procedures in this protocol are in compliance with the Animal Welfare Act, the Guide for Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. In the opinion of the study director, the study does not unnecessarily duplicate any previous work. This protocol will be reviewed by the Covance-Vienna IACUC for compliance with regulatory guidelines concerning the care and use of animals.

10.0 VETERINARY CARE/TREATMENT

In accordance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare, medical treatment necessary to prevent unacceptable pain and suffering, including euthanasia, is the sole responsibility of the attending Laboratory Animal Veterinarian. Discretionary medical treatment may be carried out based upon consensus agreement between the study director and the attending Laboratory Animal Veterinarian. The sponsor will be notified of any veterinary treatment.

11.0 MAJOR COMPUTER SYSTEMS

System[a]	Application Function

Environmental Monitoring and Control System (EMCS)	Monitors and documents facility storage conditions (e.g., refrigerators, freezers, constant room temperatures and humidity levels)
Environmental Monitoring and Control System Data Reporting (EMCSDR)	Transfers data from EMCS for reporting purposes
Cyscore	Randomizes animals and produces labels and forms
ANOVA/Program Trend	Statistical analysis
Material Tracking and Testing System (MTTS)	Test article accessioning and dispensing

a All version numbers of the applications are maintained by Covance.

12.0 RECORDS TO BE MAINTAINED

All raw data, documentation, records, the protocol and the final report generated as a result of this study will be stored in the Covance-Vienna archives for at least 1 year following finalization of the report. The Covance archives staff will contact the sponsor after at least 1 year following the report finalization to determine disposition of the archived materials (except for raw data on durable media, study correspondence, protocol, and final report which will be kept by Covance-Vienna). The Sponsor will authorize the transport of the materials to their site (or that of their designee), or authorize the transport of the materials to the archive facilities of EPL Archives, Inc., Sterling, VA (EPL). **In the event the sponsor fails to indicate disposition, these materials will be transferred to the EPL storage facilities.** Covance staff will have access to materials archived at EPL for continued research and/or regulatory audit.

COVANCE
THE DEVELOPMENT SERVICES COMPANY

Protocol Amendment No. 1

Study Title	*IN VIVO* MOUSE BONE MARROW MICRONUCLEUS ASSAY
Study Director	Yong Xu, MS. DABT
Sponsor	Daikin America, Inc. 2749 Hwy 20 Decatur, AL 35601
Study Monitor	Brad Hartong
Testing Facility	Covance Laboratories Inc. 9200 Leesburg Pike Vienna, Virginia 22182
Covance Study Number	7958-102
Genetic Toxicology Assay Number	29737-0-455OECD
Page Number	1 of 2

This amendment modifies the following portions of the protocol.

1. **4.0 Experimental Design, 4.4 Micronucleus Assay, 4.4.1 Dose Selection, 3rd paragraph, last sentence.**

   Effective 16 November 2007: The sentence is replaced with the following:

   Based on the findings of dose range-finding study, males only will be tested in definitive micronucleus assay. Doses will be 100, 200, and 400 mg/kg.

   Reason for Change: To add the dose levels and sex to be analyzed in the definitive micronucleus assay.

AMENDMENT APPROVAL

The final version of the protocol amendment was approved by the Sponsor for Study Director signature on [redacted]ebruary 2008①

Brad Hartong — 2/29/08
Brad Hartong
Study Monitor
Daikin America, Inc.
Date

Yong Xu — 04 Mar. 2008
Yong Xu, MS. DABT
Study Director
Covance Laboratories Inc.
Date

Timothy E. Lawlor — 04 MAR 2008
Timothy E. Lawlor, MA
Associate Director
Department of Genetic and Molecular Toxicology
Covance Laboratories Inc.
Date

① Written on 3/4/08. Verified per date the Study Monitor signed Amendment. JB 3/4/08

