UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND
POLLUTION PREVENTION

MEMORANDUM

Date: 6/29/2015

SUBJECT: Folpet: Data Evaluation Records (DERs) for EDSP Tier 1 Assays

PC Code: 081601
Decision No.: 461069, 464656
Petition No.: NA
Risk Assessment Type: NA
TXR No.: 0055725
MRID No.: See Table

DP Barcode: D398813, D401689
Registration No.: NA
Regulatory Action: NA
Case No.: NA
CAS No.: 133-07-3
40 CFR: NA

Ver.Apr. 2010

FROM: Greg Akerman, Ph.D.
Immediate Office
Health Effects Division (7509P)

THROUGH: Jess Rowland
Deputy Director
Health Effects Division

TO: Jolene Trujillo
Biologist/Chemical Review Manager
Risk Management and Implementation Branch V
Pesticide Re-evaluation Division (7505P)

I. ACTION REQUESTED

The Pesticide Re-evaluation Division (PRD) of OPP has requested that the Health Effects Division (HED) review the Endocrine Disruptor Screening Program (EDSP) Tier 1 assays submitted in response to the agency's Test Order for folpet: Test Order # EDSP-081601-175.

II. RESPONSE

Attached are the EDSP Tier 1 assay DERs for folpet.

III. MRID Table

Chemical: Folpet		**PC Code: 081601**
Guideline	**Assay**	**MRID**
890.1100	Amphibian Metamorphosis Assay (Frog)	49140601
890.1150	Androgen Receptor Binding (Rat Prostate)	48616901
890.1200	Aromatase Assay (Human Recombinant)	48616902
890.1250	Estrogen Receptor Binding	48616903, 48843501
890.1300	Estrogen Receptor Transcriptional Activation (Human Cell Line HeLa-9903)	48616904
890.1350	Fish Short-Term Reproduction	48684201
890.1400	Hershberger (Rat)	48616905
890.1450	Female Pubertal (Rat)	48671201
890.1500	Male Pubertal (Rat)	48671202
890.1550	Steroidogenesis (Human Cell Line – H295R)	48616906
890.1600	Uterotrophic (Rat)	48616907

Data Requirement:	EPA DP Barcode	412630
	OECD Data Point	231
	EPA MRID	49140601
	EPA Guideline	890.1100
		Amphibian Metamorphosis Assay (Frog)

Test Material:	Folpet		**Purity (%):** 97.6%
Common Name	Folpet		
Chemical Name	IUPAC	Not reported	
	CAS Name	Not reported	
	CAS No.	133-07-3	
	Synonyms	Folpan Tech	
	EPA PC Code	081601	

Primary Reviewer: John Marton, Ph.D. **Signature:**
Environmental Scientist, CDM Smith **Date:** 07/24/2013

Secondary Reviewer: Teri S. Myers, Ph.D. **Signature:**
Environmental Scientist, CDM Smith **Date:** 08/20/2013

Primary and Final Additional Reviewer: Robin Sternberg **Signature:**
Wildlife Biologist, USEPA/OCSPP/OPP/EFED/ERB1 **Date:** 09/13/2013, 05/28/2015

Digitally signed by ROBIN STERNBERG
DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=ROBIN STERNBERG, dnQualifier=0000039126
Date: 2015.06.01 16:57:55 -04'00'

Additional Reviewer: Elizabeth Donovan **Signature:**
Biologist, USEPA/OCSPP/OPP/EFED/ERB6 **Date:** 11/20/2013

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DN: cn=Elizabeth Donovan, o=EPA, ou=EFED, email=donovan.elizabeth@epa.gov, c=US
Date: 2015.06.03 08:45:43 -04'00'

Date Evaluation Completed: 05/28/2015

CITATION: Lee, M.R. 2013. Folpet- Amphibian Metamorphosis Assay with African Clawed Frog (*Xenopus laevis*) Following OPPTS Test Guideline 890.1100 and OECD Test Guideline No. 231. Unpublished study performed by Smithers Viscient, Wareham, Massachusetts. Laboratory report number 11742.6177. Study sponsored by Makhetshim Agan NA, Raleigh, North Carolina. Study completed May 22, 2013.

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Disclaimer: The guideline recommendations in this DER template are offered as a general reference to aid in preparation of the DER. The purpose of these recommendations is not to serve as substitute for the Test Guidelines, nor to provide any guidance on how the study should be conducted.

EXECUTIVE SUMMARY

The 21-day assay of folpet on amphibian metamorphosis of African clawed frog (*Xenopus laevis*) was conducted under flow-through conditions. Amphibian larvae at Nieuwkoop-Faber (NF) stage 51 (80/control and treatment group; 20/replicate) were exposed to nominal concentrations of folpet (97.6% purity) at 0 (negative and solvent [0.004 mL/L dimethylformamide (DMF)] controls), 0.0002, 0.002, and 0.02 mg a.i./L; the reviewer-calculated time-weighted average (TWA) measured concentrations were <0.000014 (<LOQ; controls), 0.000069, 0.00092, and 0.0096 mg a.i./L. The test system was maintained at 21 to 23°C and a pH of 6.8 to 7.9.

There were no significant differences ($p>0.05$) between the negative control and solvent control for any of the endpoints. Unless otherwise indicated, all effects are reported based on comparison to the negative control.

Survival rates in all treatment groups were similar to the negative control at test termination. Spinal deformities were observed in 3 to 18% of surviving tadpoles in both controls and all treatment groups, though this observation was not attributed to exposure to the test material. On Day 7, there were significant reductions ($p<0.05$) in snout-vent length of 13%, wet weight of 30%, and hind leg length (HLL) of 9% at the high treatment level relative to the negative control; on Day 21, these endpoints were not significantly different from the negative control.

Folpet did not significantly increase or decrease Day 7 or 21 normalized (for snout-vent length) HLL at any treatment level ($p>0.05$). No significant acceleration or delay of median NF developmental stage was observed after 7 or 21 days of exposure at any treatment level; no asynchronous development was observed. In the solvent control and the low and mid treatment groups, there were a total of 2, 1, and 1 late-stage tadpoles (NF>60), respectively; no late-stage tadpoles were observed in the negative control or high treatment group.

Thyroid gland histopathology findings were observed in the controls and all treatment groups, though these effects did not appear to be treatment related. Histopathological findings included mild follicular hypertrophy and hyperplasia in the controls and all treatment groups and mild follicular lumen area increase in the controls and the low and mid treatment groups.

The study met all validity and performance criteria with the exception that the coefficient of variation (CV) of the measured test concentration for the mid treatment group was 27%, exceeding the guideline performance criterion of ≤20%. This deviation did not impact the interpretation of the study.

This assay **satisfies** the EDSP Tier 1 Test Order requirements for an Amphibian Metamorphosis Assay (OCSPP Guideline 890.1100).

Results Synopsis:

Test organism NF stage at test initiation: 51
Test organism total length at test initiation (optional): Not reported
Test type: Flow-through

Table 1: Summary of Developmental and Thyroid Pathology/Histopathology Effects[1,2] in the Amphibian Metamorphosis Assay (AMA) with Folpet.

Treatment (mg a.i./L) [TWA-measured]	NF Developmental Stage		Hind Limb Length[3]		Asynchronous Development		Thyroid Gross and Histopathology
	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21	Day 21
0.000069	No	No	No	No	No	No	No
0.00092	No	No	No	No	No	No	No
0.0096	No	No	No[4]	No	No	No	No

1 A "yes" indicates a significant difference based on comparison to the negative (clean water) control, unless otherwise specified.

2 The criteria for significance are described in the Reviewer's Analysis and Statistical Verification sections of the DER. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

3 Hind-limb length is normalized to snout-vent length (SVL).

4 Hind-limb length not normalized to snout-vent length was significantly reduced.

I. MATERIALS AND METHODS

Guideline Followed: This study was conducted following guidelines outlined in the U.S. Environmental Protection Agency's Endocrine Disruptor Screening Program Test Guidelines 890.1100, Amphibian Metamorphosis (Frog); and OECD Guideline for Testing of Chemicals No. 231, Amphibian Metamorphosis Assay. The following deviations were noted:

1. The CVs for the measured concentrations for the 0.000069, 0.00092, and 0.0096 mg a.i./L treatment groups were 17, 27, and 11%, respectively. Thus, the CV for the 0.00092 mg a.i./L treatment group exceeded the guideline performance criterion of ≤20%.
2. The physical description of the test material was not reported.
3. Hardness of the dilution water (48-56 mg/L as $CaCO_3$) exceeded the recommended range (40-48 mg/L as $CaCO_3$).
4. The ammonia, fluoride, chlorate, and perchlorate concentrations in the dilution water were not reported.
5. The method for determining the highest test concentration in the range-finder was not specified.
6. The life stage of the tadpoles used in the range-finding test was not specified.
7. Test material was detected in both the negative and solvent controls on Day 14, though this was determined to be the result of sample processing errors.

These deviations do not impact the interpretation of this study.

Compliance: Signed and dated No Data Confidentiality, GLP, and Quality Assurance statements were provided. This study was conducted in compliance with all pertinent U.S. EPA (40 CFR, Part 160, U.S. EPA, 1989a and 40 CFR, Part 792; U.S. EPA, 1989b) and OECD (OECD, 1998) Good Laboratory Practice regulations with the following exceptions: routine food and water contaminant screening analyses were conducted using standard U.S. EPA procedures by GeoLabs, Inc., Braintree, Massachusetts; and the test substance was not

characterized under GLP requirements prior to its use in this study in accordance with 40 CFR, Part 160.105(a) and 40 CFR, Part 792.105(a).

A. Test Material Folpet

Description: Not reported

OECD recommends describing water solubility, melting/boiling point stability in water and light, pKa, Pow or Kow, vapor pressure of test compound, expiration date.

Lot No./Batch No. : 00138518 (Batch No.)

Purity: 97.6%

Impurities: None reported

Stability of Compound: Not reported. However, the reviewer-calculated TWA concentrations yielded recoveries of 35-48% of nominal with coefficients of variation of 11 to 27%.

Storage Conditions of Test Chemicals: Stored at room temperature in a dark, ventilated cabinet.

B. Test Organism

Table 2: General Information About the Test Species and Parental Care.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Species common name:	African clawed frog		*EPA recommends African clawed frog (Xenopus laevis). Western [Africa] clawed frog Silurana (Xenopus) tropicalis may be used as an alternate species*[1]*; however, a list of all of the necessary protocol deviations to accommodate this species is recommended for inclusion in the study report. The guideline recommends that the performance criteria used to support the reliability of the test be identified.*
Species scientific name:	*Xenopus laevis*		
Species strain (if stated):	Not reported		

[1] U.S. Environmental Protection Agency (EPA). (2011). Corrections and Clarifications on Technical Aspects of the Test Guidelines for the Endocrine Disruptor Screening Program Tier 1 Assays (OCSPP Test Guideline Series 890). March 3, 2011. Office of Chemical Safety and Pollution Prevention (OCSPP), Washington, D.C. (http://www.epa.gov/endo/pubs/assayvalidation/clarificationdoc.pdf).

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Were parents maintained as in-house stock?	Yes	Original brood stock obtained from Nasco, Fort Atkinson, Wisconsin, and maintained as in-house breeders for approximately one year prior to generating tadpoles for use in this exposure.	*EPA recommends that larvae used in the assay be derived from in-house adults.*
Were parental acclimation conditions same as definitive test?	Yes		
Acclimation period for parental frogs (if applicable):	Continuous for one year		
Details on parental feeding:	None provided		
Details on parental health:	The brood stock did not show any sign of sickness, disease, injuries, or abnormalities from the		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	day of receipt to the day of pre-exposure initiation.		

Table 3: Larval Selection and Care.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Best single spawn?	Yes	The quality of each of the three spawns was quantitatively assessed during the 6 days post-fertilization; criteria were embryo survival and the quantity of embryos.	*EPA and OECD recommend that the best 2 – 3 individual spawns, with a minimum of 1500 larvae/spawn, be evaluated to identify the best single spawn, and that the larvae selected for testing originate from the best single spawn (i.e., the spawns are not co-mixed)*
Number of spawns evaluated (if applicable):	3		
Number of eggs sampled per spawn:	Not specified	For this study, each of the three spawns produced at least 1,500 embryos.	

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
NF stage at test initiation	51		*EPA recommends that the definitive study be initiated with larvae at Nieuwkoop – Faber (NF) developmental stage 51 (≤17 days post-fertilization).*
Age at test initiation:	15 days post-fertilization (dpf)		
Mean total length at test initiation (if reported):	Not measured		
Range of total length at test initiation (if reported):	Not measured		
Was the optional size selection method used?	No		
Details on larval selection:	Best single spawn		
Loading rate (rearing density):	10 larvae/L		*EPA recommends that rearing density (loading rate) not exceed approximately 10 larvae/L culturing system for flow-through systems or 4 tadpoles/L in static-renewal exposure systems.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Type of food:	Xenopus Express tadpole food	Representative samples of the food source were periodically analyzed for the presence of pesticides, PCBs and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds have been detected in the food samples at concentrations considered toxic to the test organisms. Based on these analyses, food sources were considered to be of acceptable quality since all analyte concentrations were below levels of concern, in agreement with ASTM (2005).	*EPA recommends Sera Micron® throughout pre-exposure (after NF stage 45/46) and during the entire 21-d definitive study. If another diet is used, the study report should provide analysis of iodide content and potential contaminants, and the diet should demonstrate equal performance to Sera Micron®.*
Source of food:	Xenopus Express, Brooksville, Florida		
Iodide concentration in diet (if known):	Not reported		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Frequency of feeding:	2 times/day		*EPA recommends that feeding occur at least twice per day.*
Details on feeding regime:	Feeding regime followed guideline recommendations.		*It is recommended that food rations during the pre-exposure period be increased along with larval growth to approximately 30 mg/larva/day by test initiation. EPA and OECD recommend that food rations increase from 30 mg/larva/day at test initiation (Study Day 0-4) to 80 mg/larva/day in the last week of the test (Study Day 15-21).*

C. Exposure System

Table 4: Summary of Information on the Exposure System and Test Vessel Characteristics.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Type of exposure:	Flow-through		*EPA recommends the use of a flow-through system.*
Type of flow-through dilution system (if applicable):	Intermittent-flow proportional diluter		*Intermittent flow proportional diluters or continuous flow serial diluters are recommended.*[2]
Flow-through rate (if applicable):	61 mL/min	Flow rate provided 16 volume additions every 24 hours, with a 90% replacement time of ~3 hours. In an effort to control potential losses due to rapid hydrolysis of folpet, the exposure system was calibrated to deliver solutions at the highest turnover rate allowed by the exposure system (approximately	*Recommended flow-through rate is 25 mL/min (complete volume replacement ca. every 2.7 hrs).*

[2] Additional guidance for aquatic test design is located in OCSPP Guideline 850.1000, Special Considerations for Conducting Aquatic Laboratory Studies.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
		16 turnovers/day).	
Details on toxicant mixing for flow-through systems (if applicable):	A syringe pump delivered solution to a mixing chamber positioned over a magnetic stir plate. The mixing chamber was partially submerged within an ultrasonic water bath to aid in solubilization of the test substance into the dilution water.	Flow-splitting cells were employed to equally distribute the solutions to the four replicate vessels for each concentration or control group.	*Recommended toxicant mixing for flow-through systems: 1) Mixing chamber is recommended but not required; 2) Aeration is not recommended for mixing; 3) A demonstration that the test solution is completely mixed before introduced into the test system is recommended; 4) The recommended flow splitting accuracy is within 10%.*
Renewal period for static renewal (if applicable):	NA		*If static renewal is used, EPA recommends 24-hr renewal; renewal period is recommended not to exceed 72 hours.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Aeration?	No		*EPA recommends maintaining dissolved oxygen concentrations ≥40% air saturation (≥3.5 mg/L). Aeration may be maintained through bubblers. It is recommended to set bubblers at levels that do not cause stress on the tadpoles.*
Source of dilution water:	Dilution water was a mixture of unadulterated water from a 100-meter bedrock well and dechlorinated Town of Wareham well water.		*EPA recommends natural or reconstituted water; it is recommended that natural water be sterilized with UV and tested for pesticides, heavy metals, and other possible contaminants, including known substrates of the iodine transporter of the thyroid gland (e.g., fluoride, chlorate, perchlorate). OECD accepts any water in which the test species show control survival at least as good as indicated in the test guideline.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was dilution water analyzed for pesticides, heavy metals, and other contaminants?	Yes. No pesticides, PCBs, or toxic metals were detected at concentrations considered toxic in any of the water samples analyzed in agreement with ASTM (2005) standard practices.		
Iodide supplementation in water?	No	Iodide concentrations were analyzed in January 2013 on a representative sample of the dilution water by Galbraith Laboratories, Inc., Knoxville, Tennessee. Iodide concentrations were measured to be approximately 6.6 µg/L.	*If reconstituted water is used or if background levels of iodide in natural water are less than 0.5 µg/L, iodide supplementation is recommended. This supplementation is in addition to the recommended dietary source of iodide (e.g.. in Sera Micron).*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Test vessel type/materials:	Glass		*EPA and OECD recommend that water-contact portions of the system not compromise the study (e.g., all glass vessels or glass vessels with stainless steel frames are acceptable examples).*
Test vessel size:	30 x 15 x 20 cm	12-cm high side drain	
Fill volume:	5.5 L		
Additional details on exposure system:	The exposure system was constructed of glass, silicone sealant, stainless steel, and Teflon®.		

Table 5: Summary of Water Quality Characteristics in the Test System.

Parameter	Minimum	Maximum	Mean[1]	Measurement Interval	Guideline Recommendations
Hardness (mg/L as	48	56	52	Measured in replicate A of the negative	*EPA recommends hardness 40 to 48*

Parameter	Minimum	Maximum	Mean[1]	Measurement Interval	Guideline Recommendations
$CaCO_3$)				control, low, and high test concentrations on Day 0 and in sequentially alternating replicates weekly thereafter.	*mg/L as $CaCO_3$.*
pH	6.8	7.9	7.3	Measured in all test vessels on Day 0 and in one replicate of each concentration and control daily thereafter, sequentially alternating replicates. Temperature was also continuously measured in replicate A of the negative control.	*EPA recommends pH 7.5 ± 1, inter-replicate and inter-treatment differentials should not exceed 0.5.*
Dissolved oxygen (mg/L)	4.4	9.2	6.8		*EPA recommends dissolved oxygen (DO) >3.5 mg/L (>40% air saturation). OECD recommends DO concentration >3.5 mg/L (>40% air saturation).*
Temperature	21	23	22		*EPA recommends temperature 22±1°C; inter-replicate and inter-treatment differentials should not exceed 0.5°C.*
Iodide (µg/L)	--	--	6.6	January 2013	*EPA recommends aquatic iodide range 0.5 – 10 µg/L (supplemental iodide should not exceed 2 µg/L).*

Parameter	Minimum	Maximum	Mean[1]	Measurement Interval	Guideline Recommendations
Ammonia	Not reported				*General recommendations for frequency of measurements: EPA recommends that water quality parameters be measured in a control and at one test item concentration at least weekly. In static renewal systems, water quality parameters, including ammonia, should be measured just prior to renewal. In addition, EPA recommends that DO be measured at each concentration at least weekly and that temperature be measured continuously. OECD recommends that DO and temperature be measured at least weekly and that pH and hardness be measured at least at the beginning and end of the test.*
Fluoride	Not reported				
Perchlorate	Not reported				
Chlorate	Not reported				
Alkalinity (mg/L as $CaCO_3$)	18	24	21	Measured in replicate A of the negative control, low, and high test concentrations on Day 0 and in sequentially alternating replicates weekly thereafter.	
Conductivity (µS/cm)	290	360	325		

[1] Calculated by reviewer as mean of minimum and maximum values.

D. Study Design and Additional Experimental Conditions

Table 6: Range-Finding Study Conditions (if Applicable).

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was a range-finder conducted?	Yes		
If yes, what was the method for determining the highest test concentration in the range-finder?	Not specified		*EPA recommends that the highest test concentration is either the solubility limit of the test compound, 100 mg/L, or demonstrates adequate evidence of toxicity (e.g., ≤10% mortality), whichever concentration is lowest.*
Species:	*Xenopus laevis*		
Life stage:	Not reported		
Test duration:	96 hours		
Additional details:	The study was conducted under flow-	The study used DMF as a solvent. The turnover rate for all aquaria was approximately 12 turnovers/day.	

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	through conditions with nominal concentrations of 0 (negative and solvent controls), 0.0065, 0.013, 0.025, 0.050, and 0.100 mg a.i./L.	There were 2 replicates (10 tadpoles per replicate) per treatment and control. Water quality parameters measured during the 96-hour exposure period established a dissolved oxygen range of 8.3 to 9.6 mg/L, a pH range of 7.3 to 7.6, and a temperature range of 21 to 22 °C. After 96 hours of exposure, the LC_{50} and EC_{50} (95% C.I.) values were 0.100 (0.053-not calculated) and 0.056 (0.032-0.076) mg a.i./L, respectively. The EC_{50} value was based on sublethal effects that were not specified. At 96-hours: 40% sublethal effects at 0.050 mg/L 50% sublethal effects and 50% mortality at 0. 100 mg/L	

Table 7: Definitive Study Conditions.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Test duration:	21 days		*EPA recommends that the duration of the definitive test be 21 days.*
Method for selecting the highest test concentration in the definitive test:	Range-finder	The highest concentration in the definitive test was approximately 1/3 of the calculated EC_{50} from the range-finding test.	*EPA recommends that the highest test concentration is either the solubility limit of the test compound, 100 mg/L, or demonstrates adequate evidence of toxicity (e.g., ≤10% mortality), whichever concentration is lowest.*
Reference study citation (if applicable):	Not applicable		
Separation of test concentrations:	0.10		*EPA recommends that the maximum concentration separation be 0.1 and the minimum be 0.33.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Number of test concentrations:	3		*EPA recommends a minimum of 3 concentrations and a control, plus solvent control if appropriate.*
Are nominal concentrations adjusted for purity?	Yes		
Indicate the type of values presented for measured concentrations:	Mean-measured	The reviewer also calculated the time-weighted average (TWA) concentrations. See Reviewer's Comments.	
Limit of quantification (LOQ):	0.000012-0.000014 mg a.i./L		*EPA recommends that for chemical test concentrations below the LOQ, analyses be conducted on the stock solutions.*
Level of detection (LOD):	Not reported		
Frequency of measurement:	Samples were collected for analysis on Days 0, 3, 7, 9, 14, 16, and 21.	One replicate was analyzed per week; if anomalous results were observed, the test system would be resampled. At exposure initiation (Day 0), samples from replicate A of each treatment and control solution were	*It is recommended that test item concentration be measured in one*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
		analyzed, samples from alternating replicates were analyzed every other week thereafter. On Day 0 there was no internal standard response due to a processing error with the exposure samples. To support the use of these sample recoveries, the system was re-sampled on Day 3. The Day 7 sampling had an inconsistent internal standard response. However, sample recoveries, when calculated without the internal standard, were similar to those observed on previous sampling events. To confirm the Day 7 without internal standard results and to have a useable data set, the system was resampled on Day 9. On Day 14, the negative and solvent controls appeared to be contaminated with ~1/3 of the low concentration of folpet. The exposure system was examined and on Day 16 the negative and solvent control groups were resampled and analyzed. The reanalysis confirmed that there was an error in the processing of the exposure samples.	*tank at each treatment level at test initiation and every week thereafter.*
Number of replicates in control:	4		*EPA recommends 4 replicates.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Number of replicates in solvent control (if applicable):	4		*EPA and OECD recommend the use of a concurrent solvent control when a solubilizing agent is used. EPA recommends 4 replicates.*
Number of replicates per test item treatment level:	4		*EPA recommends 4 replicates.*
Number of larvae per treatment at test initiation:	80		
Was a solvent used?	Yes		
Solvent type (if applicable):	DMF		
Maximum solvent concentration (if applicable):	0.004 mL/L		*EPA recommends that the solvent not exceed 0.02 ml/L*[3]*. OECD recommends that solvent have no effect on survival nor produce any*

[3] Hutchinson TH, Shillabeer N, Winter MJ, Pickford DB (2006). Acute and chronic effects of carrier solvents in aquatic organisms: A critical review. Review. Aquatic Toxicology, 76, pp.69–92.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
			other adverse effects and that concentration not be greater than 0.1 ml/L[4]*.*
Was a positive control used?	No		
Positive control (if applicable):	NA		
Positive control concentration(s) (if applicable):	NA		
Photoperiod:	12 hrs light : 12 hrs dark		*EPA recommends photoperiod 12:12 (light:dark).*
Light intensity at water's surface:	0.710-1.100 Klux		*EPA recommends light intensity 0.6 – 2 Klux (at water's surface).*
Additional details:	The 5.0 mg/mL diluter stock solutions (folpet		

[4] OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. Environmental Health and Safety Publications. Series on Testing and Assessment. No. 23. Paris, France.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	+DMF) prepared every 4-5 days were observed to be clear and colorless following preparation. The 49 µL/mL solvent stock solutions (DMF) prepared as needed were observed to be clear and colorless following preparation.		

Table 8: Summary of Treatment Concentrations in the Amphibian Metamorphosis Assay with Folpet.

Treatment ID	Nominal Concentration (mg a.i./L)	Mean-Measured Concentration[1] (mg a.i./L)	Mean CV [1] (%)	Details or Remarks[2]	Guideline Recommendations
Negative Control	0	<LOQ except 0.038 on Day 14	NA	On Day 14, the negative and solvent controls appeared to be contaminated with ~1/3 of the low concentration of folpet. The exposure system was examined and on Day 16 the negative and solvent control groups were resampled and analyzed. According to the study author, the re-analysis confirmed that there was an error in the processing of the exposure samples.	*EPA and OECD recommend that test item concentrations be maintained at a coefficient of variation (CV) ≤20%.*
Solvent Control	0	<LOQ except 0.021 on Day 14	NA		
Treatment 1	0.00020	0.000068	17	TWA: 0.000069 mg a.i./L; CV: 17%	
Treatment 2	0.0020	0.00093	29	TWA: 0.00092 mg a.i./L; CV: 27%	
Treatment 3	0.020	0.0096	11	TWA: 0.0096 mg a.i./L; CV: 11%	

Abbreviations: [CV] Coefficient of variation.

LOQ=0.000012-0.000014 mg a.i./L

[1] As reported by the study author

[2] As calculated by the reviewer

E. Observations

Biological Endpoints: Day 7- NF stage, wet weight, snout-vent length (SVL), hind-limb length (HLL), normalized HLL
Day 21- NF stage, wet weight, SVL, HLL, normalized HLL, thyroid histopathology
Daily- mortality, clinical signs

Were raw (individual) data provided? Yes

EPA recommends that observations of mortality and clinical signs occur daily, at a minimum; other observations are recommended as follows: NF developmental stage (Days 7 and 21); any asynchronous development, indicated by tadpoles that cannot be assigned an NF stage (Days 7 and 21); hind limb length (Days 7 and 21); snout-vent length (Days 7 and 21); body weight (test initiation, for optional size-based larval selection); and thyroid gland gross pathology and histopathology (Day 21). Note the histopathology section of the test guideline also includes thyroid gross pathology observations.

II. RESULTS AND DISCUSSION

A. Results

Day 7 mortality was not reported. However, by test termination, survival was 99% in both controls and 100, 99, and 99% in the TWA 0.000069, 0.00092, and 0.0096 mg a.i./L treatment groups, respectively (Table 9).

Table 9: Larval Mortality in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-measured]	Larval Mortality					
	Day 7[1]			Day 21		
	n	Mortality #	Mortality %	n	Mortality #	Mortality %
Negative Control	80	NR	NR	60	1	1
Solvent Control	80	NR	NR	60	1	1
0.000069	80	0	0	60	0	0
0.00092	80	NR	NR	60	1	1
0.0096	80	NR	NR	60	1	1

Abbreviations: [NR] Not reported. (daily mortality data not reported)

[1] Sample size and cumulative mortality values at Day 7 prior to interim sacrifice.

After 7 days of exposure, median developmental stage was 54 in the controls and all treatment groups (Table 10). By test termination, median NF stage was 59 in the negative control and 58 in the solvent control and all treatment groups. The 10th and 90th percentiles of developmental stage in the controls did not differ by more than 4 NF stages.

Table 10: Larval Development in *Xenopus laevis* – Developmental Stage and Asynchronous Development.

Treatment (mg a.i./L) [TWA-measured]	Developmental Stage					
	Day 7			Day 21		
	n	Median Stage	# Asynchronous	n	Median Stage	# Asynchronous
Negative Control	20	54	0	59	59	0
Solvent Control	20	54	0	59	58	0
0.000069	20	54	0	60	58	0
0.00092	20	54	0	59	58	0
0.0096	20	54	0	59	58	0

On Day 7, mean normalized HLLs for tadpoles in the negative and solvent control groups were 0.130 and 0.125, respectively, and mean normalized HLL's for tadpoles in the TWA 0.000069, 0.00092 and 0.0096 mg/L treatment groups were 0.128, 0.125, and 0.135, respectively (Table 11).

On Day 21, mean normalized HLL's for tadpoles in the negative and solvent control groups were 0.567 and 0.569 g, respectively, and mean normalized HLL's for tadpoles in the TWA 0.000069, 0.00093 and 0.0096 mg/L treatment groups were 0.533, 0.584 and 0.478 g, respectively.

Table 11: Larval Development in *Xenopus laevis* – Hind Limb Length.

Treatment (mg a.i./L) [TWA-measured]	Hind Limb Length (HLL)							
	Day 7				Day 21			
	n	Mean (mm)	±SD	HLL: SVL[1]	n	Mean (mm)	±SD	HLL: SVL[1]
Negative Control	20	2.47	0.11	0.130	59	13.41	0.91	0.567
Solvent Control	20	2.41	0.25	0.125	59	13.16	2.38	0.570
0.000069	20	2.47	0.23	0.128	60	12.89	1.71	0.533
0.00092	20	2.46	0.13	0.126	59	14.49	2.19	0.584
0.0096	20	2.24	0.16	0.135	59	11.62	1.97	0.478

Abbreviations: SD Standard deviation.

[1] Summary results for snout-vent length (SVL) are presented in the next table (Table 12).

On Day 7, mean SVLs for tadpoles in the negative and solvent control groups were 19.02 and 19.22 mm, respectively, and mean SVLs for tadpoles in the 0.000069, 0.00092 and 0.0096 mg/L treatment groups were 19.14, 19.45, and 16.53 mm, respectively (Table 12). On Day 21, mean SVLs for tadpoles in the negative and solvent control groups were 23.81 and 23.47 mm, respectively, and mean SVLs for tadpoles in the 0.000069, 0.00093 and 0.0096 mg/L treatment groups were 24.52, 25.02, and 24.09 mm, respectively.

On Day 7, mean wet weights for tadpoles in the negative and solvent control groups were 0.428 and 0.439 g, respectively, and mean wet weights for tadpoles in the 0.000069, 0.00092 and 0.0096 mg/L treatment groups were 0.438, 0.461 and 0.300 g, respectively (Table 12). On Day 21, mean wet weights for tadpoles in the negative and solvent control groups were 0.946 and 0.875 g, respectively, and mean wet weights for tadpoles in the 0.000069, 0.00093 and 0.0096 mg/L treatment groups were 0.977, 1.072 and 0.888 g, respectively.

Table 12: Larval Growth in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-measured]	Snout-Vent Length (SVL)						Body Weight[1]					
	Day 7			Day 21			Day 7			Day 21		
	n	Mean (mm)	±SD	n	Mean (mm)	±SD	n	Mean (g)	±SD	n	Mean (g)	±SD
Negative Control	20	19.00	0.29	59	23.81	0.62	20	0.429	0.026	59	0.946	0.113
Solvent Control	20	19.22	1.49	59	23.47	0.89	20	0.439	0.096	59	0.875	0.071
0.000069	20	19.13	0.98	60	24.52	0.20	20	0.438	0.062	60	0.977	0.036
0.00092	20	19.45	0.49	59	25.03	0.85	20	0.461	0.039	59	1.072	0.078
0.0096	20	16.55	1.34	59	24.09	0.80	20	0.300	0.071	59	0.888	0.134

Abbreviations: SD Standard deviation.

[1] Also referred to as “wet weight” in the test guideline.

Incidences of mild follicular cell hypertrophy and mild follicular cell hyperplasia were observed in the negative control, solvent control, and treatment groups. Mildly increased follicular lumen area was observed in the negative control, solvent control, and 0.00092 and 0.0096 mg a.i./L treatment groups. The frequency of these observations did not appear to be treatment-related (Tables 13 and 14). Pharyngeal epithelial changes consistent with metamorphosis were present in many animals across all study groups with variable prevalence. Further, there were no treatment-related lesions and no other remarkable microscopic findings were associated with exposure to the test material.

Table 13: Gross Pathology and Histopathology of the Thyroid Gland in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-measured]	Diagnostic Observations[1]								
	Severity	Thyroid Gland Hypertrophy		Thyroid Gland Atrophy		Follicular Cell Hypertrophy		Follicular Cell Hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control	0	20	20	20	20	20	17	20	14
	1	20	0	20	0	20	3	20	6
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
Solvent Control	0	20	20	20	20	20	14	20	12
	1	20	0	20	0	20	6	20	8
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
0.000069	0	19	19	19	19	19	15	19	14
	1	19	0	19	0	19	4	19	5
	2	19	0	19	0	19	0	19	0
	3	19	0	19	0	19	0	19	0

Treatment (mg a.i./L) [TWA-measured]	Diagnostic Observations[1]								
	Severity	Thyroid Gland Hypertrophy		Thyroid Gland Atrophy		Follicular Cell Hypertrophy		Follicular Cell Hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
0.00092	0	20	20	20	20	20	14	20	18
	1	20	0	20	0	20	6	20	2
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
0.0096	0	20	20	20	20	20	14	20	16
	1	20	0	20	0	20	6	20	4
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0

[1] Thyroid gland gross pathology and histopathology are graded 0 – 3 based on severity: 0=Not remarkable, 1=Mild, 2=Moderate, 3=Severe. See OECD No. 82 for reference.

Table 14: Additional Thyroid Gland Histopathology Observations in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-measured]	Additional Qualitative Observations[1]									
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase/ Decrease)		Colloid Quality (Depletion)		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control	0	20	18	20	20	NA	NA	20	20	20	20
	1	20	2	20	0	NA	NA	20	0	20	0
	2	20	0	20	0	NA	NA	20	0	20	0
	3	20	0	20	0	NA	NA	20	0	20	0
Solvent Control	0	20	17	20	20	NA	NA	20	20	20	20
	1	20	3	20	0	NA	NA	20	0	20	0
	2	20	0	20	0	NA	NA	20	0	20	0
	3	20	0	20	0	NA	NA	20	0	20	0
0.000069	0	19	18	19	19	NA	NA	19	19	19	19
	1	19	1	19	0	NA	NA	19	0	19	0
	2	19	0	19	0	NA	NA	19	0	19	0
	3	19	0	19	0	NA	NA	19	0	19	0

Treatment (mg a.i./L) [TWA-measured]	Additional Qualitative Observations[1]									
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase/ Decrease)		Colloid Quality (Depletion)		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
0.00092	0	20	17	20	20	NA	NA	20	20	20	20
	1	20	3	20	0	NA	NA	20	0	20	0
	2	20	0	20	0	NA	NA	20	0	20	0
	3	20	0	20	0	NA	NA	20	0	20	0
0.0096	0	20	20	20	20	NA	NA	20	20	20	20
	1	20	0	20	0	NA	NA	20	0	20	0
	2	20	0	20	0	NA	NA	20	0	20	0
	3	20	0	20	0	NA	NA	20	0	20	0

[1] Thyroid histopathology is graded 0 – 3 based on severity: 0=Not remarkable, 1=Mild, 2=Moderate, 3=Severe. See OECD No. 82 for reference.

Tadpoles terminated on Day 7 were observed to have no spinal deformities. Beginning on test Day 7, several tadpoles exposed to the controls and most treatment levels were observed to be deformed (*e.g.*, spinal curvature). On Day 21, spinal deformities were observed for 12 and 15% of negative and solvent control animals and for 18, 10 and 3% of tadpoles exposed to the 0.000069, 0.00093 and 0.0096 mg/L treatment levels, respectively. According to the study author, the incidence of spinal deformities was unrelated to treatment with folpet. Based on historical control data from studies performed at Smithers Viscient, incidence of spinal deformities ranges from 0 to 63%.

Table 15: Clinical Signs in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA-measured]	Clinical Signs[1]		
	Type	n	Incidence at Study Termination
Negative Control	Spinal deformities	60	12%
Solvent Control	Spinal deformities	60	15%
0.000069	Spinal deformities	60	18%
0.00092	Spinal deformities	60	10%
0.0096	Spinal deformities	60	3%

[1] Note that asynchronous development (unable to stage) is reported previously in **Table 10** and not here.

B. **Study Author's Analysis and Conclusions**

At test termination, data for developmental stage, SVL, HLL, HLL, and wet weight were analyzed to identify significant reductions in the treatment organisms compared to the control organisms. All statistical conclusions were made at the 95% level of certainty except in the case of the basic assumption tests (e.g., Shapiro-Wilks' Test and Bartlett's Test, α=0.01).

Since a solvent was used as a carrier for the test substance, the results of the solvent control were compared to the control data. If the concentration of the carrier solvent in the solvent control caused

a statistically significant effect (t-test), either enhancement or reduction when compared to the negative control, the treatment data were compared to that of the solvent control. If the solvent concentration did not affect the measured or calculated endpoint, both controls (negative control and solvent control) were pooled for the data analysis. Pooled controls were used for analysis because the pooled data increase the replication for the control comparison thereby increasing the sensitivity of the statistical analysis for detection of folpet-related effects.

Mortality data were analyzed using the step-down Cochran-Armitage test when a monotonic trend was present and Fisher's Exact Test with the Bonferroni-Holm adjustment for non-monotonic trends. Developmental stage was analyzed using the Jonckheere-Terpstra test (step-down). Normalized HLL, SVL, and wet weight were analyzed using the Jonckheere-Terpstra test when a monotonic trend was present. When a monotonic trend was not present, data were analyzed using Dunnett's Multiple Comparison Test, Tamhane-Dunnett test, or the Mann-Whitney test with Bonferroni-Holm adjustment. All analyses were conducted using CETIS- Comprehensive Environmental Toxicity Information System™ Version 1.8.4.20 software.

Fisher's Exact Test with a Bonferroni-Holm Adjustment (C > T) determined no significant difference in Day 21 larval survival among tadpoles exposed to any of the treatment levels tested, compared to the pooled control. Dunnett's Multiple Comparison Test (C > T) determined a significant reduction in Day 7 whole body wet weight among tadpoles exposed to the 0.0096 mg/L treatment level, compared to the pooled control. Dunnett's Multiple Comparison Test (C < T) determined a significant difference in Day 21 whole body wet weight among tadpoles exposed to the 0.00092 mg/L treatment level, compared to the pooled control.

Dunnett's Multiple Comparison Test (C > T) determined a significant reduction in Day 7 snout-vent length among tadpoles exposed to the 0.0096 mg/L treatment level, compared to the pooled control. Dunnett's Multiple Comparison Test (C > T) determined a significant increase in Day 21 snout-vent length among tadpoles exposed to the 0.00092 mg/L treatment level, compared to the pooled control.

Dunnett's Multiple Comparison Test (C < T) determined no significant difference in Day 7 or 21 hind limb length among tadpoles exposed to any of the treatment levels tested, compared to the pooled control.

Dunnett's Multiple Comparison Test (C < T) determined a significant increase in Day 7 hind limb length normalized by SVL among tadpoles exposed to the 0.0096 mg/L treatment level, compared to the pooled control. Dunnett's Multiple Comparison Test (C < T) determined no significant difference in Day 21 hind limb length normalized by SVL among tadpoles exposed to any of the treatment levels tested, compared to the pooled control.

The multi-quantal procedure determined no significant difference in the Day 7 developmental stage among tadpoles exposed to any of the treatment levels tested, compared to the pooled control. The multi-quantal procedure determined a significant reduction in the Day 21 developmental stage among tadpoles exposed to the 0.0096 mg a.i./L treatment level at the 20th, 60th and 80th percentile compared to the control. The multi-quantal procedure determined no significant difference in the Day 21 developmental stage among tadpoles exposed to any of the treatment levels tested compared to the pooled control. A T-Test determined a significant difference between the 20th and 30th percentiles. To keep the analysis consistent across the range, all controls were pooled.

In summary, on Day 7, the study author detected significant reductions in wet weight and SVL in the high concentration treatment group relative to the pooled control, and a significant increase in normalized HLL in the same treatment group. Conversely, on Day 21, wet weight and SVL were significantly increased in the mid-concentration treatment group relative to the pooled control.

C. Reviewer's Analysis and Conclusions

Statistical Methods: Negative and solvent control data for each endpoint were compared using an Equal Variance Two-Sample t-test. No significant differences were observed for any of the end points tested. Subsequent comparisons to treated groups were made using only the negative control.

All data were tested for normality using Shapiro-Wilks test (α=0.01) and for homogeneity of variances using either Levene's or Bartlett's test (α=0.01). All endpoints met the assumptions of parametric statistics and were analyzed using Dunnett's Multiple Comparison test, with the exception of Day 7 Developmental Stage. This nonparametric statistic was analyzed using the Mann-Whitney U Two-sample Test. A decreasing, concentration-dependent trend was noted only for Day 7 HLL, but not for HLL normalized by SVL; the NOAEC/LOAEC for 7-day HLL was confirmed using both the Jonckheere-Terpstra and William's tests. No other concentration-dependent trends were identified. Analyses were conducted using CETIS 1.8.7.10 and backend settings implemented by EFED on 5/29/13.

At least one late-stage tadpole was found in the solvent control and the low and middle concentration treatment groups; however, in no case did the occurrence of late-stage tadpoles warrant a separate analysis (*i.e.*, no more than 20% late stage individuals in any test level). No late-stage tadpoles were observed in the negative control and highest concentration treatment group. In the solvent control and the 0.000069 and 0.0092 mg a.i./L treatment groups there were a total of 2, 1, and 1 NF=61 individuals, respectively, with no apparent pattern in their occurrence.

No asynchronous development was observed.

Unless otherwise indicated, effects were considered statistically significant at $p<0.05$.

Conclusions: On Day 7, the reviewer's analysis detected a significant reduction in HLL (Jonckheere-Terpstra, $p=0.046$), SVL (Dunnett, $p=0.0051$), and wet weight (Dunnett, $p=0.012$) at the highest treatment level, relative to the control, but no effects were detected for any other endpoints on Day 7 or at any endpoint on Day 21.

Table 16: Developmental and Thyroid Gross Pathology/Histopathology Endpoints[1,2] in the AMA with Folpet.

Treatment (mg a.i./L) [TWA-measured]	NF Developmental Stage				Hind Limb Length[3]				Asynchronous Development				Thyroid Gross and Histopathology
	Day 7		Day 21		Day 7		Day 21		Day 7		Day 21		Day 21
	Median	*p*	Median	*p*	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*	Treatment-Related Effects? (Yes/No)
Negative Control	54	NA	59	NA	NA	NA	NA	NA	NA	NA	NA	NA	No
Solvent Control	54	1.00	58	0.17	-3.6	0.17	0.44	0.97	NA	NA	NA	NA	No
0.000069	54	1.00	58	0.14	-1.16	0.94	-6.0	0.80	NA	NA	NA	NA	No
0.00092	54	1.00	58	0.31	-3.09	0.49	2.95	0.97	NA	NA	NA	NA	No
0.0096	54	1.00	58	0.31	4.44	0.23	-15.7	0.18	NA	NA	NA	NA	No
Statistical Test[4]	Mann-Whitney		Dunnett		Dunnett		Dunnett		NA		NA		NA

Abbreviations: Diff. Difference.

1 Unless otherwise indicated, effects are reported based on comparison to the clean water control. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

2 Unless otherwise specified, effects are considered statistically significant at $p<0.05$.

3 Hind-limb length is normalized to snout-vent length (SVL).

4 Statistical test for comparison of treatment groups to the negative control group. A t-test was used to compare the negative and solvent control groups.

Table 17: Growth Endpoints[1,2] in the AMA with Folpet.

Treatment (mg a.i./L) [TWA-measured]	Snout-Vent Length				Body Weight			
	Day 7		Day 21		Day 7		Day 21	
	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*	% Diff.	*p*
Negative Control	--	--	--	--	--	--	--	--
Solvent Control	1.17	0.78	-1.47	0.55	2.34	0.84	-8.09	0.33
0.000069	0.66	0.99	2.97	0.35	2.28	0.99	3.28	0.94
0.00092	2.37	0.81	5.11	0.06	7.59	0.72	13.31	0.21
0.0096	-12.89	**0.0051**	1.17	0.88	-30.05	**0.012**	-6.19	0.73
Statistical Test[3]	Dunnett		Dunnett		Dunnett		Dunnett	

Abbreviations: Diff. Difference. [1] Unless otherwise indicated, effects are reported based on comparison to the negative (clean water) control.

[2] Unless otherwise specified, effects are considered statistically significant at $p < 0.05$.

[3] Statistical test for comparison of treatment groups to the negative control group. A t-test was used to compare the negative and solvent control groups.

E. Study Deficiencies

There were deviations from the guideline as noted in Section I. Materials and Methods of the DER. All of the performance criteria and validity requirements were met with the exception of not maintaining the coefficient of variation for the measured test concentration of the mid treatment group at ≤20% (*i.e.*, 27%),. These deviations did not impact the interpretation of the study.

F. Reviewer's Comments

The reviewer's results differed from those of the study author likely due to the study author comparing treatment data to the pooled control whereas the reviewer compared all treatment data to the negative control only. Therefore, the reviewer's results are reported in the Executive Summary section of this DER.

The reviewer calculated the time-weighted average concentrations using the following equation:

$$C_{TWA} = \frac{\left(\frac{C_1 + C_0}{2}\right)(t_1 - t_0) + \left(\frac{C_2 + C_1}{2}\right)(t_2 - t_1) + \left(\frac{C_{n-1} + C_2}{2}\right)(t_{n-1} - t_2) + \left(\frac{C_n + C_{n-1}}{2}\right)(t_n - t_{n-1})}{t_n}$$

where:

C TWA is the time-weighted average concentration,

C j is the concentration measured at time interval j (j = 0, 1, 2,...n)

t j is the number of hours (or days or weeks, units used just need to be consistent in the equation) of the test at time interval j

(e.g., t 0 = 0 hours (test initiation), t 1 =24 hours, t 2 =96 hours)

On Day 0 there was no internal standard response due to a processing error with the exposure samples. To support the use of these sample recoveries, the system was re-sampled on Day 3. The recoveries from Day 3 have good internal standard response and the samples are generally similar in concentration to Day 0 (without internal standard). Therefore both the Day 0 and Day 3 sample recoveries were used in calculation of mean measured concentrations.

The Day 7 sampling had an inconsistent internal standard response. However, sample recoveries, when calculated without the internal standard, were similar to those observed on previous sampling events. To confirm the Day 7 without internal standard results and to have a useable data set, the system was resampled on Day 9. The internal standard response from the Day 9 samples was acceptable and the sample recoveries were similar to those measured on Day 7. Therefore both the Day 7 and Day 9 sample recoveries were used in calculation of mean measured concentrations.

The Day 14 sampling interval had good sample and internal standard recoveries, however, both control samples appeared to be contaminated with folpet. The measured concentration in these samples was approximately 1/3 of the low concentration. The exposure system was examined after this sampling and the addition of folpet to the control tanks did not appear to be possible, especially since folpet would need to be continuously added in order to maintain any concentration due to its rapid hydrolysis

rate. The input of folpet into the control vessels appeared to be related to a processing error with the exposure samples. To confirm the concentration in the control vessels and the concentrations in the treatment vessels, the system was resampled on Day 16. This sampling did not yield any concentration of folpet in either control vessel, which confirmed that the addition of folpet in the control samples occurred with sample processing. The results of the Day 14 and Day 16 sample recoveries were used in the calculation of mean-measured concentrations.

Analysis of nineteen out of the twenty-one QC samples prepared for this study resulted in measured concentrations which were consistent with the predetermined recovery range of 70 to 120% (Appendix 2) and ranged from 85.5 to 117% of the nominal fortified levels (0.100, 2.00 and 20.0 ng/L). Based on these results, it was demonstrated that satisfactory precision and quality control were maintained during the analysis of exposure solutions. Percent recoveries of two out of the twenty-one QC samples were below the LOQ (i.e., 52.5 and 142%). QC samples can be out of the acceptable range due to a number of factors, some of which are spiking, handling or instrument errors.

The in-life portion of the definitive toxicity test was conducted from January 30 to February 20, 2013.