Protocol Deviations

Procedure	Protocol Deviations
Dose Preparation	
Dosing Formulations	In initial dose range-finding study, olive oil without 0.5% Tween 80 was used as vehicle. As per protocol, olive oil with 0.5% Tween 80 should be the vehicle. To confirm if the observation was affected by lower bioavailibility of the test article in this vehicle, extra doses with correct vehicle was conducted. The results confirmed acceptation of the data for dose selection of definitive micronucleus assay. This deviation had no impact to the study.
Dose Administration	In the repeat dose range-finding study, due to a shortage of male mice for dosing, only two males were dosed at a dose level of 1000mg/kg instead of three as directed.
Inlife Procedures	
Clinical Observations	On 12/19/07, group 1 animal #8428 received cage side observations immediately prior to dosing but not immediately after dosing.
Body Weights	The following four animals were above the protocol specified weight range: 8443 - 40.5g 6702 - 43.7g 6704 - 41.2g 8426 - 40.8g There were no suitable replacements within the protocol specified weight range.
Disposition of Animals	For the dose range-finding study, groups 1 - 3 were sacrificed on 12/6/07 and groups 4 - 6 were sacrificed on 12/14/07. There is no documentation of sacrificed animals being discarded without necropsy. Per protocol, "All animals will be euthanized by CO2 inhalation followed by incision of the diaphragm and then disposed of without necropsy."
	On 12/5/07, dose range-finding animals in groups 2 and 3 were found dead, but there is no documentation of found dead animals being discarded without necropsy. Per protocol, "Unscheduled deaths will be discarded without necropsy."

These study deviations neither affected the overall interpretation of study findings nor compromised the integrity of the study.

Appendix 2
Test Substance Data Sheet

TEST SUBSTANCE DATA SHEET

13F-SFA

SPONSOR INFORMATION			
Sponsor	Company * : DAIKIN INDUTRIES ,LTD.		
	Name person : IKUO YAMAMOTO Ikuo Yamamoto 2/29/'08		
	Address* : 1-1 NISHI HITOTSUYA,SETTSU-SHI OSAKA,566-8585,JAPAN		
	Telephone : 81-6-6349-4425		
	Fax Number : 81-6-6349-1524	Email: iyamamot@notes.che.daikin.co.jp	

TEST SUBSTANCE IDENTITY		
Substance Name / Code * : 2-Propenoic acid, 3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl ester		
Substance Name for Report * : 13F-SFA		
Batch/Lot Number * : Lot.6X002		
Please provide the following information for your substance or, in case of mixtures, for the active ingredient(s):		
Chemical name (IUPAC) or Synonym: Propenoic acid, 3,3,4,4,5,5,6,6,7,7,8,8,8- tridecafluorooctyl ester		
Analytical method for aqueous samples* : · not applicable ~~available -YES / NO~~ ~~attached -YES / NO~~		
Molecular Formula * : C11H7F13O2		
CAS Number * : 17527-29-6	Structural Formula *:	
Molecular Weight * : 418		
Purity *(treated as 100% unless otherwise stated): 99.7 %	$CF_3(CF_2)_5CH_2CH_2OC(=O)CH=CH_2$	
Composition*:		

PHYSICO-CHEMICAL DATA		
Physical State at Room Temp.* ~~: Powder / Granules / Crystals / Solid / Paste~~ / Liquid / ~~Vapour /~~		
Appearance / Colour * : Colorless liquid		Specific Gravity:1.55 at 25degC
Boiling Point: 76 - 80deg C at 1.07kPa	Vapour Pressure: Pa at K	Hygroscopic Yes / No
Melting Point: -33deg C	pH at conc. of %	Homogeneous Yes / No

STORAGE	
The substance will be **stored at room temperature in the dark** unless specified otherwise.	
Specific storage instructions :	~~-fridge / freezer (≤ -20 ºC) / under nitrogen / continuously protected from all light / other:~~
Expiry Date (day-month-year) * : 30 - Oct. - 2008	

HANDLING	
The substance will be **handled in a standard laboratory environment** unless specified otherwise. If applicable, dosing of animals will be performed under normal animal room conditions.	
Specific handling instructions :	
Stable at higher temperatures (above 40 °C): NO / ~~YES- Maximum temperature: Maximum duration:~~	

1 of 2 page

13F-SFA

STABILITY, SOLUBILITY AND MISCIBILITY IN VEHICLE (mark with X when applicable)

VEHICLE	STABLE *						SOLUBLE			MISCIBLE	
	No	for at least (hours)				Un-known	No	Yes	Value or range	No	Yes
		4	24	48	96						
Water						X					
1% Aq carboxy methyl cellulose						X					
Corn oil						X					
Propylene glycol						X					
Polyethylene glycol						X					
Methyl ethyl ketone						X					X
Dimethyl sulphoxide						X					X
Ethanol						X					X
Acetone						X					X
Olive oil						X					
Dimethyl formamide						X					