III. REFERENCES

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OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	09-9411-9250	**Endpoint:**	07d Developmental Stage	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Nonparametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	9.6	>9.6	NA	

Mann-Whitney U Two-Sample Test

Control	vs	C-µg ai/L	Test Stat	Critical	Ties	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	8	NA	1	6	1.0000	Exact	Non-Significant Effect
		0.92	8	NA	1	6	1.0000	Exact	Non-Significant Effect
		9.6	8	NA	1	6	1.0000	Exact	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0	0	3	65540	<0.0001	Significant Effect
Error	0	0	12			
Total	0		15			

07d Developmental Stage Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	54	54	54	54	54	54	0	0.0%	0.0%
0.069		4	54	54	54	54	54	54	0	0.0%	0.0%
0.92		4	54	54	54	54	54	54	0	0.0%	0.0%
9.6		4	54	54	54	54	54	54	0	0.0%	0.0%

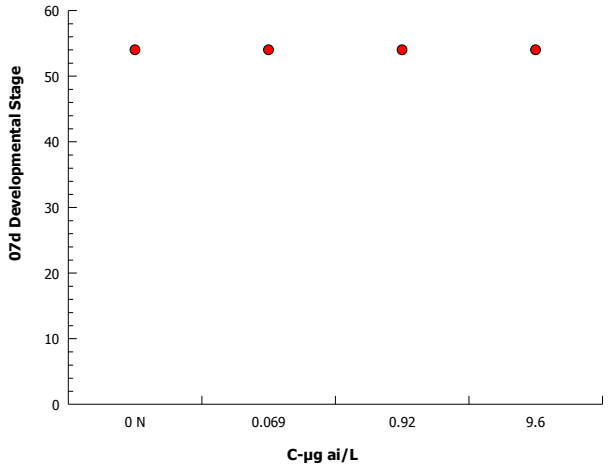
07d Developmental Stage Detail

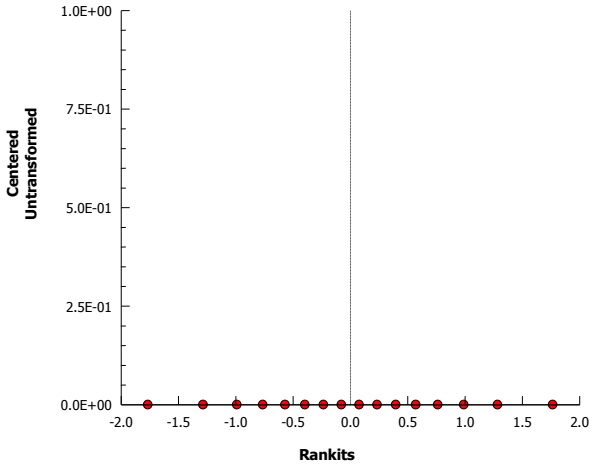
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	54	54	54	54
0.069		54	54	54	54
0.92		54	54	54	54
9.6		54	54	54	54

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	09-9411-9250	**Endpoint:**	07d Developmental Stage	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Nonparametric-Two Sample	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) — **Smithers Viscient**

Analysis ID:	02-1642-2111	**Endpoint:**	07d Developmental Stage	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	Test Result
Untransformed	NA	C <> T	NA	NA	Passes 07d developmental stage

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0	2.447		6	1.0000	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0	0	1	65540	<0.0001	Significant Effect
Error	0	0	6			
Total	0		7			

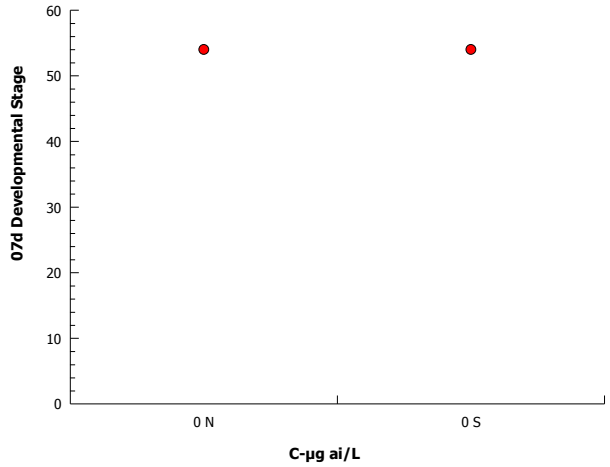
07d Developmental Stage Summary

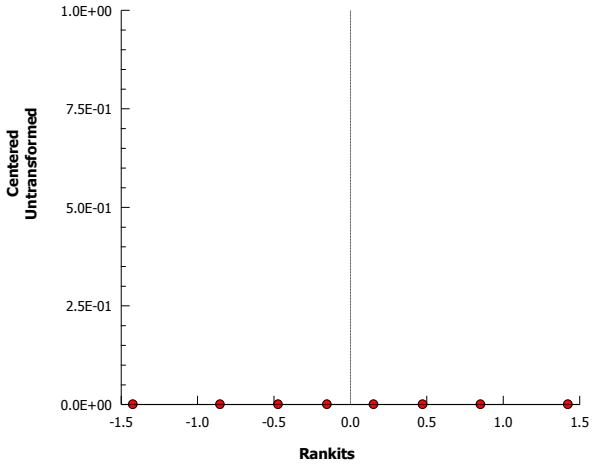
C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	54	54	54	54	54	54	0	0.0%	0.0%
0	Negative Control	4	54	54	54	54	54	54	0	0.0%	0.0%

07d Developmental Stage Detail

C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Solvent Blank	54	54	54	54
0	Negative Control	54	54	54	54

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	07-0929-5297	**Endpoint:**	07d HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	12.8%	9.6	>9.6	NA	

Dunnett Multiple Comparison Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	0.02129	2.683	0.315	6	1.0000	CDF	Non-Significant Effect
		0.92	0.04257	2.683	0.315	6	0.9999	CDF	Non-Significant Effect
		9.6	1.937	2.683	0.315	6	0.1784	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.151925	0.05064165	3	1.836	0.1944	Non-Significant Effect
Error	0.3310499	0.0275875	12			
Total	0.4829749		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	1.537	11.34	0.6738	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9517	0.8408	0.5163	Normal Distribution

07d HLL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	2.467	2.288	2.647	2.445	2.36	2.62	0.05648	4.58%	0.0%
0.069		4	2.465	2.099	2.831	2.46	2.22	2.72	0.1151	9.34%	0.1%
0.92		4	2.462	2.25	2.675	2.455	2.32	2.62	0.06688	5.43%	0.2%
9.6		4	2.24	1.98	2.5	2.225	2.08	2.43	0.08175	7.3%	9.22%

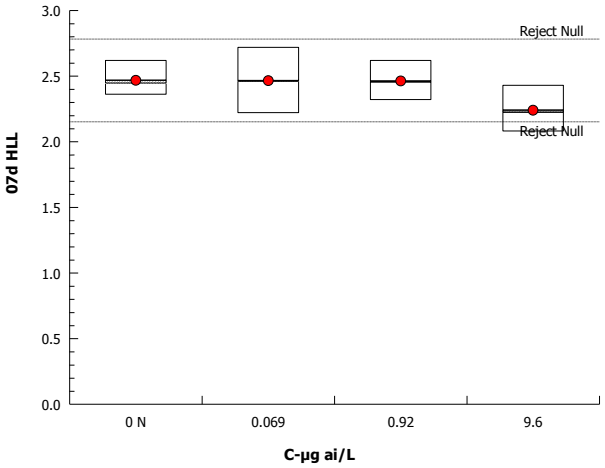
07d HLL Detail

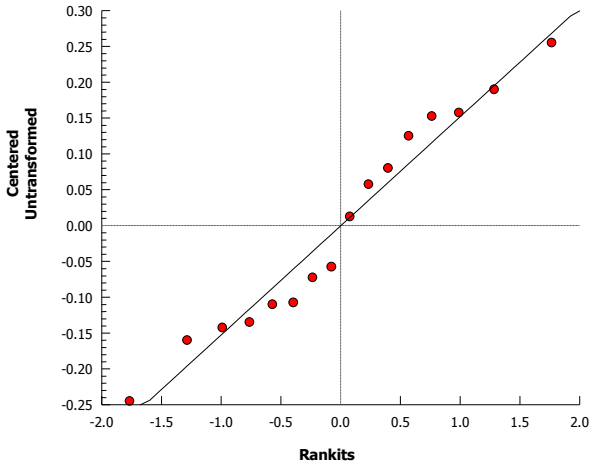
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	2.41	2.48	2.36	2.62
0.069		2.72	2.59	2.33	2.22
0.92		2.39	2.62	2.52	2.32
9.6		2.32	2.08	2.43	2.13

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	07-0929-5297	**Endpoint:**	07d HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	08-5287-9643	**Endpoint:**	07d HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	13.7%	Passes 07d hll

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.4515	2.447	0.339	6	0.6675	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.007812485	0.007812485	1	0.2038	0.6675	Non-Significant Effect
Error	0.229975	0.03832916	6			
Total	0.2377874		7			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	5.008	47.47	0.2187	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9581	0.6451	0.7916	Normal Distribution

07d HLL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	2.405	2.003	2.807	2.35	2.19	2.73	0.1264	10.51%	0.0%
0	Negative Control	4	2.467	2.288	2.647	2.445	2.36	2.62	0.05648	4.58%	-2.6%

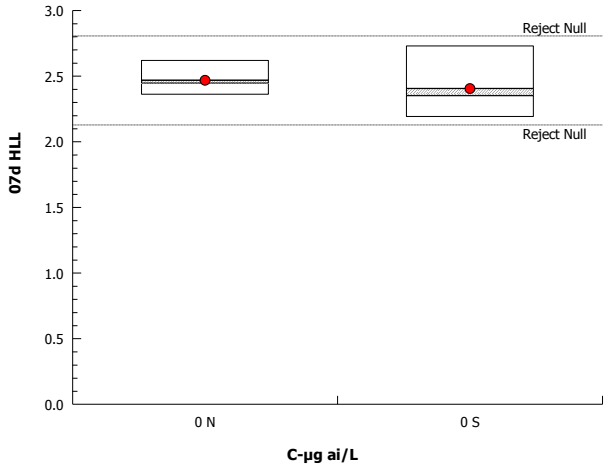
07d HLL Detail

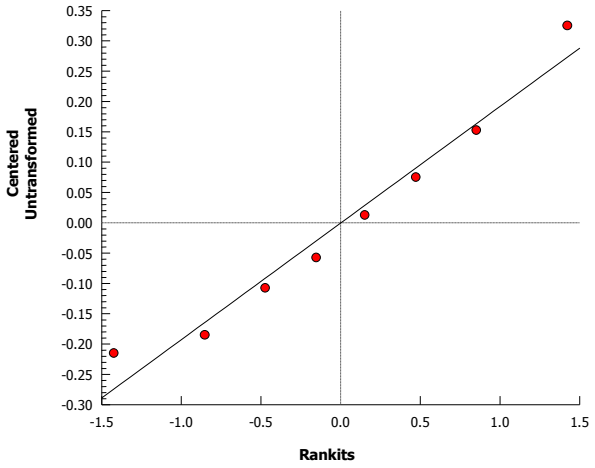
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Solvent Blank	2.19	2.48	2.73	2.22
0	Negative Control	2.41	2.48	2.36	2.62

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	08-5287-9643	**Endpoint:**	07d HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	17-2435-7396	**Endpoint:**	07d HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:48	**Analysis:**	Nonparametric-Control vs Ord. Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	NOEL	LOEL	TOEL	TU
Untransformed	NA	C > T	NA	NA	0.92	9.6	2.972	

Jonckheere-Terpstra Step-Down Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	9	NA		-2	0.4429	Exact	Non-Significant Effect
		0.92	0.2202	1.645	1	-2	0.4129	Asymp	Non-Significant Effect
		9.6*	1.685	1.645	2	-2	0.0460	Asymp	Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.151925	0.05064165	3	1.836	0.1944	Non-Significant Effect
Error	0.3310499	0.0275875	12			
Total	0.4829749		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	1.537	11.34	0.6738	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9517	0.8408	0.5163	Normal Distribution

07d HLL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	2.467	2.288	2.647	2.445	2.36	2.62	0.05648	4.58%	0.0%
0.069		4	2.465	2.099	2.831	2.46	2.22	2.72	0.1151	9.34%	0.1%
0.92		4	2.462	2.25	2.675	2.455	2.32	2.62	0.06688	5.43%	0.2%
9.6		4	2.24	1.98	2.5	2.225	2.08	2.43	0.08175	7.3%	9.22%

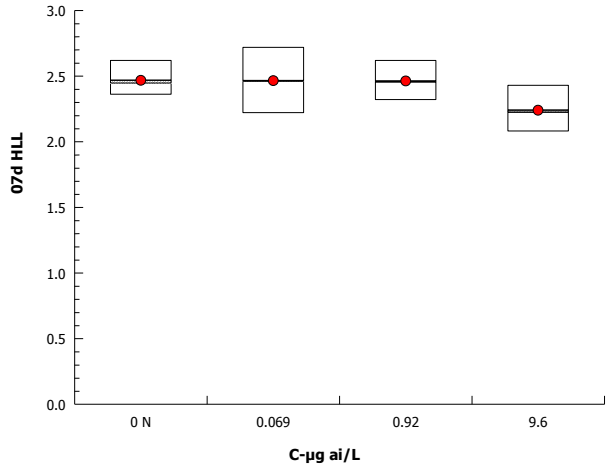
07d HLL Detail

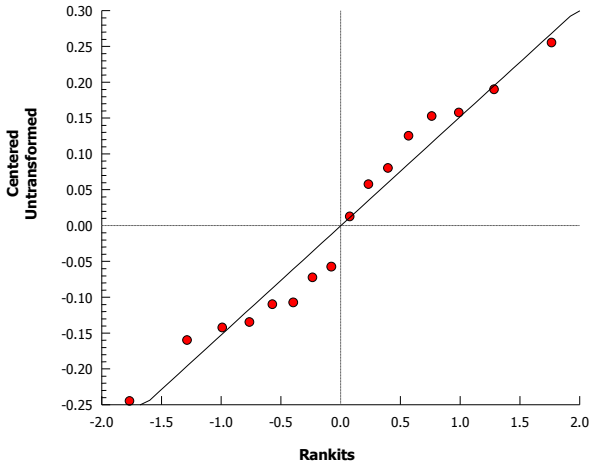
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	2.41	2.48	2.36	2.62
0.069		2.72	2.59	2.33	2.22
0.92		2.39	2.62	2.52	2.32
9.6		2.32	2.08	2.43	2.13

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	17-2435-7396	**Endpoint:**	07d HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:48	**Analysis:**	Nonparametric-Control vs Ord. Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	02-2300-1371	**Endpoint:**	07d HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:49	**Analysis:**	Parametric-Control vs Ord.Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C > T	NA	NA	9.06%	0.92	9.6	2.972	

Williams Multiple Comparison Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	0.02129	1.782	0.209	6	>0.05	CDF	Non-Significant Effect
		0.92	0.04257	1.873	0.22	6	>0.05	CDF	Non-Significant Effect
		9.6*	1.937	1.903	0.224	6	<0.05	CDF	Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.151925	0.05064165	3	1.836	0.1944	Non-Significant Effect
Error	0.3310499	0.0275875	12			
Total	0.4829749		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	1.537	11.34	0.6738	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9517	0.8408	0.5163	Normal Distribution

07d HLL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	2.467	2.288	2.647	2.445	2.36	2.62	0.05648	4.58%	0.0%
0.069		4	2.465	2.099	2.831	2.46	2.22	2.72	0.1151	9.34%	0.1%
0.92		4	2.462	2.25	2.675	2.455	2.32	2.62	0.06688	5.43%	0.2%
9.6		4	2.24	1.98	2.5	2.225	2.08	2.43	0.08175	7.3%	9.22%

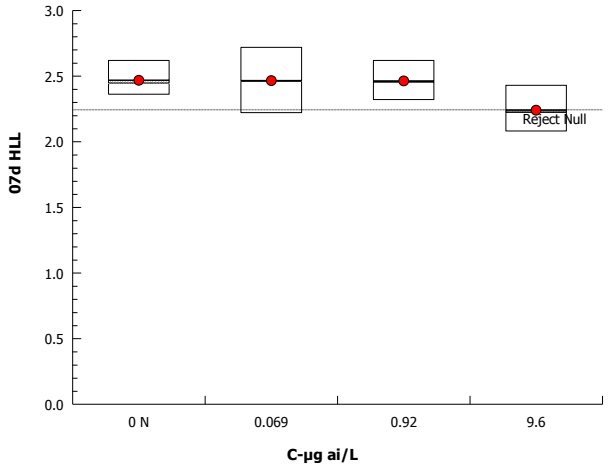
07d HLL Detail

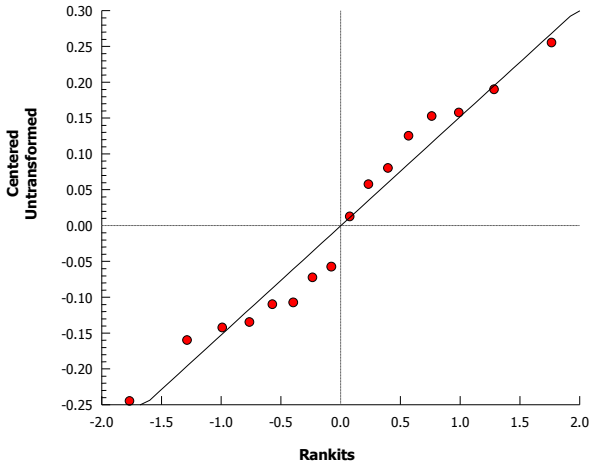
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	2.41	2.48	2.36	2.62
0.069		2.72	2.59	2.33	2.22
0.92		2.39	2.62	2.52	2.32
9.6		2.32	2.08	2.43	2.13

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	02-2300-1371	**Endpoint:**	07d HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:49	**Analysis:**	Parametric-Control vs Ord.Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	01-4284-4598	**Endpoint:**	07d Normalized HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	6.72%	9.6	>9.6	NA	

Dunnett Multiple Comparison Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	0.4622	2.683	0.009	6	0.9377	CDF	Non-Significant Effect
		0.92	1.233	2.683	0.009	6	0.4910	CDF	Non-Significant Effect
		9.6	1.772	2.683	0.009	6	0.2314	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.0002051874	6.839581E-05	3	3.247	0.0601	Non-Significant Effect
Error	0.0002527501	2.106251E-05	12			
Total	0.0004579376		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	0.07617	11.34	0.9945	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9456	0.8408	0.4239	Normal Distribution

07d Normalized HLL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	0.1295	0.1221	0.1369	0.1285	0.125	0.136	0.002327	3.59%	0.0%
0.069		4	0.128	0.1201	0.1359	0.1285	0.122	0.133	0.002483	3.88%	1.16%
0.92		4	0.1255	0.1188	0.1322	0.126	0.12	0.13	0.002102	3.35%	3.09%
9.6		4	0.1352	0.1281	0.1424	0.1365	0.129	0.139	0.00225	3.33%	-4.44%

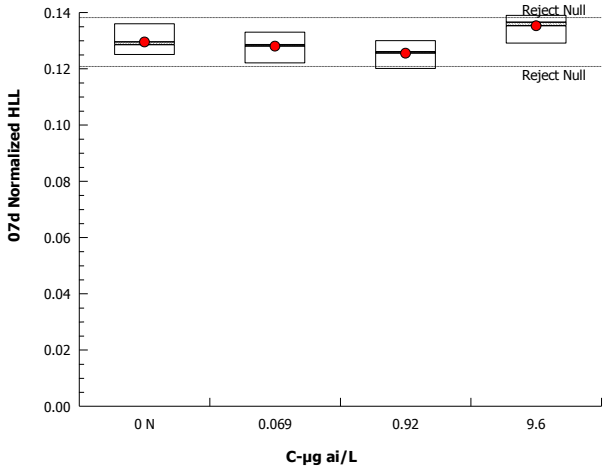
07d Normalized HLL Detail

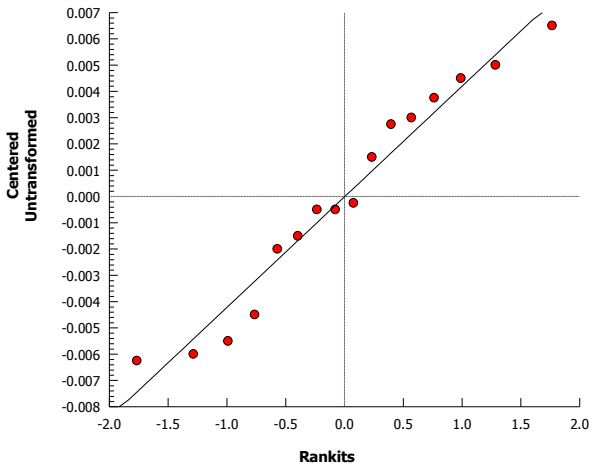
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	0.128	0.129	0.125	0.136
0.069		0.133	0.131	0.126	0.122
0.92		0.125	0.13	0.127	0.12
9.6		0.129	0.135	0.139	0.138

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	01-4284-4598	**Endpoint:**	07d Normalized HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	02-8765-4746	**Endpoint:**	07d Normalized HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	5.43%	Passes 07d normalized hll

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	1.567	2.447	0.007	6	0.1682	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	4.050003E-05	4.050003E-05	1	2.455	0.1682	Non-Significant Effect
Error	9.900001E-05	0.0000165	6			
Total	0.0001395		7			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	1.912	47.47	0.6079	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.8477	0.6451	0.0902	Normal Distribution

07d Normalized HLL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	0.125	0.1196	0.1304	0.1235	0.123	0.13	0.001683	2.69%	0.0%
0	Negative Control	4	0.1295	0.1221	0.1369	0.1285	0.125	0.136	0.002327	3.59%	-3.6%

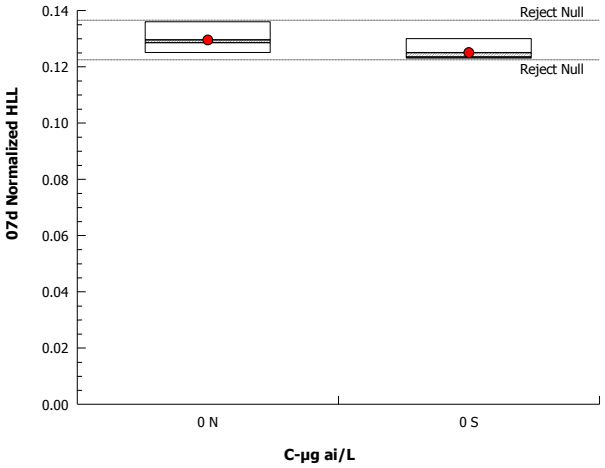
07d Normalized HLL Detail

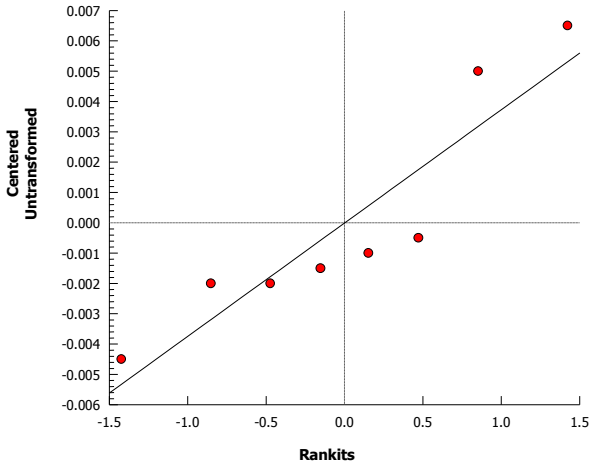
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Solvent Blank	0.123	0.124	0.13	0.123
0	Negative Control	0.128	0.129	0.125	0.136

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	02-8765-4746	**Endpoint:**	07d Normalized HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	17-9185-0261	**Endpoint:**	07d SVL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	8.76%	0.92	9.6	2.972	

Dunnett Multiple Comparison Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	0.2016	2.683	1.664	6	0.9940	CDF	Non-Significant Effect
		0.92	0.7257	2.683	1.664	6	0.8109	CDF	Non-Significant Effect
		9.6*	3.951	2.683	1.664	6	0.0051	CDF	Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	21.36688	7.122294	3	9.262	0.0019	Significant Effect
Error	9.227503	0.7689586	12			
Total	30.59439		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	5.982	11.34	0.1125	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.961	0.8408	0.6793	Normal Distribution

07d SVL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	19	18.53	19.47	19	18.7	19.3	0.1472	1.55%	0.0%
0.069		4	19.13	17.57	20.68	19.05	18.2	20.2	0.4888	5.11%	-0.66%
0.92		4	19.45	18.67	20.23	19.45	18.9	20	0.2466	2.54%	-2.37%
9.6		4	16.55	14.42	18.68	16.45	15.4	17.9	0.669	8.08%	12.89%

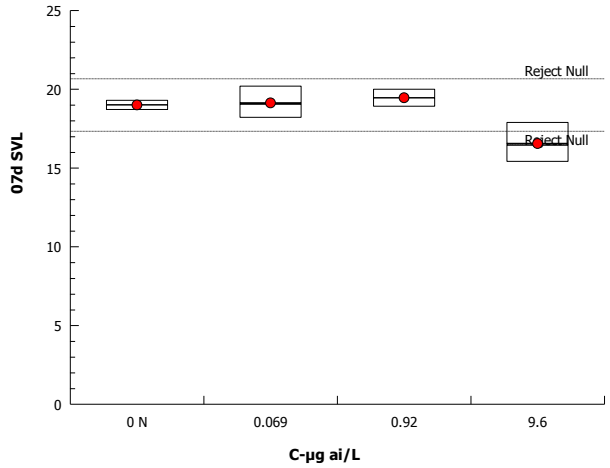
07d SVL Detail

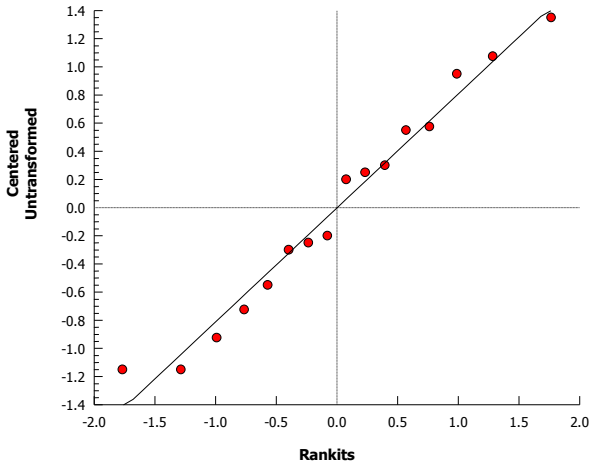
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	18.8	19.2	18.7	19.3
0.069		20.2	19.7	18.4	18.2
0.92		18.9	20	19.7	19.2
9.6		17.9	15.4	17.5	15.4

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	17-9185-0261	**Endpoint:**	07d SVL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	07-5181-0358	**Endpoint:**	07d SVL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	9.76%	Passes 07d svl

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.297	2.447	1.854	6	0.7765	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.1012499	0.1012499	1	0.0882	0.7765	Non-Significant Effect
Error	6.887499	1.147917	6			
Total	6.988749		7			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	25.49	47.47	0.0246	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.961	0.6451	0.8194	Normal Distribution

07d SVL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	19.22	16.86	21.59	19	17.9	21	0.7432	7.73%	0.0%
0	Negative Control	4	19	18.53	19.47	19	18.7	19.3	0.1472	1.55%	1.17%

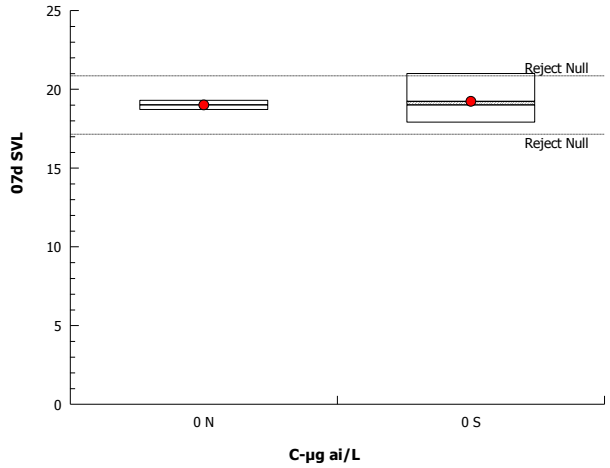
07d SVL Detail

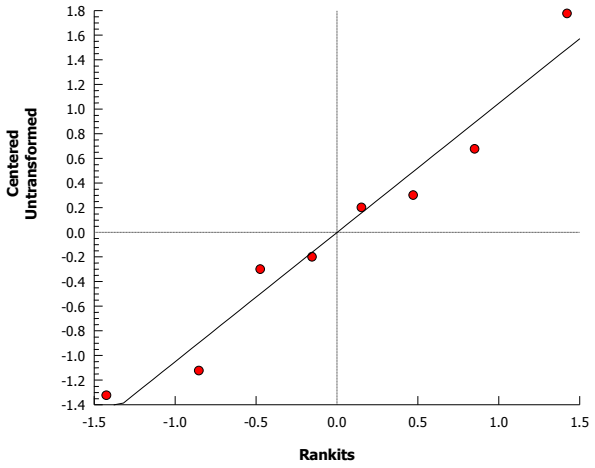
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Solvent Blank	17.9	19.9	21	18.1
0	Negative Control	18.8	19.2	18.7	19.3

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	07-5181-0358	**Endpoint:**	07d SVL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	18-1275-4718	**Endpoint:**	07d Wet Weight	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	23.3%	0.92	9.6	2.972	

Dunnett Multiple Comparison Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	0.2617	2.683	0.1	6	0.9872	CDF	Non-Significant Effect
		0.92	0.8724	2.683	0.1	6	0.7205	CDF	Non-Significant Effect
		9.6*	3.456	2.683	0.1	6	0.0124	CDF	Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.06342925	0.02114308	3	7.618	0.0041	Significant Effect
Error	0.0333065	0.002775542	12			
Total	0.09673575		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	2.821	11.34	0.4200	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9365	0.8408	0.3086	Normal Distribution

07d Wet Weight Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	0.4285	0.3872	0.4698	0.428	0.398	0.46	0.01298	6.06%	0.0%
0.069		4	0.4382	0.3388	0.5377	0.4315	0.379	0.511	0.03123	14.25%	-2.28%
0.92		4	0.461	0.3982	0.5238	0.4635	0.417	0.5	0.01973	8.56%	-7.59%
9.6		4	0.2997	0.1876	0.4119	0.2945	0.232	0.378	0.03525	23.52%	30.05%

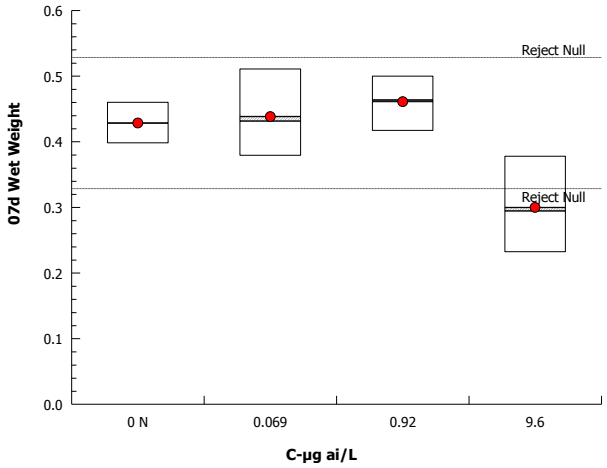
07d Wet Weight Detail

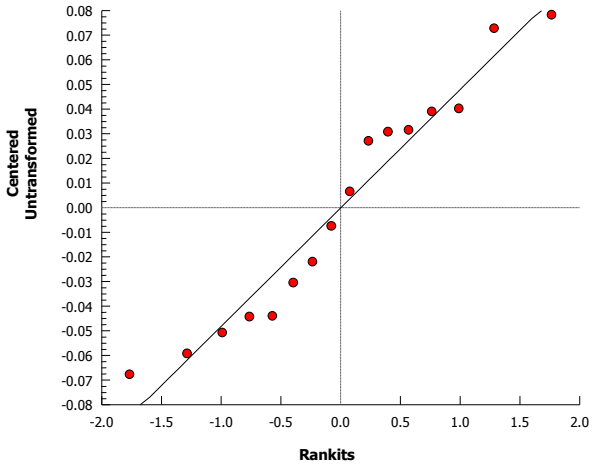
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	0.421	0.435	0.398	0.46
0.069		0.511	0.469	0.379	0.394
0.92		0.417	0.5	0.488	0.439
9.6		0.378	0.232	0.34	0.249

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	18-1275-4718	**Endpoint:**	07d Wet Weight	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	03-6676-0414	**Endpoint:**	07d Wet Weight	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	28.3%	Passes 07d wet weight

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.2066	2.447	0.121	6	0.8432	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.0002101248	0.0002101248	1	0.04267	0.8432	Non-Significant Effect
Error	0.02954575	0.004924291	6			
Total	0.02975587		7			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	13.62	47.47	0.0595	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9837	0.6451	0.9787	Normal Distribution

07d Wet Weight Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	0.4387	0.2863	0.5912	0.428	0.347	0.552	0.04789	21.83%	0.0%
0	Negative Control	4	0.4285	0.3872	0.4698	0.428	0.398	0.46	0.01298	6.06%	2.34%

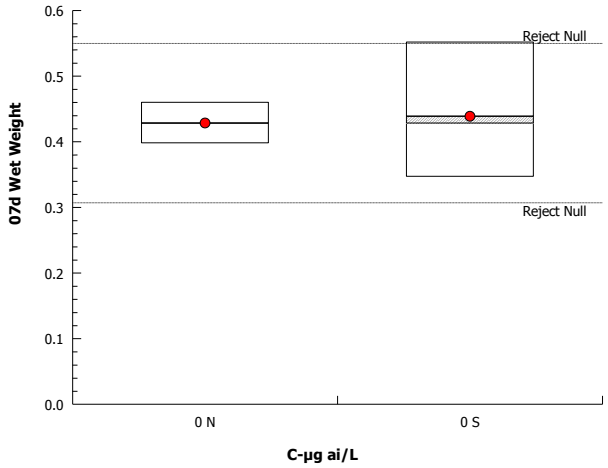
07d Wet Weight Detail

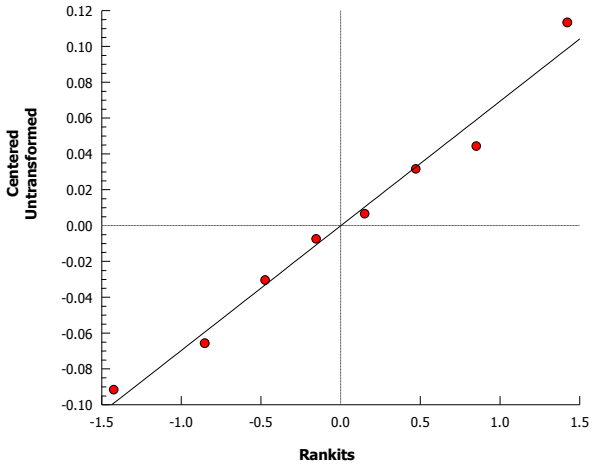
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Solvent Blank	0.347	0.483	0.552	0.373
0	Negative Control	0.421	0.435	0.398	0.46

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	03-6676-0414	**Endpoint:**	07d Wet Weight	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	04-4003-8086	**Endpoint:**	21d Developmental Stage	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	2.18%	9.6	>9.6	NA	

Dunnett Multiple Comparison Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	2.089	2.683	1.284	6	0.1393	CDF	Non-Significant Effect
		0.92	1.567	2.683	1.284	6	0.3142	CDF	Non-Significant Effect
		9.6	1.567	2.683	1.284	6	0.3142	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	2.25	0.75	3	1.636	0.2331	Non-Significant Effect
Error	5.5	0.4583333	12			
Total	7.75		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	1.219	11.34	0.7484	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.8772	0.8408	0.0352	Normal Distribution

21d Developmental Stage Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	59	57.7	60.3	59	58	60	0.4082	1.38%	0.0%
0.069		4	58	56.7	59.3	58	57	59	0.4082	1.41%	1.7%
0.92		4	58.25	57.45	59.05	58	58	59	0.25	0.86%	1.27%
9.6		4	58.25	57.45	59.05	58	58	59	0.25	0.86%	1.27%

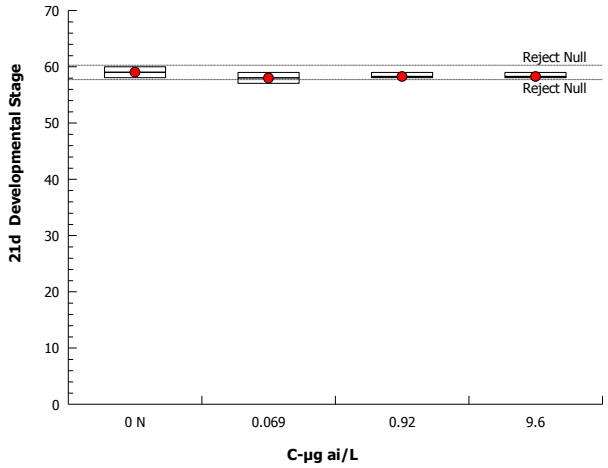
21d Developmental Stage Detail

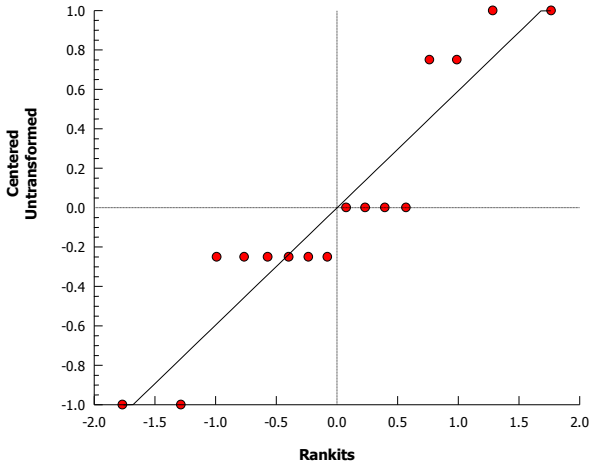
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	58	59	60	59
0.069		59	58	57	58
0.92		58	59	58	58
9.6		59	58	58	58

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	04-4003-8086	**Endpoint:**	21d Developmental Stage	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	07-8437-7858	**Endpoint:**	21d Developmental Stage	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	1.99%	Passes 21d developmental stage

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	1.567	2.447	1.171	6	0.1682	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	1.125	1.125	1	2.455	0.1682	Non-Significant Effect
Error	2.75	0.4583333	6			
Total	3.875		7			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	2.667	47.47	0.4419	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9128	0.6451	0.3739	Normal Distribution

21d Developmental Stage Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	58.25	57.45	59.05	58	58	59	0.25	0.86%	0.0%
0	Negative Control	4	59	57.7	60.3	59	58	60	0.4082	1.38%	-1.29%

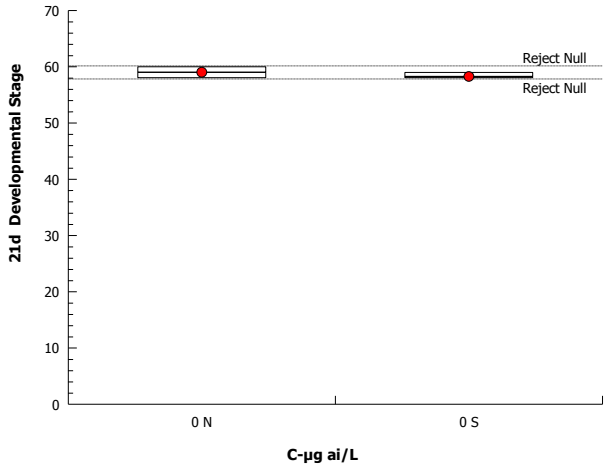
21d Developmental Stage Detail

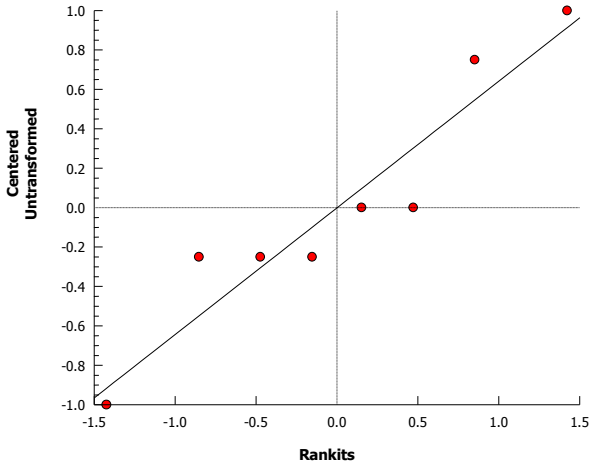
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Solvent Blank	58	58	59	58
0	Negative Control	58	59	60	59

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	07-8437-7858	**Endpoint:**	21d Developmental Stage	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	20-9625-6810	**Endpoint:**	21d No LS HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	24.9%	9.6	>9.6	NA	

Dunnett Multiple Comparison Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	0.4173	2.683	3.343	6	0.9526	CDF	Non-Significant Effect
		0.92	0.8706	2.683	3.343	6	0.7217	CDF	Non-Significant Effect
		9.6	1.434	2.683	3.343	6	0.3782	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	17.07662	5.692206	3	1.833	0.1949	Non-Significant Effect
Error	37.27237	3.106031	12			
Total	54.34899		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	1.907	11.34	0.5919	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9241	0.8408	0.1967	Normal Distribution

21d No LS HLL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	13.41	11.96	14.86	13.29	12.48	14.58	0.4559	6.8%	0.0%
0.069		4	12.89	10.17	15.61	12.77	10.94	15.08	0.8545	13.26%	3.88%
0.92		4	14.49	11.01	17.98	14.24	12.09	17.4	1.096	15.12%	-8.09%
9.6		4	11.62	8.493	14.75	10.93	10.13	14.49	0.9835	16.92%	13.33%

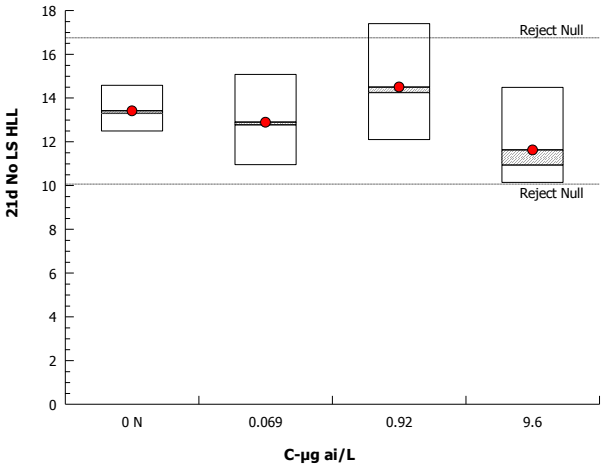
21d No LS HLL Detail

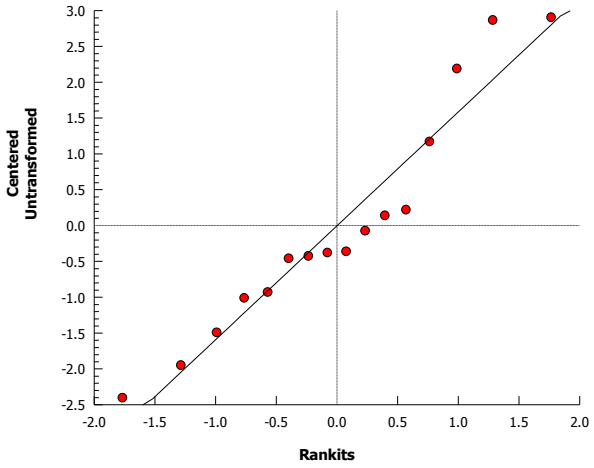
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	12.48	14.58	12.95	13.63
0.069		15.08	13.03	10.94	12.51
0.92		14.07	17.4	12.09	14.42
9.6		14.49	10.13	11.26	10.61

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	20-9625-6810	**Endpoint:**	21d No LS HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:45	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	20-4941-8995	**Endpoint:**	21d No LS HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	23.2%	Passes 21d no ls hll

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.1965	2.447	3.114	6	0.8507	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.125	0.125	1	0.0386	0.8507	Non-Significant Effect
Error	19.4324	3.238734	6			
Total	19.5574		7			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	6.792	47.47	0.1499	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9826	0.6451	0.9747	Normal Distribution

21d No LS HLL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	13.16	9.379	16.94	12.95	10.78	15.95	1.188	18.06%	0.0%
0	Negative Control	4	13.41	11.96	14.86	13.29	12.48	14.58	0.4559	6.8%	-1.9%

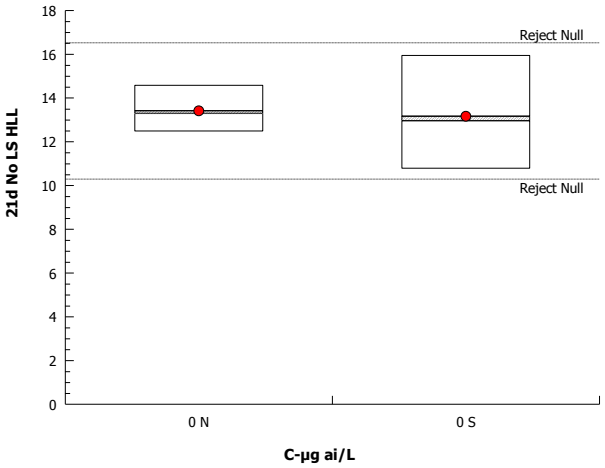
21d No LS HLL Detail

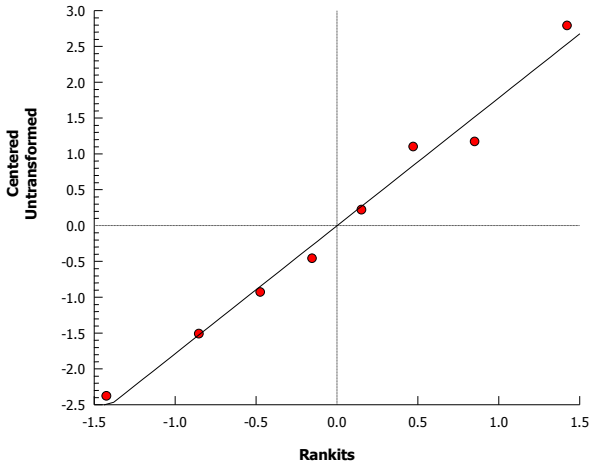
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Solvent Blank	14.26	11.65	15.95	10.78
0	Negative Control	12.48	14.58	12.95	13.63

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	20-4941-8995	**Endpoint:**	21d No LS HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	19-0120-4412	**Endpoint:**	21d No LS Normalized HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:44	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	21.7%	9.6	>9.6	NA	

Dunnett Multiple Comparison Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	0.7429	2.683	0.123	6	0.8009	CDF	Non-Significant Effect
		0.92	0.366	2.683	0.123	6	0.9669	CDF	Non-Significant Effect
		9.6	1.939	2.683	0.123	6	0.1779	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.0260165	0.008672168	3	2.07	0.1578	Non-Significant Effect
Error	0.0502755	0.004189624	12			
Total	0.076292		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	3.429	11.34	0.3301	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9244	0.8408	0.1984	Normal Distribution

21d No LS Normalized HLL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	0.567	0.5295	0.6045	0.566	0.541	0.595	0.01177	4.15%	0.0%
0.069		4	0.533	0.4235	0.6425	0.5265	0.457	0.622	0.0344	12.91%	6.0%
0.92		4	0.5838	0.4535	0.714	0.577	0.492	0.689	0.04093	14.02%	-2.95%
9.6		4	0.4782	0.3683	0.5882	0.463	0.412	0.575	0.03454	14.44%	15.65%

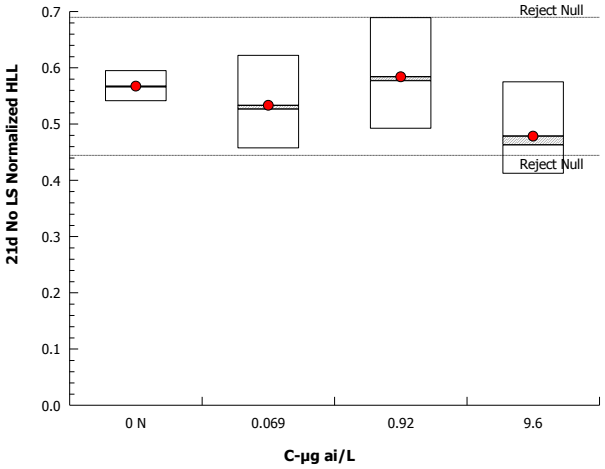
21d No LS Normalized HLL Detail

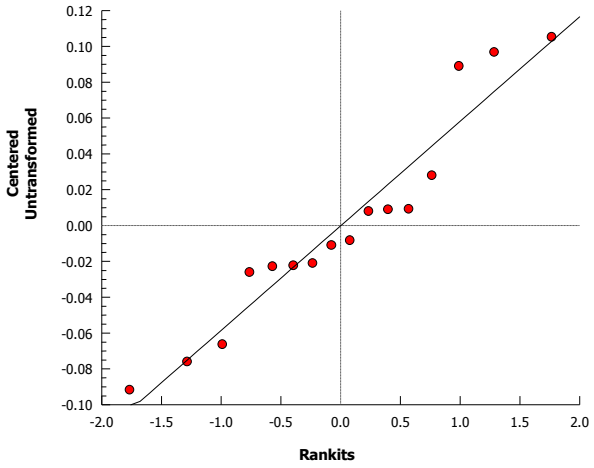
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	0.541	0.595	0.556	0.576
0.069		0.622	0.541	0.457	0.512
0.92		0.593	0.689	0.492	0.561
9.6		0.575	0.412	0.47	0.456

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	19-0120-4412	**Endpoint:**	21d No LS Normalized HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:44	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	15-5549-6334	**Endpoint:**	21d No LS Normalized HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	28.3%	Passes 21d no ls normalized hll

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.03814	2.447	0.160	6	0.9708	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	1.249998E-05	1.249998E-05	1	0.001454	0.9708	Non-Significant Effect
Error	0.051571	0.008595168	6			
Total	0.0515835		7			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	30.03	47.47	0.0195	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9416	0.6451	0.6273	Normal Distribution

21d No LS Normalized HLL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	0.5695	0.3643	0.7747	0.5435	0.459	0.732	0.06449	22.65%	0.0%
0	Negative Control	4	0.567	0.5295	0.6045	0.566	0.541	0.595	0.01177	4.15%	0.44%

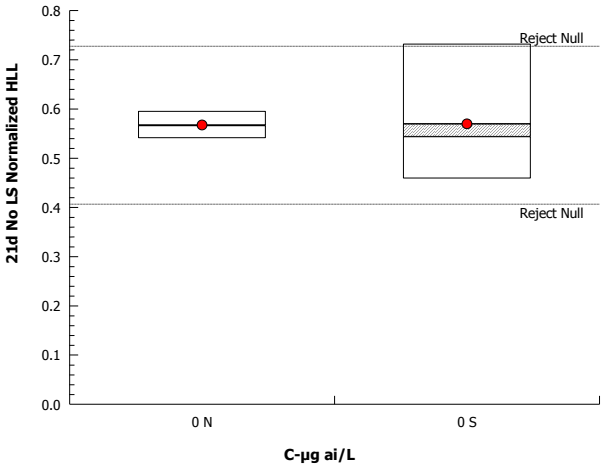
21d No LS Normalized HLL Detail

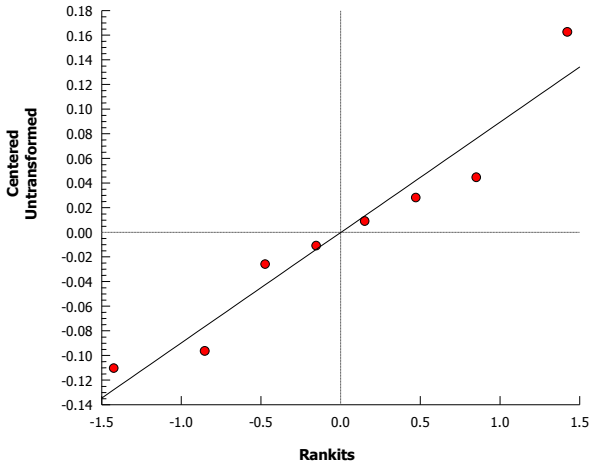
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Solvent Blank	0.614	0.473	0.732	0.459
0	Negative Control	0.541	0.595	0.556	0.576

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	15-5549-6334	**Endpoint:**	21d No LS Normalized HLL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	20-3407-9679	**Endpoint:**	21d No LS SVL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:44	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	5.31%	9.6	>9.6	NA	

Dunnett Multiple Comparison Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	1.5	2.683	1.265	6	0.3455	CDF	Non-Significant Effect
		0.92	2.581	2.683	1.265	6	0.0599	CDF	Non-Significant Effect
		9.6	0.5911	2.683	1.265	6	0.8833	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	3.381734	1.127244	3	2.536	0.1059	Non-Significant Effect
Error	5.3336	0.4444667	12			
Total	8.715334		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	4.48	11.34	0.2141	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9573	0.8408	0.6139	Normal Distribution

21d No LS SVL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	23.81	22.82	24.8	23.6	23.34	24.7	0.3099	2.6%	0.0%
0.069		4	24.52	24.2	24.84	24.52	24.31	24.72	0.1008	0.82%	-2.97%
0.92		4	25.03	23.68	26.37	25.05	24.16	25.85	0.4234	3.38%	-5.11%
9.6		4	24.09	22.82	25.36	24.17	23.06	24.95	0.3988	3.31%	-1.17%