HAZARDS

	No	Yes	Comments		No	Yes	Comments
Toxic			LD50 >2000mg/kg ,oral,rat	Carcinogenic			?
Irritant			mild to rabbit skin	Genotoxic			?
Corrosive			?	Teratogenic			?
Flammable			Flash point 104deg C	Volatile		X	
Explosive			?	Other hazards:			

2 of 2 page

Appendix 3
Historical Control Data

Mouse Micronucleus - 1/2006 through 12/2006

		% Micronucleated PCEs From 2000 PCEs per Animal Mean ± S.D. Males	PCE:NCE Ratio Mean ± S.D. Males
Pooled Vehicle Controls			
24 hour harvest	Minimum	0.00	0.21
	Maximum	0.40	1.14
	Average	0.053 ± 0.062	0.691 ± 0.185
	N	194	194
48 hour harvest	Minimum	0.00	0.19
	Maximum	0.30	1.40
	Average	0.051 ± 0.055	0.718 ± 0.212
	N	130	130
Positive Controls – Cyclophosphamide, 80 mg/kg			
24 hour harvest	Minimum	0.50	0.21
	Maximum	5.20	1.33
	Average	2.241 ± 1.032	0.608 ± 0.176
	N	190	190

PCE = Polychromatic erythrocyte
NCE = Normochromatic erythrocyte
N = Number of animals
Staining of slides performed using May-Grünwald solution and Giemsa

GLOSSARY

The following lists of references, abbreviations, and comments on the data are used by Covance.

References

Dunnett, C. W., "A Multiple Comparisons Procedure for Comparing Several Treatments with a Control," *Journal of the American Statistical Association*, 50:1096-1121 (1955).

Dunnett, C. W., "New Tables for Multiple Comparisons with a Control," *Biometrics*, 20:482-491 (1964).

Heddle, J. A., Hite, M., Kirkhart, B., Mavournin, K. H., MacGregor, J. T., Newell, G. W. and Salamone, M. F., "The Induction of Micronuclei as a Measure of Genotoxicity," *Mutation Research*, 123:61-118 (1983).

Heddle, J. A., Cimino, M. C., Hayashi, M., Romagna, F., Shelby, M. D., Tucker, J. D., Vanparys, Ph., and MacGregor, J. T., "Micronuclei as an Index of Cytogenetic Damage: Past, Present, and Future," *Environmental and Molecular Mutagenesis*, 18:277-291 (1991).

OECD, "Mammalian Erythrocyte Micronucleus Test," *OECD Guidelines for Testing of Chemicals*, Section 4, Guideline 474, updated and adopted 21 July 1997.

Salamone, M. F. and Mavournin, K. H., "Bone Marrow Micronucleus Assay: a Review of the Mouse Stocks Used and Their Published Mean Spontaneous Micronucleus Frequencies," *Environmental and Molecular Mutagenesis, 23*:239-273 (1994).

Schmid, W., "The Micronucleus Test," *Mutation Research*, 31:9-15 (1975).

Schmid, W., "The Micronucleus Test for Cytogenetic Analysis," *Chemical Mutagens, Principles and Methods for Their Detection*, (ed.) Hollaender, A., Vol. 4, pp. 31-53, Plenum Press (1976).

Winer, B. J., *Statistical Principles in Experimental Design*, Second Ed., McGraw-Hill: New York (1971).

Abbreviations

General

Mean; MEAN	Average
SD; S.D.; STAND DEV; STANDARD DEV; sd; STD.DEV	Standard deviation
SE; S.E.	Standard error
-; NA	No value; not applicable; not present
#; N; No.	Number
Obs.	Observations
IPD	Immediate postdose
PD	Postdose
a.m.	Ante meridian
p.m.	Post meridian
M	Male
F	Female
ID	Identification

Units of Measure

G, g	Gram
KG, kg	Kilogram
MG, mg	Milligram
ML, mL	Milliliter
UL, µL	Microliter
HR, hr	Hours
min	Minute

Major Computer Systems

EMCS	Environmental Monitoring and Control System
EMCSDR	Environmental Monitoring and Control System Data Reporting
MTTS	Material Tracking and Testing System

Comments on the Data

Various models of calculators, computers, and computer programs were used to analyze data in this study. Because different models round off or truncate numbers differently, values in some tables (*e.g.*, means, standard deviations, or individual values) may differ slightly from those in other tables, from individually calculated data, or from statistical analysis data. Neither the integrity nor the interpretation of the data was affected by these differences.