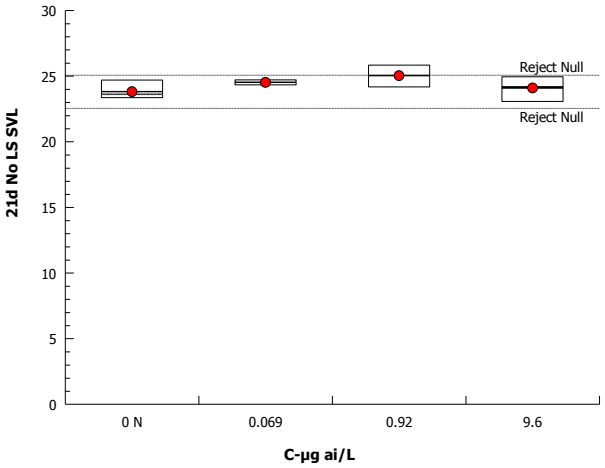
21d No LS SVL Detail

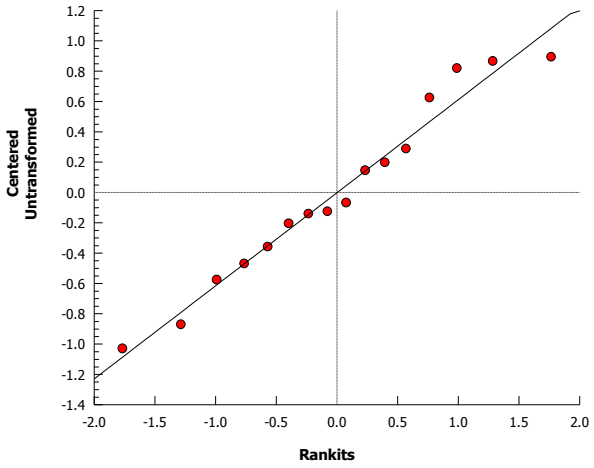
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	23.45	24.7	23.34	23.74
0.069		24.66	24.31	24.38	24.72
0.92		24.45	25.65	24.16	25.85
9.6		24.95	24.38	23.96	23.06

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	20-3407-9679	**Endpoint:**	21d No LS SVL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:44	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	12-8693-7448	**Endpoint:**	21d No LS SVL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	5.57%	Passes 21d no ls svl

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.6367	2.447	1.325	6	0.5478	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.2377405	0.2377405	1	0.4054	0.5478	Non-Significant Effect
Error	3.518819	0.5864698	6			
Total	3.756559		7			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	2.054	47.47	0.5694	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9456	0.6451	0.6669	Normal Distribution

21d No LS SVL Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	23.47	22.05	24.88	23.44	22.41	24.57	0.4441	3.79%	0.0%
0	Negative Control	4	23.81	22.82	24.8	23.6	23.34	24.7	0.3099	2.6%	-1.47%

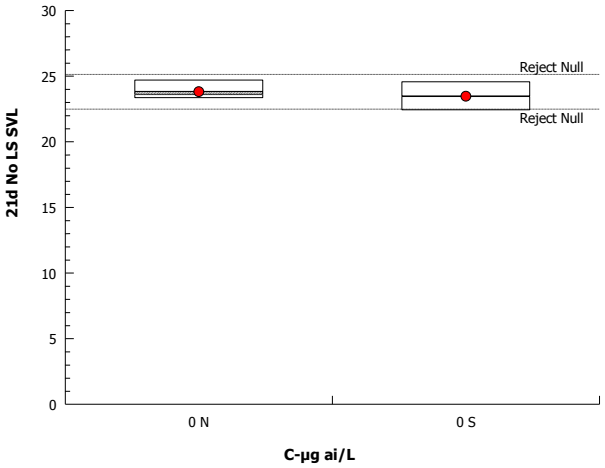
21d No LS SVL Detail

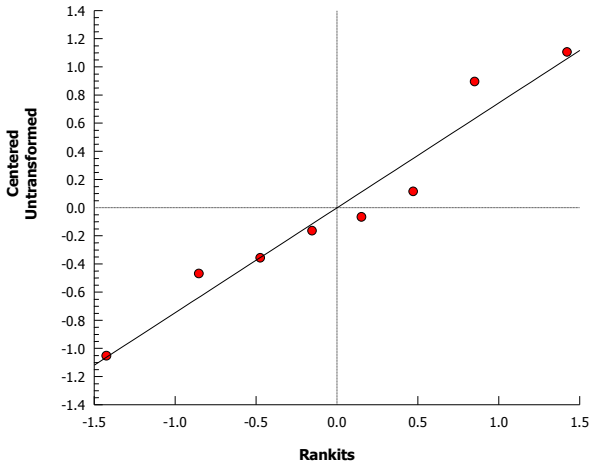
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Solvent Blank	23.58	24.57	22.41	23.3
0	Negative Control	23.45	24.7	23.34	23.74

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	12-8693-7448	**Endpoint:**	21d No LS SVL	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	16-6412-0912	**Endpoint:**	21d No LS Wet Weight	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:44	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	19.5%	9.6	>9.6	NA	

Dunnett Multiple Comparison Test

Control	vs	C-µg ai/L	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.069	0.4499	2.683	0.185	6	0.9420	CDF	Non-Significant Effect
		0.92	1.827	2.683	0.185	6	0.2123	CDF	Non-Significant Effect
		9.6	0.8498	2.683	0.185	6	0.7349	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.07128694	0.02376231	3	2.503	0.1089	Non-Significant Effect
Error	0.1139252	0.009493763	12			
Total	0.1852121		15			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	4.048	11.34	0.2563	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9478	0.8408	0.4563	Normal Distribution

21d No LS Wet Weight Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	0.9461	0.7662	1.126	0.9298	0.8262	1.099	0.05653	11.95%	0.0%
0.069		4	0.9771	0.9205	1.034	0.9757	0.935	1.022	0.01778	3.64%	-3.28%
0.92		4	1.072	0.9483	1.196	1.076	0.994	1.142	0.03886	7.25%	-13.31%
9.6		4	0.8875	0.6747	1.1	0.8618	0.7536	1.073	0.06687	15.07%	6.19%

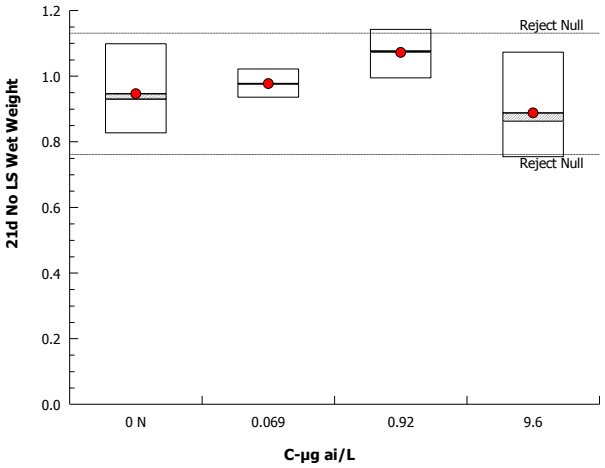
21d No LS Wet Weight Detail

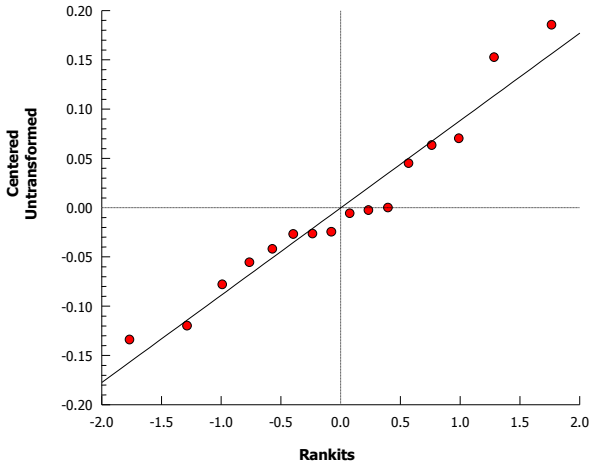
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	0.9402	1.099	0.8262	0.9195
0.069		1.022	0.9745	0.935	0.9769
0.92		1.016	1.135	0.994	1.142
9.6		1.073	0.8629	0.8607	0.7536

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	16-6412-0912	**Endpoint:**	21d No LS Wet Weight	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:44	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes

Graphics





OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	04-1344-0051	**Endpoint:**	21d No LS Wet Weight	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	08-2562-8980	**Test Type:**	EDSP AMA Tier 1	**Analyst:**	
Start Date:	30 Jan-13	**Protocol:**	OCSPP 890.1100 Tier I AMA	**Diluent:**	Laboratory Water
Ending Date:	20 Feb-13	**Species:**	Xenopus laevis	**Brine:**	Not Applicable
Duration:	21d 0h	**Source:**	Nasco, Fort Atkinson, WI	**Age:**	
Sample ID:	03-5444-1785	**Code:**	49140601	**Client:**	EPA OCSPP EFED
Sample Date:	30 Jan-13	**Material:**	Folpet	**Project:**	Fungicide
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Batch Note: Flow-through study; MRID # 49140601
Sample Note: MRID # 49140601

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	17.3%	Passes 21d no ls wet weight

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	1.061	2.447	0.163	6	0.3295	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.01003236	0.01003236	1	1.126	0.3295	Non-Significant Effect
Error	0.05346991	0.008911652	6			
Total	0.06350227		7			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	2.535	47.47	0.4650	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.9791	0.6451	0.9581	Normal Distribution

21d No LS Wet Weight Summary

C-µg ai/L	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	0.8753	0.7623	0.9883	0.8744	0.8004	0.9519	0.0355	8.11%	0.0%
0	Negative Control	4	0.9461	0.7662	1.126	0.9298	0.8262	1.099	0.05653	11.95%	-8.09%

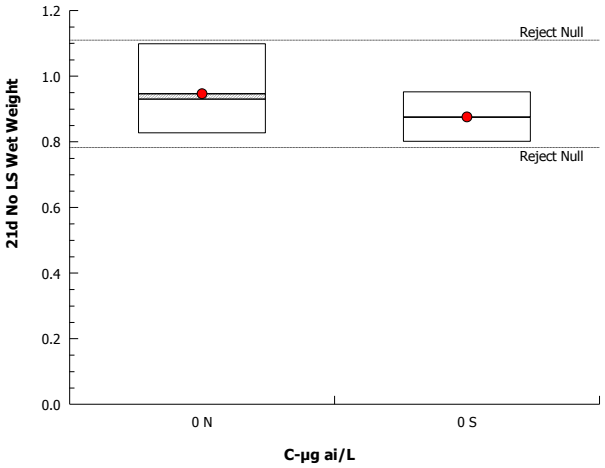
21d No LS Wet Weight Detail

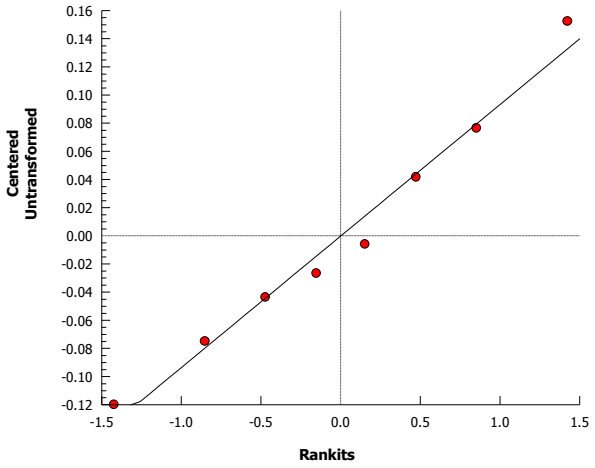
C-µg ai/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Solvent Blank	0.9171	0.9519	0.8317	0.8004
0	Negative Control	0.9402	1.099	0.8262	0.9195

OPPTS 890.1100 EDSP Amphibian Metamorphosis (Frog) **Smithers Viscient**

Analysis ID:	04-1344-0051	**Endpoint:**	21d No LS Wet Weight	**CETIS Version:**	CETISv1.8.7
Analyzed:	15 Aug-13 12:47	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes

Graphics





DATA EVALUATION RECORD

FOLPET

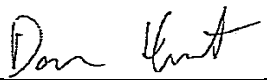
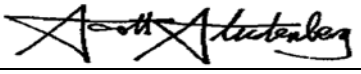
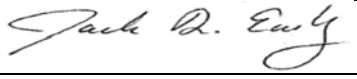
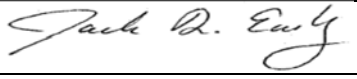
Study Type: OCSPP 890.1150, Androgen Receptor Binding (Rat Prostate Cytosol)

EPA Contract No. EP10H001452
Task Assignment No. 3-06-2012

(Revisions to MRID 48616901 to include Saturation binding data;
Main study was originally reviewed under TA 2-41-2012)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road
Building 100, Suite B
Durham, NC 27713

Primary Reviewer:	Signature:	
Daniel R. Hunt, M.S.	Date:	5/08/2012
Secondary Reviewer:	Signature:	
Scott D. Studenberg, Ph.D., D.A.B.T.	Date:	7/16/2012
Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	7/26/2012
Quality Assurance:	Signature:	
Jack D. Early, M.S.	Date:	7/26/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Ayaad Assaad, D.V.M., Ph.D. **Signature:** [signature] **Date:** 6/2/2015
Health Effects Division, Office of Pesticide Programs
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:** [signature] **Date:** 6/10/15
Health Effects Division, Office of Pesticide Programs

DATA EVALUATION RECORD

STUDY TYPE: Androgen Receptor Binding (Rat Prostate Cytosol); OCSPP 890.1150

PC CODE: 081601 **DP BARCODE:** D398813

TXR#: 0055725 **CAS No.:** 133-07-3

TEST MATERIAL (PURITY): Folpet (94.5% a.i.)

SYNONYMS: 2-[(trichloromethyl)thio]-1H-isoindole-1,3(2H)-dione, Folpan Technical

CITATION: Willoughby, J.A. (2012). Folpet: Androgen Receptor Binding Assay Using Rat Prostate Cytosol. CeeTox, Inc., Kalamazoo, MI. Laboratory Study No: 9141V-100357ARB, January 5, 2012. MRID 48616901. Unpublished.

SPONSOR: Makhteshim Chemical Works Ltd. c/o Makhteshim Agan of North America, Inc., 4515 Falls of Neuse Road, Suite 300, Raleigh, NC

TEST ORDER #: EDSP 081601-175

EXECUTIVE SUMMARY: In an androgen receptor (AR) binding assay (MRID 48616901), ventral prostate cytosol from Sprague Dawley rats was used as the source of AR to conduct a competitive binding experiment, which measured the binding of a single concentration of [^{3}H]-R1881 (1 nM) in the presence of increasing concentrations of folpet (94.5% purity, Lot# 00138518). Folpet was tested at concentrations from 10^{-10} to 10^{-3} M in Run 1 or 10^{-11} to 10^{-4} M in Runs 2 and 3. DMSO was used as the solvent vehicle at a final assay concentration of approximately 3.2%. A total of three runs were performed, and each run included dexamethasone as a weak positive control, and R1881 as the ligand reference standard.

A saturation binding experiment was conducted to demonstrate that the AR in the rat prostate cytosol was present in reasonable numbers and was functioning with appropriate affinity for the radiolabeled reference androgen (R1881) prior to routinely conducting AR competitive binding experiments. Saturation binding data were not originally provided in the study report; however, summarized saturation binding data (MRID 48843501) from the performing laboratory were submitted following a request by the Agency. The mean dissociation constant (K_d) for [^{3}H]-R1881 was 0.613±0.041 nM and the estimated B_{max} was 0.817±0.049 fmol/100 µg protein for the single batch of prostate cytosol that was used for this assay. The mean and individual K_d and B_{max} values were below the expected ranges reported in the EPA validation program (K_d 0.685 to 1.57 nM and B_{max} 7 to 16 fmol/100 µg protein). Confidence in these numbers is high based on the goodness of fit (R^2 = 0.957-0.984) and the small variation among runs.

In the competitive binding experiment, the estimated mean log IC_{50}s for R1881 (−9.0 M) and the weak positive control, dexamethasone (−4.6 M), were within the expected ranges. The mean relative binding affinity (RBA) for the weak positive control was 0.004%. All performance criteria were generally met; however, potential drift in the study assay could not be assessed because all solvent control tubes were analyzed at the beginning of each run.

Substantial precipitation of folpet was visually observed at a concentration of 10^{-3} M in Run 1; therefore, the second and third runs were performed at a maximum test concentration of 10^{-4} M. The specific [^{3}H]-R1881 binding was >75% at all soluble concentrations of folpet (10^{-11} to 10^{-4} M) in all runs. A log IC_{50} and RBA could not be calculated for folpet.

Based on the results from the three runs, folpet is classified as a Non-Binder in the Androgen Receptor Binding Assay.

The assay **satisfies** the EDSP Tier 1 guideline requirements for an Androgen Receptor Binding assay (OCSPP 890.1150).

COMPLIANCE: Signed and dated GLP Compliance, Data Confidentiality, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

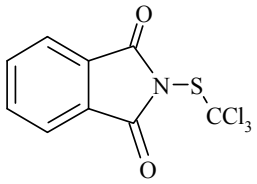
A. MATERIALS

1. Test Facility: CeeTox, Inc.

Location: Kalamazoo, MI
Study Director: Jamin A. Willoughby
Other Personnel: Karen Rutherford, Director of Laboratory Operations
David Blakeman, Senior Scientist/Endocrine Group Leader
Cameron Haines, Scientist
Steven McColley, Scientist
Benjamin Meyer, Scientist
Colleen Toole, Director of Project Management
Study Period: June 6, 2011 to January 5, 2012

2. Test substance: Folpet

Description: Off-white powder
Source: Makhteshim Chemical Works Ltd., Beer Sheva, Israel
Batch #: 00138518
Purity: 94.5%
Solubility: Not reported (NR), but soluble in DMSO up to 30 mM (stock concentration)
Volatility: NR
Stability: NR
Storage conditions: Room temperature
CAS #: 133-07-3
Molecular weight: 296.56 g/mol
Structure:



3. Non-labeled ligand: R1881

Supplier: Sigma Aldrich, St. Louis, MO
Catalog #: R0908
Lot #: 060M4638
Purity: 98%
CAS #: 965-93-5

4. Radioactive ligand: [^{3}H]-R1881

Supplier: Perkin-Elmer, Boston, MA
Catalog and Lot #: NET590 (653698)
Date of production: February 24, 2011
Date of use: October 25, 2011 to October 31, 2011
Radiochemical purity: NR
Specific activity: 85.1 Ci/mmol
Concentration of stock: 10 nM

5. Positive control: Dexamethasone

Supplier:	Sigma Aldrich, St. Louis, MO
Catalog #	D1756
Lot #:	1419230
Purity:	98.9%
CAS # :	50-02-2

6. Solvent/vehicle control: Dimethyl sulfoxide (DMSO)

Justification for choice of solvent:	DMSO is one of the recommended solvents according to the EPA Guideline (OPPTS 890.1150)
Final Concentration:	*ca*. 3.2%

B. METHODS

1. Preparation of Rat Ventral Prostate Cytosol: Male Sprague Dawley rats (number not specified) from Charles River Laboratories were castrated at 90 days of age and the ventral prostates were excised <1 day following castration, weighed, and placed in ice-cold TEDG (Tris, EDTA, DTT, glycerol) buffer (inclusion of protease inhibitor not reported), homogenized, and centrifuged for 30 min at 30,000 × *g* at 4ºC. Supernatant was pooled, and the protein concentration of the cytosol was determined to be 8.8 mg/mL using a commercially available protein kit compatible with DTT in the TEDG buffer (Bradford Method). Cytosol was divided into aliquots and stored frozen until use.

2. Saturation Radioligand Binding Experiment: A saturation binding experiment measuring total and non-specific binding of [^{3}H]-R1881 was performed to demonstrate that the AR was present in reasonable concentrations and had the appropriate affinity for the R1881 ligand (MRID 48843501). The conditions for the saturation binding experiment are summarized in Table 1.

TABLE 1. Summary of Conditions for Saturation Binding Experiment [a]

Source of receptor		Rat ventral prostate cytosol
Concentration of radioligand (as serial dilutions)		0.25 to 10 nM
Concentration of non-labeled ligand (100X [radioligand])		2 to 1000 nM
Optimization of receptor concentration		Sufficient to bind 25 to 35% of radioligand at 0.25 nM
Temperature		4º C
Incubation time		16 to 20 hours
Composition of assay buffer (TEDG)	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride (PMSF)	1 mM
	DTT	1 mM
	Sodium Molybdate	1 mM

a Data were obtained from page 2 of the study report (MRID 48843501).

On the day of each assay, the specific activity of the stock solution [^{3}H]-R1881 (originally 85.1 Ci/mmol as manufactured on February 24, 2011) was adjusted for decay over time (adjusted specific activities were not reported), and serial dilutions in low-salt TEDG+PMSF buffer were prepared to achieve the final concentrations in cytosol of 0.25,

0.50, 0.70, 1.0, 1.5, 2.5, 5.0, and 10 nM to determine total binding. To determine non-specific binding, solutions of non-labeled R1881 were prepared in a similar manner to achieve concentrations that were 100-fold greater than each respective radiolabeled concentration, resulting in final concentrations in cytosol of 25, 50, 70, 100, 150, 250, 500, and 1000 nM.

In the absence of cytosol, the radiation found in 7.5, 15, 21, 30, or 45 µL of 10 nM [^{3}H]-R1881 and 7.5, 15, or 30 µL of 100 nM [^{3}H]-R1881 was measured. For each batch of cytosol, the optimal protein concentration was determined by calculating specific binding to differing amounts of protein per tube, using 0.25 nM radiolabeled R1881. The optimal protein concentration was determined to be 1.86 mg protein/assay tube, which resulted in the binding of approximately 25-35% of the total radioactivity added. Cytosolic protein used in this assay was thawed fresh for this experiment at 4°C, and maintained at 4°C during the binding assay. Each saturation binding experiment consisted of three non-current runs (conducted on September 24, 25, and 26, 2011, respectively). Each run contained three replicates at each concentration, resulting in the 72 samples depicted in Table 2.

TABLE 2. Saturation Binding Experiment Run[a,b]

Total Binding	Non-Specific Binding		Radioligand alone	
Tubes 1-24[c]	Tubes 25-48[d]		Tubes 49-72[e]	
[^{3}H]-R1881 Final conc. (nM)	[^{3}H]-R1881 Final conc. (nM)	R1881 Final conc. (nM)	[^{3}H]-R1881 Initial conc. (nM)	[^{3}H]-R1881 (µL)
0.25	0.25	25	10	7.5
0.50	0.50	50	10	15
0.70	0.70	70	10	21
1.0	1.0	100	10	30
1.5	1.5	150	10	45
2.5	2.5	250	100	7.5
5.0	5.0	500	100	15
10	10	1000	100	30

a Data were obtained from page 3 of the study report (no MRID).
b Each concentration was run in triplicate for a total of 72 samples.
c Tubes 1-24 contained 50 µL of triamcinolone acetonide and 7.5-45 µL [^{3}H]-R1881. Samples were dried, and 300 µL of prostate cytosol were added.
d Tubes 25-48 contained 50 µL of triamcinolone acetonide and 7.5-45 µL [^{3}H]-R1881. R1881 was added in a 100-fold molar excess of [^{3}H]-R1881 in a volume of 7.5-45 µL. Samples were dried, and 300 µL of prostate cytosol were added.
e Tubes 49-72 contained only 7.5, 15, 21, 30, or 45 µL of 10 nM [^{3}H]-R1881 or 7.5, 15, 21, or 30 µL of 100 nM [^{3}H]-R1881 without cytosol or other components to determine the total counts added.

Following addition of triamcinolone acetonide, [^{3}H]-R1881, and/or R1881, the tubes were dried, dissolved in diluted prostate cytosol (300 µL), and incubated in a rotor for 20 hours at approximately 4°C. Samples were maintained at temperatures of approximately 4°C except during whole rack vortexing. To separate bound from free R1881, hydroxyapatite (HAP) slurry was added to each tube and vortexed 5 times with 4-minute intervals over 20 minutes. The samples were then centrifuged, and the supernatant was aspirated and discarded. The samples were washed 4 times in 2 mL of ice cold TEDG+PMSF buffer, followed by vortexing and centrifugation for 3 minutes at 700 x g. Following the last wash and

decanting of the Tris buffer pellets were then extracted by additional of 2 mL of ethanol. The samples were vortexed and centrifuged for 10 minutes at 1000 x g. Samples were maintained on ice at all times between vortexing. After the final centrifugation, the ethanol supernatants were decanted into scintillation vials that each contained 14-mL portions of scintillation cocktail, and the radiation was quantified by liquid scintillation counting. A total of three runs were performed.

3. **Competitive Binding Experiment:** A summary of the assay conditions for the competitive binding experiment is included in Table 3.

TABLE 3. Summary of Conditions for Competitive Binding Experiment[a]		
Source of receptor		Rat ventral prostate cytosol
Concentration of radioligand		1 nM
Optimization of receptor concentration		Sufficient to bind 10-15% of radioligand[b]
Concentration of test substance (as serial dilutions)		10^{-10} to 10^{-3} M (Run 1) 10^{-11} to 10^{-4} M (Runs 2 and 3)
Incubation Temperature		4±2 ºC
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	DTT	1 mM
	Sodium molybdate	1 mM
	Protease Inhibitor (PI)[c]	0.5%

a Data were obtained from pages 14-16, and 56 of the study report.
b The protein concentration was not reported.
c The recommended PI in the Guideline is 1 mM PMSF. The PI was identified in the study protocol as Calbiochem Protease Inhibitor Cocktail, Set III, EDTA free. It is a mixture of six PIs that includes 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). AEBSF has similar specificity to PMSF with greater stability at lower pH.

The competitive binding experiment was performed according to the protocol provided in the EPA Test Guidelines OCSPP 890.1150. The competitive binding experiment measures the binding of a single concentration of [^{3}H]-R1881 (adjusted specific activity of 82.0 Ci/mmol for Runs 1 and 2; 81.9 Ci/mmol for Run 3) to the AR in the presence of increasing concentrations of a test substance.

DMSO was used as the solvent, and precipitation of folpet was visually observed at concentrations 10^{-3} and 10^{-4} M in the first run; no precipitation was observed in subsequent runs performed over the concentration range of 10^{-11} to 10^{-4} M. Therefore, the highest concentration of folpet used in evaluating the data was 10^{-5} for the first run and 10^{-4} M for the second and third runs. The protein concentration used in the competitive binding experiment was not reported; however, it was reported to be sufficient to bind 10-15% of the [^{3}H]-R1881.

Dilutions of the test substance, reference standard (R1881), weak positive control (dexamethasone), and solvent control (DMSO) were prepared to achieve the concentrations shown in Table 4. Each assay consisted of three independent runs on three different days,

and each run contained three replicates at each concentration plus six replicates of the [^{3}H]-R1881, NSB, and solvent controls, resulting in a total of 81 samples per run.

TABLE 4. Competitor Final Molar (M) Concentrations in Competitive Binding Assay [a, b]

Solvent Control	Reference standard	Weak positive control	Test Chemical		None
DMSO	R1881	Dexamethasone	Folpet		
Tubes 7-12	Tubes 13-33 [c]	Tubes 37-60	Tubes 61-84		Tubes 1-6
			Run 1	Runs 2 & 3[d]	
	--	1×10^{-3}	1×10^{-3}	1×10^{-4}	
	--	1×10^{-4}	1×10^{-4}	1×10^{-5}	
	1×10^{-6}	1×10^{-5}	1×10^{-5}	1×10^{-6}	
	1×10^{-7}	1×10^{-6}	1×10^{-6}	1×10^{-7}	
	1×10^{-8}	1×10^{-7}	1×10^{-7}	1×10^{-8}	
	1×10^{-9}	1×10^{-8}	1×10^{-8}	1×10^{-9}	
	1×10^{-10}	1×10^{-9}	1×10^{-9}	1×10^{-10}	
	1×10^{-11}	1×10^{-10}	1×10^{-10}	1×10^{-11}	

a Data were obtained from pages 12-14, and 16 of the study report.
b Each concentration of each chemical was run in triplicate for a total of 81 tubes per run (tubes were numbered 1-84,but tubes 34-36 were excluded from the sequence). Tubes 1-84 contained 50 µL of triamcinolone acetonide and 30 µL [^{3}H]-R1881. Samples were dried, and 300 µL of prostate cytosol were added. Tubes 7-84 also contained 10 µL of the solvent control, reference standard (non-radiolabeled R-1881), weak positive control, or test substance, with the exception of Tubes 13-18 that contained 30 µL of non-radiolabeled R1881 (used to evaluate non-specific binding). Tubes 1-6 contained only 30 µL of [^{3}H]-R1881.
c Tubes 13-18 were used to evaluate non-specific binding by adding 100X of cold (non-radiolabeled) R1881.
d Test concentrations used in Runs 2 and 3 were altered due to precipitation observed at 10^{-3} M in Run 1.

To separate bound from free R1881, HAP slurry was added to each tube and vortexed once every 5 minutes for 20 minutes. The samples were then centrifuged, and the supernatant was discarded. The samples were washed 4 times in 50 mM TRIS buffer. Following the last wash and decanting of the Tris buffer, the pellets were then extracted by addition of 2-mL portions of ethanol. The samples were vortexed at 5 minute intervals for *ca*. 15-20 minutes. Samples were centrifuged for 10 minutes at 700 × *g*. Supernatants were radio-assayed by scintillation counting.

4. **Data Analysis:** For the saturation binding assay, the maximal binding capacity (B_{max}), dissociation constant (K_d), and inhibition concentration (IC_{50}) were calculated with nonlinear regression analysis by using Graph Pad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA). Scatchard plots were also plotted for the binding data (GraphPad Software, Inc.). Automatic outlier elimination for binding data was performed using the method of Motulsky and Brown (2006)[1] with a Q value of 1.0, implemented by using the ROUT procedure of Prism v. 5. Receptor binding data plots were corrected for ligand depletion with the method of Swillens (1995)[2] by using Prism v. 5. Mean and standard deviation were calculated for each run of the saturation and competitive binding experiments using Microsoft Excel 2007 (v. 12.0.6557.5000;

[1] Motulsky, H.J. and Brown, R.E. (2006) Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics, Vol 7, pp 123-142.
2 Swillens, S. (1995) Interpretation of binding curves obtained with high receptor concentrations: practical aid for computer analysis. *Molec. Pharmacol.* 47(6):1197-1203.

Microsoft Corporation, Redmond, WA), and mean and standard error were calculated for the composite three runs using Microsoft Excel 2010.

5. Definitions

a. Classification of test material

If the data fit a 4-parameter nonlinear regression model, the test chemical is classified as follows:

Binder: The average curve for the test chemical across runs crosses 50% of radioligand bound.

Equivocal: The average lowest portion of curves across runs is between 50% and 75% radioligand binding (*i.e.* radioligand displacement is at least 25% but less than 50%), or the curve falls outside the range for the weak positive control (−0.6 to −1.4).

Non-Binder: The average lowest portion of curves across runs is greater than 75% activity (*i.e.* less than 25% displacement of radioligand), or the data do not fit the model.

Untestable: If the test compound is not soluble above 1×10^{-6} M and the binding curve does not cross 50%, the chemical is judged to be untestable.

b. Descriptors for receptor binding

B_{max}: maximal binding capacity
K_d: dissociation constants
IC_{50}: Concentration of the test substance at which 50% of radioligand is displaced from the AR by the competitor
Relative Binding Affinity (RBA): IC_{50} of R1881 × 100 ÷ IC_{50} of test substance
Log RBA: Log_{10} (IC_{50} of R1881 × 100 ÷ IC_{50} of test substance)

II. RESULTS

A. SATURATION BINDING EXPERIMENT: Saturation binding experiment parameters are presented in Table 5. The mean K_d for [^{3}H]-R1881 was 0.613±0.041 nM and the estimated B_{max} was 0.817±0.049 fmol/100 µg protein for the single batch of prostate cytosol that was prepared. The mean and individual K_d values for each run were below the range reported in the EPA validation program (0.685 to 1.57 nM). However, confidence in these numbers is high according to the goodness of fit (R^2 = 0.957-0.984) and the small variation among runs.

TABLE 5. Saturation Binding Experiment of [^{3}H]-R1881 with Androgen Receptor from Rat Prostate Cytosol[a]

Parameter	Run 1	Run 2	Run 3	Mean ± SE[b]
R^2 (unweighted)	0.984	0.977	0.957	0.957-0.984
B_{max} (nM)	0.011	0.010	0.011	0.011±0.001
B_{max} (fmol/100 µg protein)	0.809	0.773	0.870	0.817±0.049
K_d (nM)	0.565	0.638	0.635	0.613±0.041

a Data were obtained from page 4 of the study report (no MRID).
b The range of R^2 is reported and the mean ± SE is reported for the other parameters.
R^2 = Goodness of fit for curve calculated for specific binding

Figure 1 illustrates the non-specific, specific, and total binding curves for [^{3}H]-R1881 to the androgen receptor for the three independent runs. The specific binding reached a plateau in each run, and non-specific binding was less than 20% of total binding. Figure 2 contains the Scatchard plots that illustrate the binding of [^{3}H]-R1881 to the androgen receptor. The data fits resulted in linear plots.

FIGURE 1. Binding of [^{3}H]-R1881 to the Androgen Receptor during the Saturation Binding Experiment.

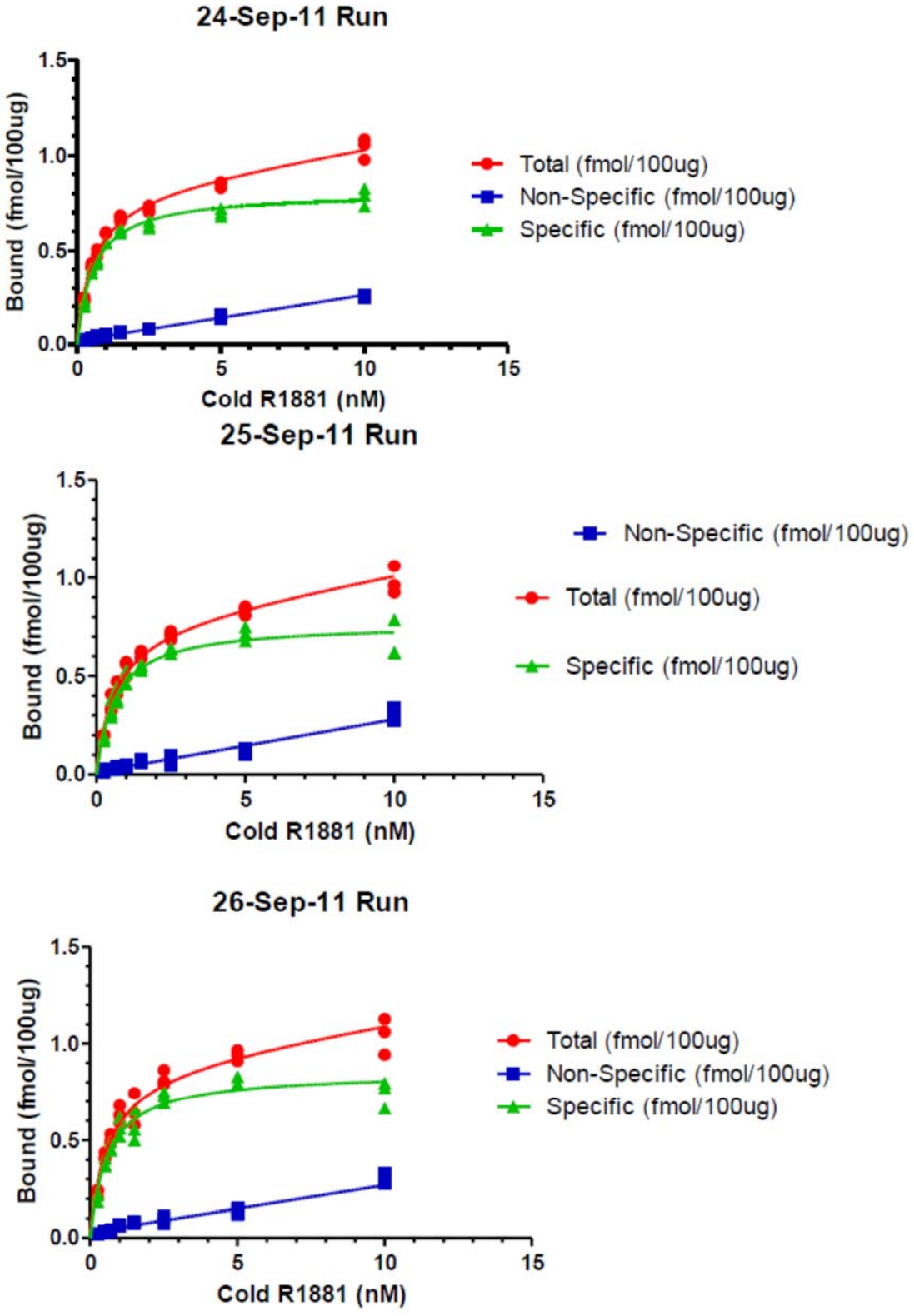
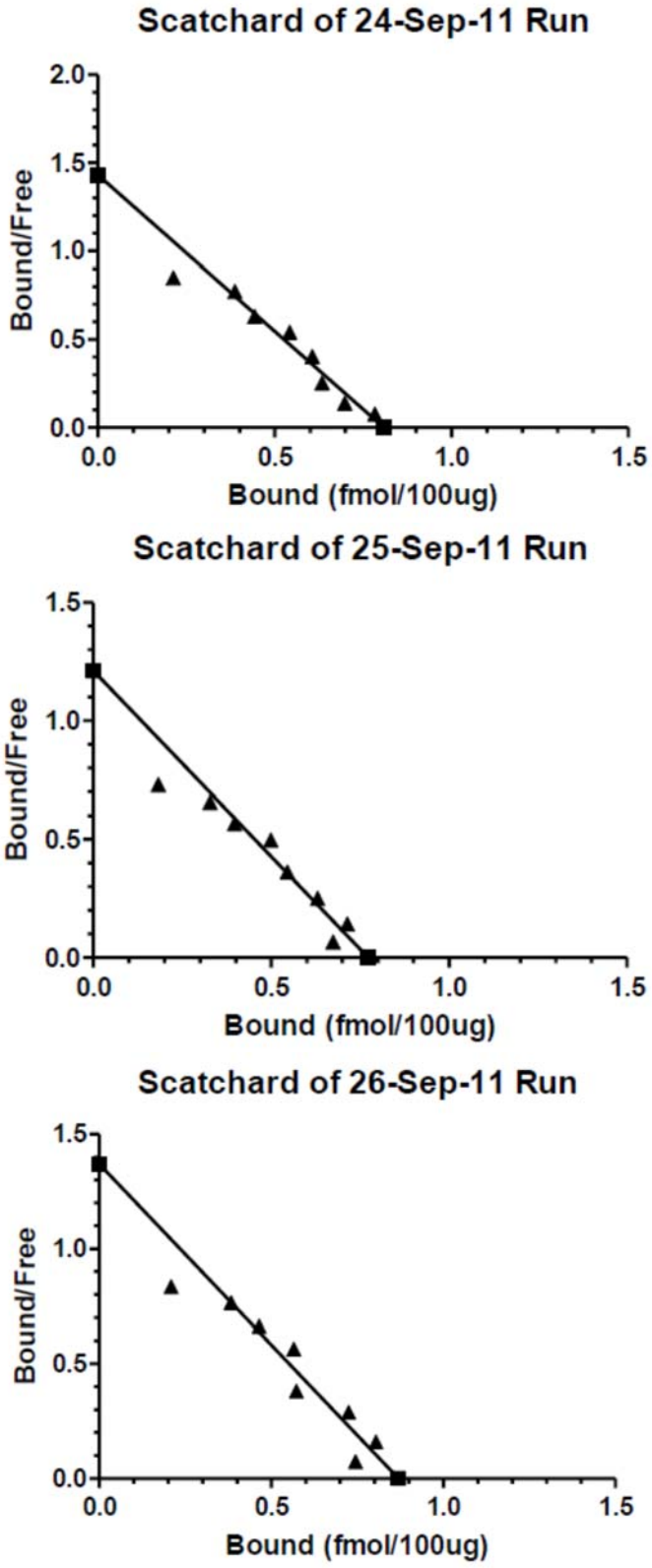


FIGURE 2. Scatchard Plots of the Binding of [^{3}H]-R1881 to the Androgen Receptor.



B. COMPETITIVE BINDING EXPERIMENT: Competitive binding experiment parameters are presented in Table 6 and shown graphically in Figures 3-5.

The estimated average log IC_{50} was −9.0 M for R1881 and −4.6 M for the weak positive control (dexamethasone). Compared to R1881, the mean RBA for the positive control was 0.004%. Confidence in these numbers is high due to the small variation. All solvent control tubes were run at the beginning of the experiment, preventing the reviewer from determining potential drift in the assay from the beginning to the end of the run.

Substantial precipitation of folpet was visually observed at a concentration of 10^{-3} M in Run 1 and slight precipitation was observed at 10^{-4} M in Run 1, therefore the highest concentration used for data evaluation was 10^{-5} M in the first run. No precipitation was observed in subsequent runs performed over the concentration range of 10^{-11} to 10^{-4} M.

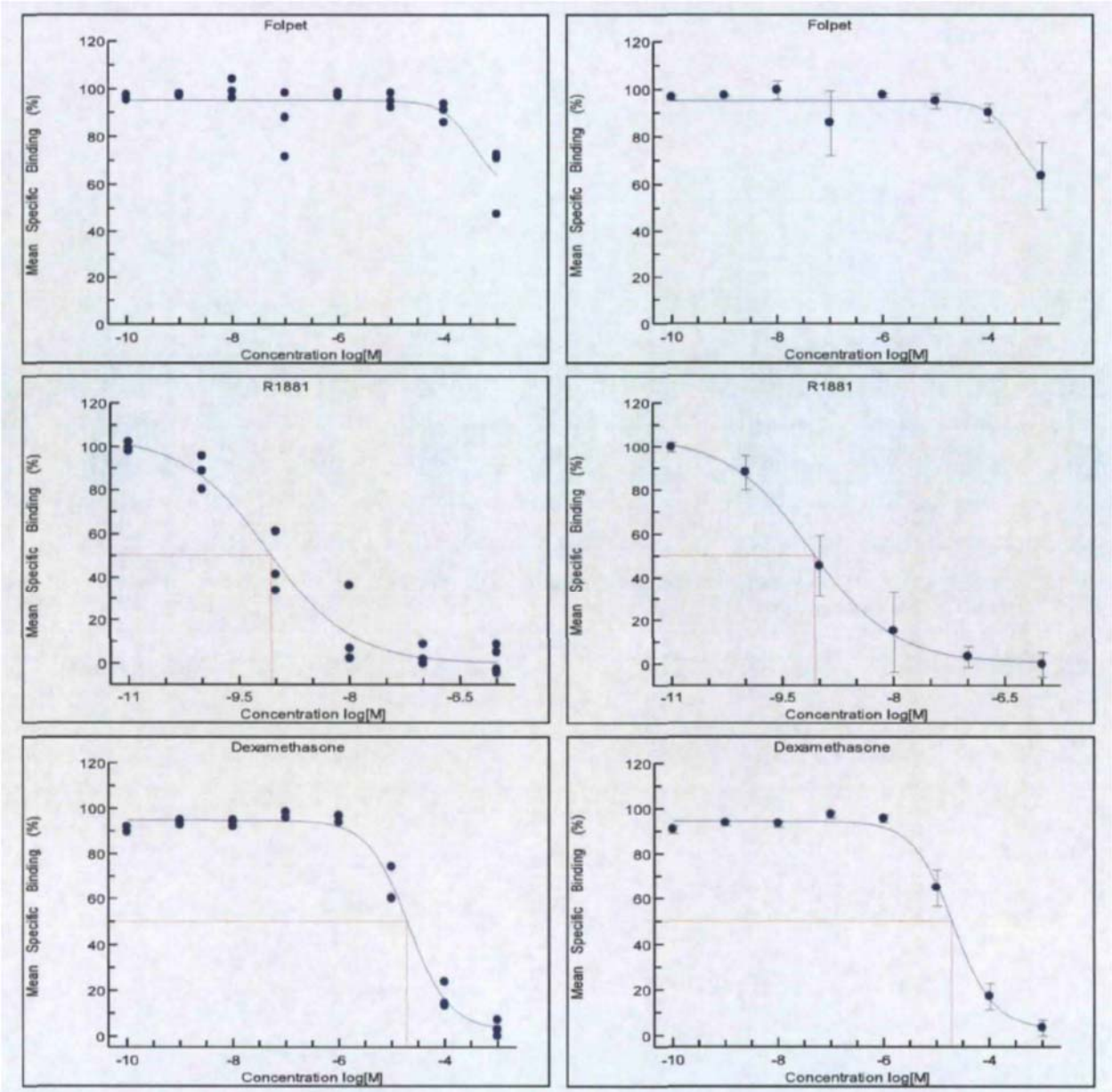
The specific [^{3}H]-R1881 binding was >75% at all soluble concentrations of folpet (10^{-11} to 10^{-4} M) in all runs. A log IC_{50} and RBA for folpet could not be determined as exposure to folpet did not result in 50% reduction in [^{3}H]-ligand binding at any concentration of folpet. Based on the results of the three valid assays, folpet is classified as a "non-binder" with the androgen receptor under the conditions of this assay.

TABLE 6. Competitive Binding Assay of Folpet with AR from Rat Prostate Cytosol[a]

Parameter		Run 1	Run 2	Run 3	Mean ± SE[b]
R^2 (unweighted)	R1881	NR	NR	NR	NA
	Dexamethasone	NR	NR	NR	NA
	Folpet	NR	NR	NR	NA
Log IC_{50} (M)	R1881	−9.1	−9.0	−9.0	−9.0 ± 0.033
	Dexamethasone	−4.7	−4.6	−4.6	−4.6 ± 0.033
	Folpet	NA	NA	NA	NA
IC_{50} (M)[b]	R1881	7.94×10^{-10}	1.00×10^{-9}	1.00×10^{-9}	9.31×10^{-10} (± 0.69)
	Dexamethasone	2.00×10^{-5}	2.51×10^{-5}	2.51×10^{-5}	2.34×10^{-5} (± 0.17)
	Folpet	NA	NA	NA	NA
Log RBA[b]	Dexamethasone	−4.4	−4.4	−4.4	−4.4 ± 0.0
	Folpet	NA	NA	NA	NA
RBA (%)[b]	Dexamethasone	0.004	0.004	0.004	0.004 ± 0.000
	Folpet	NA	NA	NA	NA

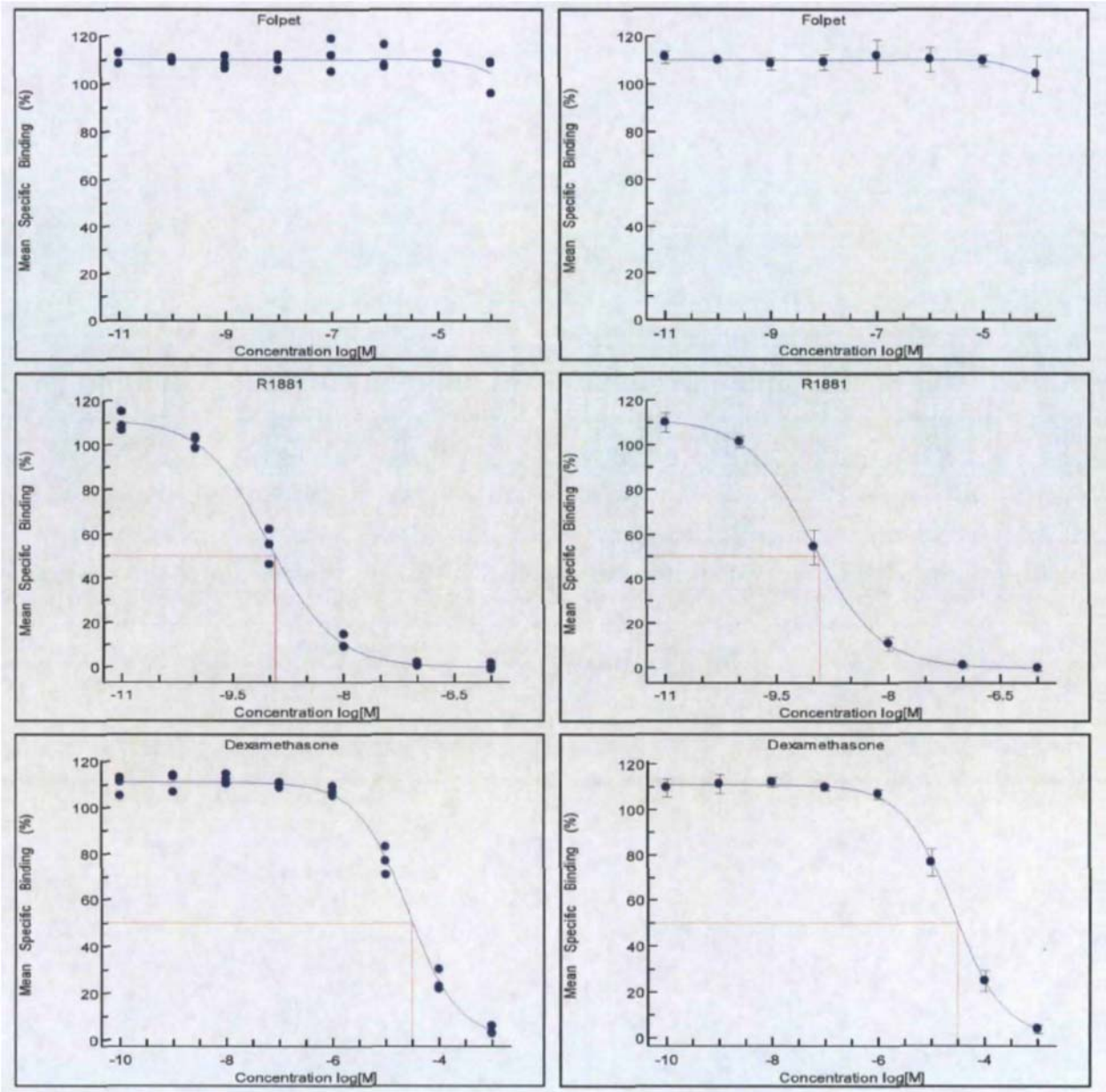
a Data were obtained from page 20 of the study report.
b All values are reviewer-calculated from the log IC_{50} values reported in the study report. For means expressed in scientific notation, the SE values in parentheses are presented in the same order of magnitude as the mean value.
SE Standard Error
NA Not applicable.
NR Not reported
R^2 Goodness of fit
RBA (%) Relative binding affinity

FIGURE 3. Percentage [^{3}H]-R1881 Bound to the Androgen Receptor in the Presence of Radioinert R1881, Dexamethasone, or Folpet (Run 1).



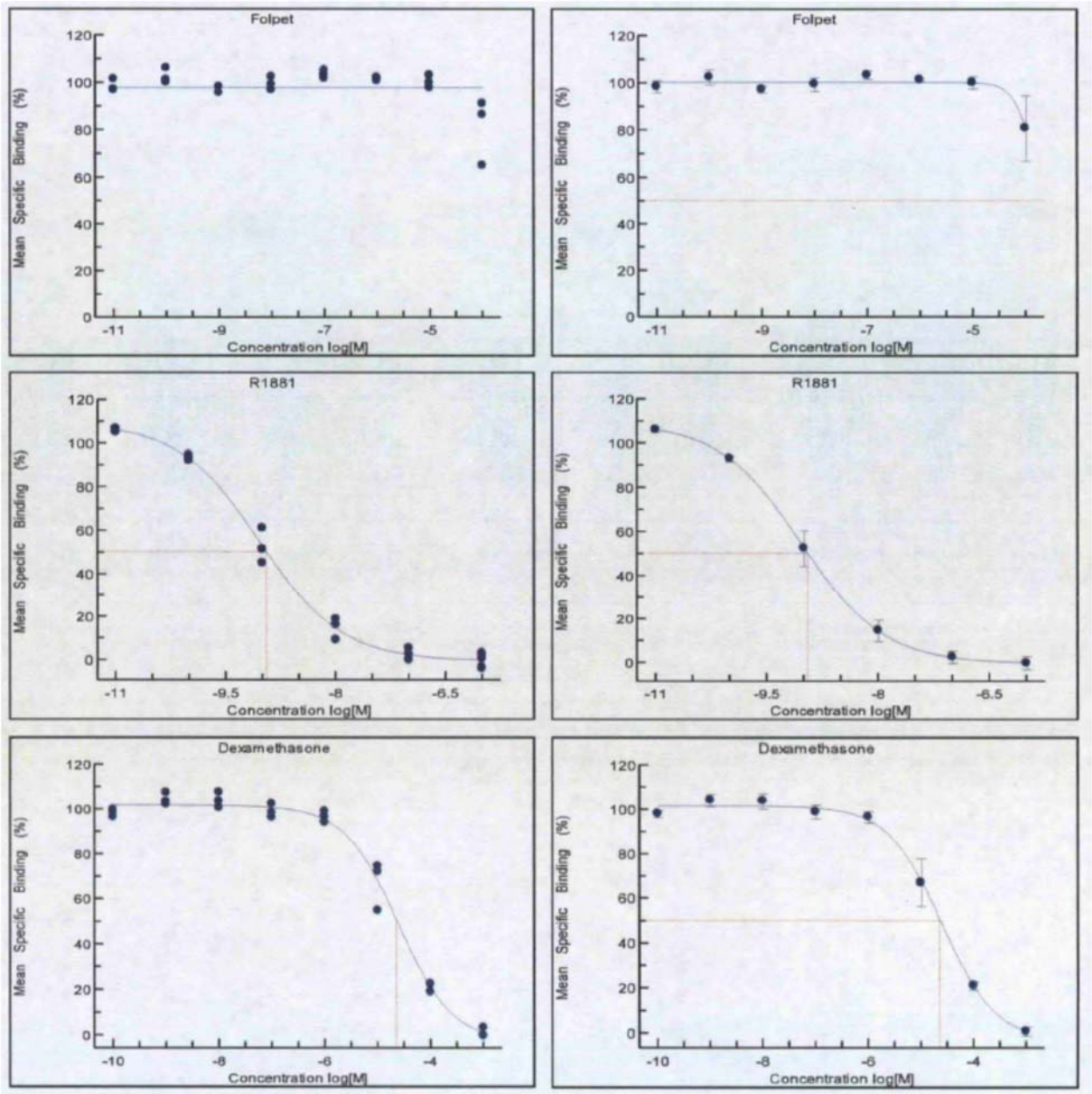
The graphs on the left show individual replicates while the graphs on the right show the mean values while error bars represent ± standard deviation.

FIGURE 4. Percentage [^{3}H]-R1881 Bound to the Androgen Receptor in the Presence of Radioinert R1881, Dexamethasone, or Folpet (Run 2).



The graphs on the left show individual replicates while the graphs on the right show the mean values while error bars represent ± standard deviation.

FIGURE 5. Percentage [^{3}H]-R1881 Bound to the Androgen Receptor in the Presence of Radioinert R1881, Dexamethasone, or Folpet (Run 3).



The graphs on the left show individual replicates while the graphs on the right show the mean values while error bars represent ± standard deviation.

C. PERFORMANCE CRITERIA: To ensure that the competitive binding assay was functioning properly, each run was evaluated using the criteria shown in Table 7.

TABLE 7. Criterion [a]	Tolerance Limit(s) [b]	Value	Yes	No
Ligand depletion is minimal. The recommended ratio of total binding in the absence of competitor to total amount of [^{3}H]-R1881 added per assay tube.	≤15%	≤12.7%	X	
Test chemical Top (% binding)	80 to 115	96.8 to 111.5	X	
R1881 fitted curve parameters				
Top (% binding)	82 to 114	105 to 112	X	
Bottom (% binding)	−2.0 to 2.0	0	X	
Hill Slope	−1.2 to −0.8	-1.0 to -0.8	X	
Weak positive control (dexamethasone) fitted curve parameters				
Top (% binding)	87 to 106	95 to 111[c]		X
Bottom (% binding)	−12 to 12	-3 to 3	X	
Hill Slope	−1.4 to −0.6	-1.1 to -0.9	X	
Saturation Binding Experiment K_d (nM)	0.685 to 1.57	0.565 to 0.638[d]		X
Non-specific binding (%)	≤10.0	0.36 to 0.58[e]	X	

a Data were obtained from pages 23-28, and 34-36 of the main study report.
b These values represent ranges from the validation study.
c The top % binding was high (111%) in Run 2, but within the recommended range in the other two runs.
d MRID 48843501
e Calculated by the study reviewer.
NR Not reported

Additionally, the curve for the reference material showed that increasing concentrations of unlabeled R1881 displaced [^{3}H]-R1881 in a manner consistent with one-site binding, as indicated by a descent from 88.3-101.5% to 11.0-15.1% binding over approximately an 81-fold increase in concentration of R1881 (i.e., covering approximately 2 log units). Examination across the runs indicated consistency of the Hill slope, placement along the X-axis, and top and bottom plateaus.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Folpet was classified as a "non-binder" in all three valid independent runs and thus has a final classification of "non-binder."

B. AGENCY COMMENTS: In the saturation binding experiments, the mean K_d for [^{3}H]-R1881 was 0.613 nM and the mean estimated B_{max} was 0.817 fmol/100 µg protein for the single batch of prostate cytosol that was used. The mean and individual K_d values were below the range reported in the EPA validation program (0.685 to 1.57 nM). Confidence in these numbers is high according to the goodness of fit (R^2 = 0.957-0.984) and the small variation among runs.

In the competitive binding experiment, the estimated mean log IC_{50}s for R1881 (−9.0 M) and the weak positive control, dexamethasone (−4.6 M), were within the expected ranges.

The mean RBA for the weak positive control was 0.004%. Confidence in these numbers is high due to the small variation. All performance criteria were generally met.

Substantial precipitation of folpet was visually observed at a concentration of 10^{-3} M in Run 1, therefore the second and third runs were performed at a maximum test concentration of 10^{-4} M. Folpet is classified as a non-binder as specific [^{3}H]-R1881 binding to the androgen receptor was >75% for all three runs at concentrations up to 10^{-4} M.

C. STUDY DEFICIENCIES: The following deficiencies were noted that were not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- Curves were not provided showing the average binding of each test substance across all three runs.
- All solvent control tubes were run at the beginning of the experiment, preventing the reviewer from determining drift in the assay from the beginning to the end of the run.
- The protein concentration used in the assay was not reported.

DATA EVALUATION RECORD

FOLPET

Study Type: OCSPP 890.1200, Aromatase Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-41-2012 (MRID 48616902)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
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Primary Reviewer Joan Harlin, M.S.	Signature: Date:	Joan Harlin 4/30/2012
Secondary Reviewer Rebecca L. Bryan, B.S.	Signature: Date:	Rebecca L. Bryan 5/14/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	Jack D. Early 5/25/2012
Quality Assurance: Steven Brecher, Ph.D., D.A.B.T.	Signature: Date:	 5/25/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Ayaad Assaad, D.V.M., Ph.D. **Signature:**
Health Effects Division, Office of Pesticide Programs **Date:**
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:**
Health Effects Division, Office of Pesticide Programs **Date:**

DATA EVALUATION RECORD

STUDY TYPE: Aromatase (Human Recombinant); OCSPP 890.1200

PC CODE: 081601 **DP BARCODE:** D398813

TXR#: 0055725 **CAS No.:** 133-07-3

TEST MATERIAL (PURITY): Folpet (94.5% a.i.)

SYNONYMS: 2-[(trichloromethyl)thio]-1H-isoindole-1,3(2H)-dione; Folpan Tech

CITATION: Wilga P.C. (2012). Folpet: Human Recombinant Aromatase Assay. CeeTox, Inc., Kalamazoo, MI. Laboratory Report No.: 9141V-100357AROM, January 4, 2012. MRID 48616902. Unpublished.

SPONSOR: Makhteshim Chemical Works Ltd. c/o Makhteshim Agan of North America, Inc., Raleigh, NC

TEST ORDER #: EDSP-081601-175

EXECUTIVE SUMMARY: In an *in vitro* aromatase (CYP19) assay (MRID 48616902), folpet (94.5% a.i. Batch # 00138518) was incubated with human recombinant aromatase and tritiated androstenedione ([1β-^{3}H(N)]-androst-4-ene-3,17-dione; [^{3}H]ASDN) in dimethyl sulfoxide (DMSO) at final log concentrations of 10^{-11} to 10^{-5} M for 15 minutes to assess the potential of folpet to inhibit aromatase activity. In Run 1, the two highest concentrations (10^{-3} and 10^{-4} M) of folpet tested showed precipitation during the 15-minute incubation period and were not used in data interpretation. Therefore, the highest test concentration used for Runs 2 and 3 was 10^{-5} M.

Aromatase activity was determined by measuring the amount of tritiated water produced at the end of a 15-minute incubation for each concentration of chemical. Tritiated water was quantified using liquid scintillation counting (LSC). Three runs were conducted and each run included a full activity control, a background activity control, a positive control series (10^{-10} to 10^{-5} M) using a known inhibitor (4-hydroxyandrostenedione; 4-OH ASDN), and the folpet series (10^{-11} to 10^{-5} M) with three repetitions per concentration.

Aromatase activity in the full activity controls was 0.608 ± 0.042 nmol·mg-protein^{-1}·min^{-1}. The response of each full activity control within a run was between 90 to 110% of the average full activity. Activity in the background controls ranged 0.24 to 0.37% and averaged 0.30% of the full activity controls.

For the positive control substance (4-OH ASDN), aromatase activity results were within the recommended ranges for the performance criteria. The estimated log IC_{50} for 4-OH ASDN averaged −7.27 M and the slope was −0.94.

For folpet, aromatase activity at the lowest and highest tested concentrations evaluated, 10^{-11} and 10^{-5} M, was 97.03% and 57.32% of the control, respectively. Since the average lowest portion of the response curve across runs was between 50 and 75% activity, the response was considered equivocal at soluble concentrations.

Based on data from the average response curve, folpet is classified as Equivocal for aromatase inhibition in this assay.

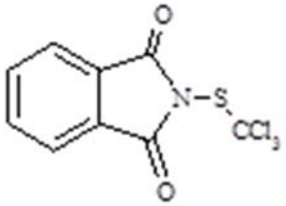
The assay **satisfies** the EDSP Tier 1 Test Order requirements for an Aromatase assay (OCSPP 890.1200).

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Substance: Folpet

Description:	Powder
Source:	Makhteshim Chemical Works, Ltd.
Lot # (expiration date):	00138518 (May 26, 2012)
Purity:	94.5%
Volatility:	Not reported
Storage conditions:	Room temperature (e.g. ambient)
Stability:	Not reported
Solvent:	DMSO
Solubility (in test solvent):	Not reported
Highest Concentration Tested:	10^{-3} M
Stock Solution Preparation:	Serial dilution
Molecular weight:	296.6 g/mol
CAS #:	133-07-3
Structure:	

2. Non-Labeled Substrate: Androstenedione (ASDN)

CAS # :	63-05-8
Source:	Steraloids, Inc. (Catalog # A6030-100)
Batch # (expiration date):	L1712 (April 2016)
Purity:	99.8%

3. Radiolabeled Substrate: 1-β [^{3}H(N)]-Androst-4-ene-3,17-dione; ([^{3}H]ASDN)

Source:	Perkin Elmer (Catalog #NET-926)
Batch # (expiration date):	619344 (January 10, 2012)
Radiochemical Purity (Supplier):	>97%
Specific activity:	26.3 mCi/mmol
Radiochemical Purity (In-lab determination):	Not determined

4. Positive Control: 4-hydroxyandrostenedione (4-OH ASDN)

CAS #	566-48-3
Source:	Sigma-Aldrich (Catalog # F2552)
Batch # (expiration date):	081K2133 (March 2015)
Purity:	99.6%

5. Solvent (Vehicle Control): Dimethyl sulfoxide (DMSO)

Source:	Not reported
Batch # (expiration date):	Not reported
Justification for choice of solvent:	Not reported
Concentration (% of total volume in assays):	≤1%

6. Test Microsomes: Human recombinant aromatase (CYP19) microsomes

Source:	BD Gentest™, Woburn, MA (Catalog # 456260)
Lot # (expiration date):	19701 (July 2014)
Protein concentration:	3.7 mg/mL
Cytochrome C reductase activity:	540 nmole /mg protein/minute
Aromatase activity:	5.7 pmol/pmol P450/min

B. METHODS

1. Assay Components and Preparations: A mixture of non-labeled and radiolabeled [^{3}H]ASDN was prepared such that the final concentration of ASDN in the assay was approximately 0.1 µM, and the amount of tritium added to each incubation tube was 0.1 µCi.

The test chemical was formulated in DMSO such that the volume of DMSO used per assay was no more than 1% v/v of the total assay volume. The rationale for selecting DMSO as the solvent was not reported.

A stock solution of the positive control substance, 4-OH ASDN, was formulated in DMSO. Fresh dilutions of the stock solution were prepared in the same solvent as the stock solution on the day of use. Dilutions were prepared such that the target concentrations of the positive control substance (10^{-10} to 10^{-5} M; Table 4) were achieved by the addition of 20 µL of the dilution for a final assay volume of 2 mL.

Human recombinant microsomes were purchased from BD Gentest™, and stored at −80 ± 10ºC. Microsomes were portioned into individual vials based on the protein concentration of the batch (0.004 mg/mL microsomal protein per tube). Before use of the microsomes, the batch was thawed and sub-aliquoted into individual vials before being refrozen to minimize freeze-thaw cycles to no more than one.

Other assay components sodium phosphate buffer, propylene glycol, and NADPH are reported in Table 1.

TABLE 1. Assay Components and Conditions

Assay Factor	Values
0.1M sodium phosphate buffer (pH 7.4)	
Microsomal Protein	0.004 mg/mL
NADPH	0.3 mM
[^{3}H]ASDN	100 nM
Propylene Glycol	5%
Temperature	37°C
Incubation Time	15 min

2. Suitability Assessments: The protein concentration of the microsomes (Lot #19701) was supplied by the vendor as 3.7 mg/mL. Aromatase activity of the microsomes was also provided by the vendor as 5.7 pmol/min/pmol P450. The mean aromatase activity in the full activity control samples was determined to be 0.608 nmol·mg-protein^{-1}·min^{-1}, which was greater than the minimum recommended aromatase activity of 0.1 nmol·mg-protein^{-1}·min^{-1}.

3. Aromatase Assay: Each assay run contained 4 tubes for the full enzyme activity and 4 tubes for the background activity controls. Two tubes of each control were run at the beginning of the assay, and two of each control were run at the end of the assay. A full concentration curve in duplicate for the positive control, and a full concentration curve in triplicate for the test substance were established. The aromatase assay was conducted generally according to the procedures described in OCSPP 890.1200 (Section h, pp. 9-10).

The amount of ^3H_2O in the aqueous fraction was quantified for each assay tube by LSC, and aromatase activity was reported in units of pmol·mg-protein^{-1}·min^{-1}.

4. Demonstration of Proficiency: It was stated that proficiency testing of the CYP19 aromatase assay was conducted. Each of the proficiency chemicals (atrazine, econazole, fenarimol and nitrofen) were incubated alongside the positive control inhibitor (4-OH ASDN) on three separate occasions using the same methods employed in the current study. However, the actual data from the proficiency testing were not included in the study report.

a. Positive Control

(1) Initial Demonstration of Laboratory Proficiency: The positive control data for laboratory met the following criteria:

- Mean aromatase activity in the absence of an inhibitor was at least 0.1 nmol/mg-protein/min.
- Mean background control activity (0.3%) was ≤ 15% of the full activity control.
- Coefficient of variation (CV) for replicates within each sample type and concentration of 4-OH ASDN was <15%.
- Performance criteria (Table 2) were met, and served as guidance in identifying runs that provided parameters in the preferred ranges.

(2) Demonstration of Proficiency of New Technician for Conducting Assay (when applicable): The positive control data for slope, top and bottom percent met the criteria as listed in section (i) of OCSPP 890.1200.

TABLE 2. Performance Criteria for the Positive Control

Parameter	Lower Limit Criteria	Upper Limit Criteria	Actual Lower Limit	Actual Upper Limit
Slope	−1.2	−0.8	−1.00	−0.89
Top (%)	90	110	96.23	102.39
Bottom (%)	−5	+6	-0.68	0.66
Log IC_{50} (M)	−7.3	−7.0	−7.28	−7.25

Data were obtained from pages 17 and 29 of the study report.

b. Proficiency Chemicals: It was stated that the proficiency of the laboratory was demonstrated prior to running any test chemicals by conducting three full scale test runs on each of the proficiency chemicals as listed in Table 3. The data were not included in the study report. It was stated that the four proficiency chemicals were correctly classified as non-inhibitor or inhibitor.

TABLE 3. Proficiency Chemicals			
Compound	**CAS#**	**Class**	**Concentrations**
Econazole	24169-02-6	Inhibitor	Not reported
Fenarimol	60168-88-9	Inhibitor	Not reported
Nitrofen	1836-75-5	Inhibitor	Not reported
Atrazine	1912-24-9	Non-inhibitor	Not reported

5. Determination of Aromatase Activity with Test Chemical(s): The response of aromatase activity to the presence of eight concentrations of folpet per run, in triplicate, was tested during three independent runs (Table 4). Solubility was assessed (presence of cloudiness or a precipitate). If insolubility was observed at the highest test concentration (10^{-3} M) for the first run, then the highest test concentration would be adjusted for the second and third runs at the highest test concentration that appeared soluble using log or half-log concentrations.

The full enzymatic activity was obtained at the two lowest concentrations of folpet, defining the top of the concentration-response curve.

TABLE 4. Test Chemical Study Design for each Test Run[a]			
Sample Type	**Repetitions (Tubes)**	**Description**	**Reference or Chemical (M)**
Full Activity Control	4	All test components[b] plus solvent vehicle	N/A
Bkgd Activity Control	4	Same as above without NADPH	N/A
4-OH ASDN Conc 1	2	All test components plus 4-OH ASDN	1×10^{-5}
4-OH ASDN Conc 2	2	All test components plus 4-OH ASDN	1×10^{-6}
4-OH ASDN Conc 3	2	All test components plus 4-OH ASDN	$1\times10^{-6.5}$
4-OH ASDN Conc 4	2	All test components plus 4-OH ASDN	1×10^{-7}
4-OH ASDN Conc 5	2	All test components plus 4-OH ASDN	$1\times10^{-7.5}$
4-OH ASDN Conc 6	2	All test components plus 4-OH ASDN	1×10^{-8}
4-OH ASDN Conc 7	2	All test components plus 4-OH ASDN	1×10^{-9}
4-OH ASDN Conc 8	2	All test components plus 4-OH ASDN	1×10^{-10}
Folpet Conc 1[c, d]	3	All test components plus Folpet	1×10^{-3} or 1×10^{-4}
Folpet Conc 2[c, e]	3	All test components plus Folpet	1×10^{-5}
Folpet Conc 3[c]	3	All test components plus Folpet	1×10^{-6}
Folpet Conc 4[c]	3	All test components plus Folpet	1×10^{-7}
Folpet Conc 5[c]	3	All test components plus Folpet	1×10^{-8}
Folpet Conc 6[c]	3	All test components plus Folpet	1×10^{-9}
Folpet Conc 7[c, f]	3	All test components plus Folpet	1×10^{-10}
Folpet Conc 8[c, g]	2	All test components plus Folpet	1×10^{-11}

a Data were obtained from page 19 of the study report.
b The complete assay contained buffer, propylene glycol, microsomal protein, [^{3}H]ASDN, and NADPH.
c Test chemical.
d Two highest concentrations used in Run #1; not used in Runs #2 and 3 due to precipitation.
e Highest concentration in Runs #2 and 3.
f Lowest concentration in Run #1.
g Lowest concentration in Runs #2 and 3.

C. DATA ANALYSIS

1. Raw Data: Raw data were converted to aromatase activity (nmol/mg protein/min) and percent control for the positive control and test chemical. The following raw data and calculated endpoints for each run were included in the report (Table 5).

TABLE 5. Raw and Calculated Data	
Raw/Calculated Data	**Included (X)**
DPM/mL for each portion of extracted aqueous incubation mixture	X
Average DPM/mL for each aqueous portion (after extraction)	X
Total DPM for each aqueous portion (after extraction)	X
The total DPM present in the assay tube at initiation	X
The percentage of substrate converted to product	X
Total DPM after extraction corrected for background	X
Aromatase activity expressed in nmol/mg protein/min	X
Average aromatase activity in the full activity control tubes	X
Percentage of control activity remaining in the presence of various inhibitor concentrations	X

DPM Disintegrations per minute

2. **Statistical Methods:** For data generated at CeeTox, basic statistical analysis was performed on the data, which included means of replicates, standard deviation of the mean, standard error of the mean, and coefficient of variation. Analysis of variance (ANOVA) was not performed.

   The response curve was fitted by weighted nonlinear regression analysis using a 4-parameter regression model (XLfit; IDBS; Version 5.2.0.0; Fit Model 208) and Tukey's Bi-Weight statistical analysis for outlier analysis. For each independent run of the test substance, the individual percent of control values were plotted versus logarithm of the test chemical concentration. The fitted concentration response curves were superimposed on the plot, with individual plots prepared for each run, and with plotted means. The fitted concentration response curves for each run were superimposed on the plots. On separate plots, the average percent of control values for each run were plotted versus logarithm of test substance concentration. The average concentration response curve across runs was superimposed on the same plot.

   Assay drift was assessed by comparing the % full activity and background activity at values at the beginning of each run to the end of each run.

3. **Interpretation of Results:** Interpretation of the assay results was based on the average of three runs, using the categories presented in Table 6.

TABLE 6. Interpretation of Results		
Criteria		**Interpretation**
Data fit 4-parameter nonlinear regression model	Average curve across runs crossed 50%[a]	Inhibitor
	Average lowest portion of curves across runs is between 50% and 75% activity[b]	Equivocal
	Average lowest portion of curves across runs is greater than 75% activity[b]	Non-inhibitor
Data do not fit model	---	

a Ordinarily, an inhibition curve will fall from 90% to 10% over 2 log units with a slope near −1. Unusually steep curves may indicate protein denaturing or solubility issues. If the slope of the curve is steeper than −2.0, the result is classified as equivocal.

b If the test compound was not soluble above 10^{-6} M and the inhibition curve does not cross 50%, the chemical is typically determined to be untestable in the aromatase assay.

II. RESULTS

A. CONTROL ACTIVITY: Aromatase activity in the full activity controls ranged from 0.507-0.668 nmol·mg-protein^{-1}·min^{-1} for the 3 test runs, with a mean and standard deviation of 0.608 ± 0.042 nmol·mg-protein^{-1}·min^{-1}. The response of each full activity control within a run was between 90 to 110% of the average full activity. Activity in the background controls ranged 0.24 to 0.37% and averaged 0.30% of the full activity controls.

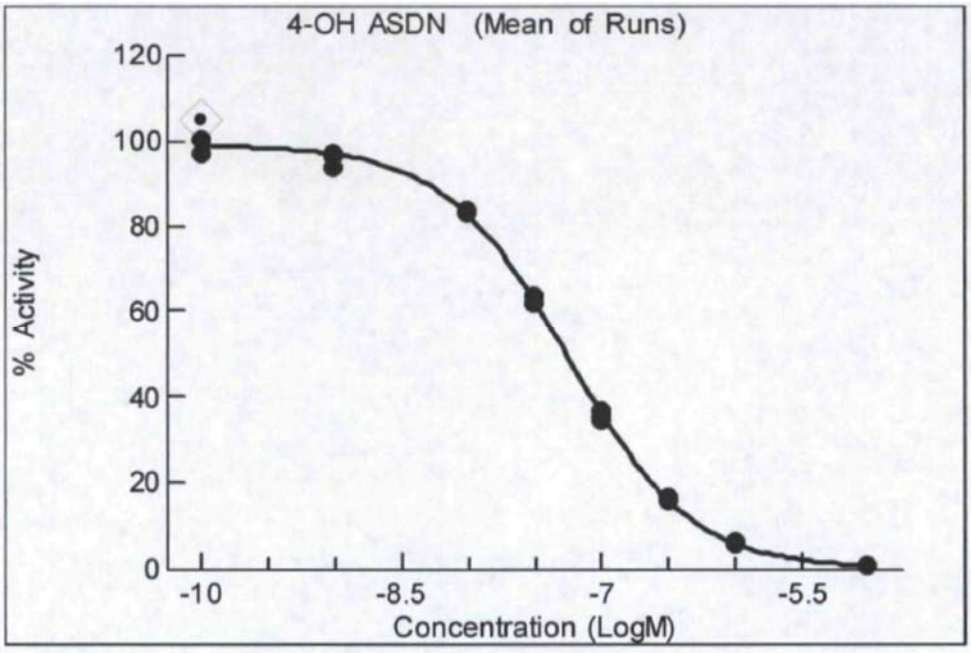
B. POSITIVE CONTROL: For the positive control substance (4-OH ASDN), aromatase activity averaged 0.619 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration 10^{-10} M and 0.005 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration 10^{-5} M. The mean aromatase activity of the positive control (expressed as % full control activity) for each concentration tested across all 3 runs is presented in Table 7, along with the overall standard deviation, SEM, and % CV. The average inhibition response curve for the positive control is shown in Figure 1.

TABLE 7. Effect of Folpet on Aromatase Activity (as percent of control) from Independent Runs[a]

Chemical	Concen. (Log M)	# Runs	Overall Mean[b]	Overall SD[b]	Overall SEM[b]	Overall % CV[b]
4-OH ASDN (positive control)	−5	3	0.75	0.02	0.01	2.5
	−6	3	6.20	0.36	0.21	5.8
	−6.5	3	16.06	0.12	0.07	0.8
	−7	3	35.37	1.15	0.66	3.2
	−7.5	3	62.18	0.82	0.47	1.3
	−8	3	83.15	0.46	0.27	0.6
	−9	3	95.48	1.76	1.02	1.8
	−10	3	100.64	3.83	2.21	3.8
Folpet	−5	3	57.32	1.61	0.93	2.8
	−6	3	85.42	3.16	1.82	3.7
	−7	3	97.13	1.27	0.73	1.3
	−8	3	99.48	3.28	1.90	3.3
	−9	3	101.00	1.79	1.03	1.8
	−10	3	100.23	3.29	1.90	3.3
	−11[c]	2	97.03	5.70	4.03	5.9

a Data were obtained from Appendix 1, pp. 41, 43, and 45 of the study report
b Calculated by the reviewers from data presented in this table.
c Data obtained for Runs 2 and 3; test concentration was not used for Run 1.
SD Standard Deviation
SEM Standard error of the mean
CV Coefficient of Variance

FIGURE 1. Average Inhibition Response Curve for 4-OH ASDN.



C. <u>TEST SUBSTANCE</u>: For folpet, aromatase activity averaged 0.606 ± 0.022 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration 10^{-11} M and 0.387 ± 0.105 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration used for study interpretation, 10^{-5} M. The 10^{-3} M and 10^{-4} M test concentrations used in Run 1 showed precipitation; therefore, the highest test concentration used in Runs 2 and 3 was 10^{-5} M. The mean aromatase activity of folpet (expressed as % full control activity) for each concentration tested across all 3 runs is presented in Table 7 (presented above), along with the overall standard deviation, SEM, and % CV. Inhibition response curves for folpet from each run are shown in Figure 2, and the average inhibition response curve across all runs is shown in Figure 3.

FIGURE 2. Inhibition Response Curves for Folpet From Each Test Run.

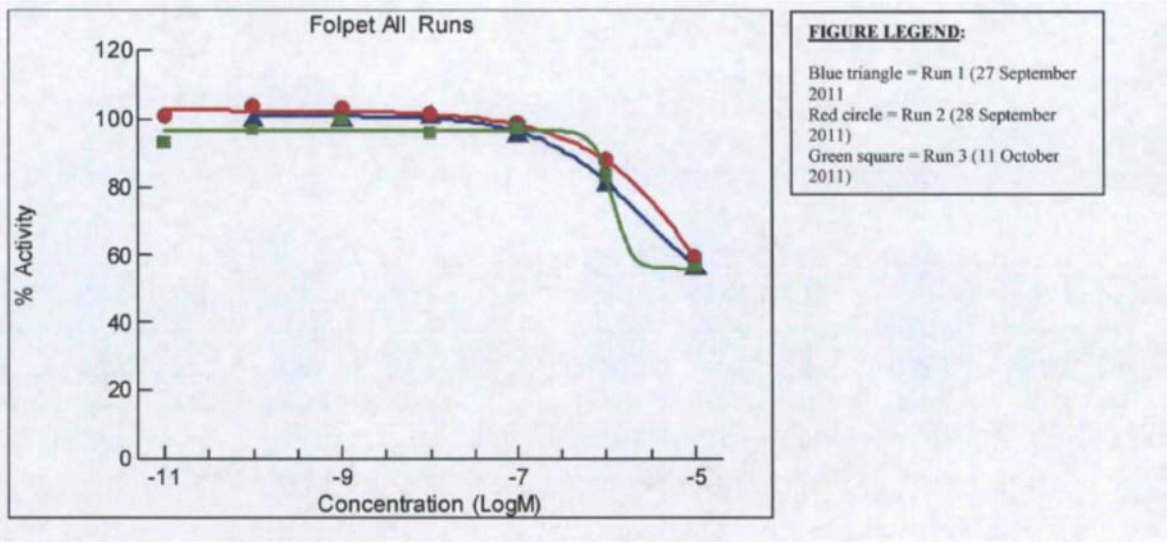
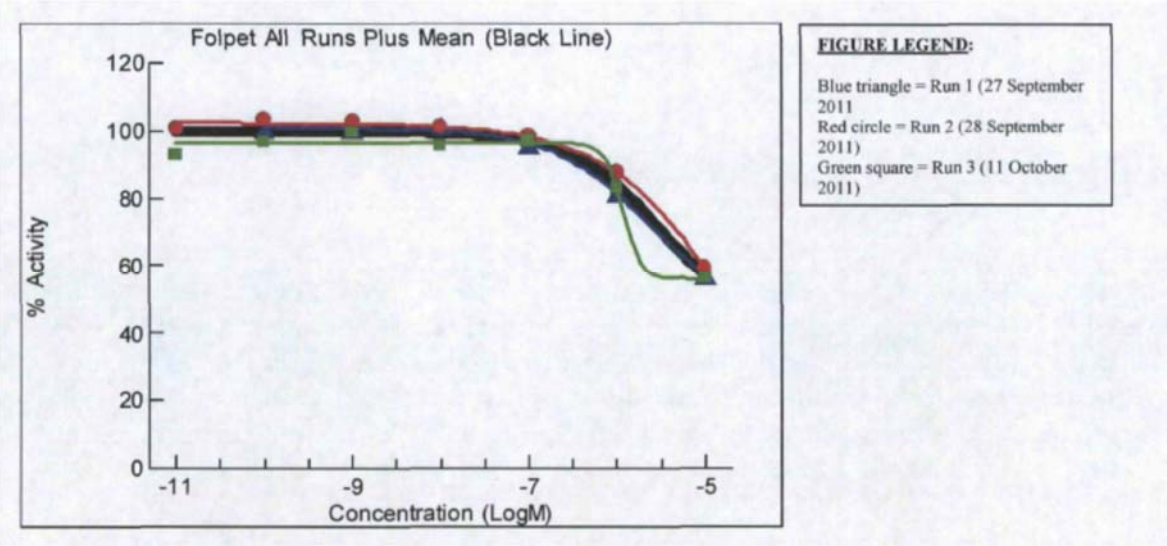


FIGURE 3. Mean Inhibition Response Curves for Folpet.



The data for folpet could not be fit to the 4-parameter nonlinear regression model; therefore, log IC_{50} and Hill slope estimates were not determined for folpet. For 4-OH ASDN, the estimated log IC_{50} averaged −7.27 M and the slope was −0.94 (Table 8). The variation in the positive control values was acceptable (<15% CV).

TABLE 8. Effect of Folpet on Aromatase Activity (as Percent of Control) From Independent Runs[a]						
Chemical	**Run 1**	**Run 2**	**Run 3**	**Mean[b]**	**SD[b]**	**%CV[b]**
Log IC_{50} (M)						
Folpet	NA	NA	NA	NA	NA	NA
4-OH ASDN	−7.28	−7.28	−7.25	−7.27	0.02	−0.24
Hill Slope						
Folpet	NA	NA	NA	NA	NA	NA
4-OH ASDN	−0.94	−0.89	−1.00	−0.94	0.06	−5.84

a Data were obtained from Table 14, page 29 of the study report
b Calculated by the reviewers from data presented in this table.
SD Standard Deviation
CV Coefficient of Variance
NA Not applicable

Based on the data from the average response curve and the criteria listed above in Table 8, the results indicate that folpet is an equivocal inhibitor of aromatase activity under the conditions of this assay. Mean aromatase activity for folpet was 57.32 ± 1.61% at the highest soluble test concentration of 10^{-5} M.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATORS CONCLUSIONS: Folpet was tested at final concentrations of 10^{-11} to 10^{-5} M. The 10^{-3} and 10^{-4} M concentrations from run 1 were not included in the analysis because of observed precipitation. Folpet at the highest soluble concentration of 10^{-5} M was determined to be 57.3% (+ 1.6% SD) of control activity, classifying it as equivocal for aromatase activity inhibition according to EDSP guideline OPPTS 890.1200.

B. AGENCY COMMENTS: Aromatase activity in the full activity controls was 0.608 ± 0.042 nmol·mg-protein^{-1}·min^{-1}. The response of each full activity control within a run was between 90 to 110% of the average full activity. Activity in the background controls ranged 0.24 to 0.37% and averaged 0.30% of the full activity controls.

For the positive control substance (4-OH ASDN), aromatase results were within the recommended ranges for the top of the curve, bottom curve, Hill slope, log IC_{50}, and %CV for replicates of each concentration within runs. The estimated log IC_{50} for 4-OH ASDN averaged −7.27 M and the Hill slope was −0.94.

For folpet, aromatase activity at the lowest and highest tested concentrations evaluated, 10^{-11} and 10^{-5} M, was 97.03 ± 5.70% and 57.32 ± 1.61% of the control. The data could not be fit to the 4-parameter nonlinear regression model. Since the average lowest portion of the response curve across runs was between 50 and 75% activity, folpet is classified as equivocal for aromatase inhibition in this assay.

C. STUDY DEFICIENCIES: The following deficiencies were noted that are not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- As recommended in the test guideline, mid-log concentrations (i.e. $10^{-5.5}$ M) of folpet should have been added in order to better define the response curve at concentrations approaching where precipitation was observed (see section (j) of OCSPP 890.1200).

- No proficiency data were provided. It was stated in the protocol (p. 70) that any available proficiency data would be provided as an appendix.

DATA EVALUATION RECORD

FOLPET

Study Type: OCSPP 890.1250, Estrogen Receptor Binding Assay

EPA Contract No. EP10H001452
Task Assignment No. 3-06-2012

(Revisions to MRID 48616903 to include Saturation binding data;
Main study was originally reviewed under TA 2-41-2012)

Prepared for
Health Effects Division
Office of Pesticide Programs
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2777 South Crystal Drive
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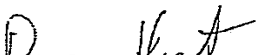
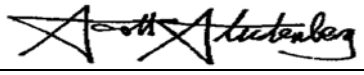
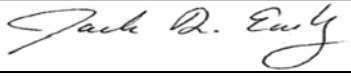
Primary Reviewer:	Signature:	
Daniel R. Hunt, M.S.	Date:	5/10/2012
Secondary Reviewer:	Signature:	
Scott D. Studenberg, Ph.D., D.A.B.T.	Date:	7/20/2012
Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	7/30/2012
Quality Assurance:	Signature:	
Jack D. Early, M.S.	Date:	7/30/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Ayaad Assaad, D.V.M., Ph.D. **Signature:**
Health Effects Division **Date:**
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:**
Health Effects Division **Date:**

Template version 08/2011

DATA EVALUATION RECORD

STUDY TYPE: Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC); OCSPP 890.1250

PC CODE: 081601 **DP BARCODE:** D398813

TXR#: 0055725 **CAS No.:** 133-07-3

TEST MATERIAL (PURITY): Folpet (94.5%)

SYNONYMS: 2-[(trichloromethyl)thio]-1H-isoindole-1,3(2H)-dione, Folpan Technical

CITATION: Willoughby, J.A. (2012). Folpet: Estrogen Receptor Binding Assay Using Rat Uterine Cytosol. CeeTox, Inc., Kalamazoo, MI. Laboratory Study No: 9141V-100357ERB, January 5, 2012. MRID 48616903. Unpublished.

Willoughby, J.A. (2012). Supplemental Information - Laboratory Proficiency Data for ERTA assays and Saturation Binding Data for AR and ER Binding Assays for Assorted Chemicals. CeeTox, Inc., Kalamazoo, MI. July 19, 2012. MRID 48843501. Unpublished.

SPONSOR: Makhteshim Chemical Works Ltd. c/o Makhteshim Agan of North America, Inc., 4515 Falls of Neuse Road, Suite 300, Raleigh, NC

TEST ORDER #: EDSP 081601-175

EXECUTIVE SUMMARY: In an estrogen receptor (ER) binding assay (MRID 48616903) for folpet (94.5% purity, Lot#: 00138518), uterine cytosol from Sprague Dawley rats was used as the source of ER for a competitive binding experiment, which measured the binding of a single concentration of [^{3}H]-17β-estradiol (1 nM) in the presence of increasing concentrations (10^{-11} to 10^{-4} M) of folpet. DMSO was used as the solvent vehicle at a final concentration of 4%. A total of three valid runs were performed, and each run included 19-norethindrone as a weak positive control, octyltriethoxysilane as a negative control, and 17-β-estradiol as the natural ligand reference material.

The saturation binding experiment was conducted to demonstrate that the ER in the rat uterine cytosol was present in reasonable numbers and was functioning with appropriate affinity for the radiolabeled reference estrogen prior to routinely conducting ER competitive binding experiments. Saturation binding data were not originally provided in the study report; however, summarized saturation binding data (MRID 48843501) from the performing laboratory were submitted following a request by the Agency. The protein concentrations used in the saturation

binding runs varied between each run, and were approximately 3- to 6-fold greater than recommended (160 to 320 µg versus 50 ±10 µg). The mean dissociation constant (K_d) for [^{3}H]-17β-estradiol was 0.331 ± 0.061 nM and the estimated B_{max} was 74.55 ± 3.03 fmol/100 µg protein for the prepared rat uterine cytosol. The K_d for each run was within the expected Guideline range of 0.03 to 1.5 nM. Although the Scatchard plots fit straight lines to the data, the concavity observed in all of the data sets may indicate issues with ligand depletion.

In the competitive binding experiment, the estimated mean log IC_{50} was −9.0 M for 17β-estradiol and −5.5 M for the weak positive control, 19-norethindrone. The mean relative binding affinity (RBA) was 0.0295% for the weak positive control. Performance criteria were generally met; however, octyltriethoxysilane displaced more than 25% of [^{3}H]-17β-estradiol from the ER at the highest concentration tested (10^{-3} M) in two of the three assay runs. One run was repeated.

The specific [^{3}H]-ligand binding was >75% at all folpet concentrations tested in all three runs. A log IC_{50} and RBA could not be calculated for folpet.

Based on the results from the three runs, folpet is classified as Not Interactive in the Estrogen Receptor Binding Assay.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for an Estrogen Receptor Binding assay (OCSPP 890.1250).

COMPLIANCE: Signed and dated GLP Compliance, Data Confidentiality, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

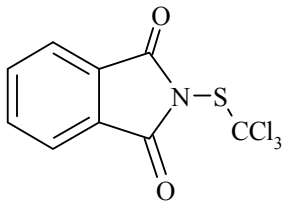
A. MATERIALS

1. Test Facility: CeeTox, Inc.

Location: Kalamazoo, MI
Study Director: Jamin A. Willoughby, Sr.
Other Personnel: Karen Rutherford, Director of Laboratory Operations
David Blakeman, Senior Scientist/Endocrine Group Leader
Cameron Haines, Scientist
Steven McColley. Scientist
Benjamin Meyer, Scientist
Colleen Toole, Director of Project Management
Study Period: June 6, 2011 to January 5, 2012

2. Test substance: Folpet

Description: Off-white powder
Source: Makhteshim Chemical Works Ltd., Beer Sheva, Israel
Batch #: 00138518
Purity: 94.5%
Solubility: Not reported (NR), but soluble in DMSO up to 100 mM (stock concentration)
Volatility: NR
Stability: NR
Storage conditions: Room temperature
CAS #: 133-07-3
Molecular weight: 296.56 g/mol
Structure:



3. Non-labeled ligand: 17β-estradiol

Supplier: Sigma-Aldrich, St. Louis, MO
Catalog #: E8875
Lot #: 110M0138V
Purity: 100%
CAS #: 50-28-2

4. Radioactive ligand: [^{3}H]-17β-estradiol

Supplier: NR
Catalog #: NR
Batch#: NR
Radiochemical purity: NR
Specific activity: 130.2 Ci/mmol (May 6, 2011)
Concentration of stock: 50 nM

5. Positive control: 19-Norethindrone

Supplier: Sigma-Aldrich, St. Louis, MO
Catalog #: N4128
Lot #: 030M1359V
Purity: 99%
CAS #: 68-22-4

6. Negative control: Octyltriethoxysilane

Supplier:	Sigma-Aldrich, St. Louis, MO
Catalog #:	440213
Lot #:	24996KK
Purity:	99.34%
CAS #:	2943-75-1

7. Solvent/vehicle control: Dimethyl sulfoxide (DMSO)

Justification for choice of solvent:	DMSO is one of the recommended solvents according to the EPA Guideline (OPPTS 890.1250)
Final Concentration:	4%

B. METHODS

1. Preparation of Rat Uterine Cytosol (RUC): Female Sprague Dawley rats (number not specified) from Harlan Laboratories were 12-13 weeks old at the time of euthanasia, and rats had been ovariectomized 7 days prior to tissue harvest. The uteri were weighed, and placed in ice-cold TEDG (Tris, EDTA, DTT, glycerol) + Protease Inhibitor (PI, unspecified) buffer, homogenized, and centrifuged for 10 min at 2,500 × *g* at 4ºC. Supernatant was transferred and centrifuged for 60 minutes at 105,000 × *g*, discarding the resulting pellets. Protein concentration of the cytosol was determined to be 1.10 mg/mL using a protein kit compatible with DTT in the TEDG buffer (Bradford Method). Cytosol was divided into 1- to 6-mL portions for immediate use or storage at −80ºC until use.

2. Saturation (Radioligand) Binding Experiment: A saturation binding experiment measuring total and non-specific binding of [^{3}H]-17β-estradiol was performed to demonstrate that the ER was present in reasonable concentrations and had the appropriate affinity for the native ligand (MRID 48843501). The conditions for the saturation binding are summarized in Table 1.

TABLE 1. Summary of Conditions for Saturation Binding Experiment[a]

Source of receptor		Rat uterine cytosol
Concentration of radioligand (as serial dilutions)		0.03 to 3 nM
Concentration of non-labeled ligand (100X [radioligand])		3 to 300 nM
Concentration of receptor		Sufficient to bind approximately 25 to 35% of radioligand at 0.03 nM
Temperature		4ºC
Incubation time		16 to 20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride (PMSF)	1 mM
	DTT	1 mM

a Data were obtained from page 1 of the study report (MRID 48843501).

On the day of the assay, the specific activity of the stock solution [^{3}H]-17β-estradiol (originally 130.2 Ci/mmol as manufactured on May 6, 2011) was adjusted for decay over time (adjusted specific activities were not reported), and serial dilutions in TEDG+PMSF

buffer were prepared to achieve the final concentrations of 0.03, 0.06, 0.08, 0.1, 0.3, 0.6, 1, and 3 nM. Solutions of non-labeled 17β-estradiol were prepared in a similar manner to achieve concentrations that were 100-fold greater than each respective radiolabeled concentration to result in final concentrations of 3, 6, 8, 10, 30, 60, 100, and 300 nM.

For each batch of cytosol, the optimal protein concentration was determined by testing serial amounts of protein per tube, using 0.03 nM radiolabeled estradiol, until a concentration was reached that bound approximately 25 to 35% of the total radioactivity added. The final protein concentrations were 320 µg, 192 µg and 160 µg per assay tube for the first, second and third saturation binding experiments, respectively (*Note: typically 50 ± 10 µg protein per tube)*. Each assay consisted of three non-concurrent runs (conducted on August 5, 6, and 7, 2011, respectively). Each run included three replicates of each test substance at each concentration, resulting in the 72 samples depicted in Table 2.

TABLE 2. Saturation Binding Experiment Run[a]

Total binding[b]	Non-specific binding[c]	Radioligand alone[d]	Assay Components
Tubes 1-24	Tubes 25-48	Tubes 49-72	
350 µL	300 µL	---	TEDG+PMSF buffer
50 µL	50 µL	50 µL	[^{3}H]-17β-estradiol (8 serial dilutions)[e]
---	50 µL	---	Non-labeled 17β-estradiol (8 serial dilutions, 100x each respective labeled concentration)[f]
100 µL	100 µL	---	Uterine cytosol (diluted to appropriate concentration)
500 µL	500 µL	50 µL	Total volume in each assay tube

a Data were obtained from page 2 of the study report (no MRID).
b Total binding = [^{3}H]-17β-estradiol bound to ER
c Non-specific binding = [^{3}H]-17β-estradiol and 100-fold greater non-labeled bound to ER
d Total [^{3}H]-17β-estradiol alone for dpm determination at each concentration
e Final concentrations of [^{3}H]-17β-estradiol = 0.03, 0.06, 0.08, 0.1, 0.3, 0.6, 1, and 3 nM.
f Final concentrations of non-labeled 17β-estradiol = 3, 6, 8, 10, 30, 60, 100, and 300 nM.

Tubes were incubated with gentle vortexing for 17.5-19 hours at approximately 4°C. To separate bound from free estradiol, hydroxyapatite (HAP) slurry was added to each tube and vortexed (3 times with 5-min intervals). Subsequently, the contents of each tube were washed three times as follows: 2-mL portions of TEDG+PMSF buffer were added, vortexed, centrifuged for 10 min at 1000 x *g*, and the supernatant decanted and discarded. After the final centrifugation, ethanol (1.5 mL) was added to the HAP pellet remaining in each tube to extract the [^{3}H]-17β-estradiol, followed by vortexing, and centrifugation for 10 min at 1000 x *g*. Aliquots (1 mL) of supernatant were radioassayed by scintillation counting. The temperature was maintained at approximately 4ºC throughout the assay prior to extraction with ethanol.

3. Competitive Binding Experiment: A summary of the experimental conditions for the Competitive Binding Experiment is included in Table 3.

TABLE 3. Summary of Conditions for Competitive Binding Experiment[a]		
Source of receptor		Rat Uterine Cytosol
Concentration of radioligand		1 nM
Concentration of receptor		Sufficient to bind 10-15% of radioligand[b]
Concentration of test substance (as serial dilutions)		10^{-11} to 10^{-4} M
Temperature		4±2 ºC
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	DTT	1 mM
	Protease Inhibitor (PI)[c]	0.5%

a Data were obtained from pages 14-15 of the study report.
b The protein concentration was 80.0 µg/assay tube (pp. 35, 38, and 41).
c The protease inhibitor was not specified, but the Guideline specifies the inclusion of 1 mM PMSF.

Solubility of folpet in DMSO and assay buffer was evaluated visually and no precipitation was noted. On the day of the assay, the specific activity of the stock solution [^{3}H]-17β-estradiol was adjusted for decay over time (adjusted specific activity of 126.6 Ci/mmol for Run 1; 126.5 Ci/mmol for Run 2, and 126.1 Ci/mmol for Run 3), and diluted in TEDG buffer to achieve a final concentration of 1 nM. The protein concentration used in the competitive binding experiment was 80.0 µg/assay tube, and was reported to be sufficient to bind 10-15% of the radioligand. Serial dilutions of the test substance, weak positive control (19-norethindrone), negative control (octyltriethoxysilane), and reference material (non-labeled 17β-estradiol) were prepared to achieve the concentrations shown in Table 4. Each assay consisted of three independent runs on three different days (one run was repeated because the positive and weak positive control data were too far outside of the acceptable range). Each run contained three replicates at each concentration plus six replicates of the [^{3}H]-17β-estradiol only control (total radioactivity) and triplicate replicates of the non-specific binding (NSB) and solvent controls at the beginning of each run, resulting in a total of 102 samples per run.

TABLE 4. Molar (M) concentrations in Competitive Binding Assay Run [a,b]			
Folpet	Positive control	Negative control	Reference Chemical
	19-Norethindrone	Octyltriethoxysilane	Non-labeled 17β-estradiol
Tubes 79-102 [c]	Tubes 31-54 [c]	Tubes 55-78 [c]	Tubes 7-30 [c]
10^{-11}	$10^{-8.5}$	10^{-10}	Solvent control [d]
10^{-10}	$10^{-7.5}$	10^{-9}	10^{-11}
10^{-9}	10^{-7}	10^{-8}	10^{-10}
10^{-8}	$10^{-6.5}$	10^{-7}	$10^{-9.5}$
10^{-7}	10^{-6}	10^{-6}	10^{-9}
10^{-6}	$10^{-5.5}$	10^{-5}	$10^{-8.5}$
10^{-5}	$10^{-4.5}$	10^{-4}	10^{-8}
10^{-4e}	10^{-4}	10^{-3}	10^{-7}

a Data were obtained from pages 12-14, 16, and 35-43 of the study report.
b Each tube contains: 10µL of either the test substance, positive control, negative control, solvent control, or non-labeled 17β-estradiol; 390 µL of TEDG+PI buffer with [^{3}H]-17β-estradiol; and 100 µL of uterine cytosol (with ER), for a total of 500 µL.
c Each concentration of each chemical was run in triplicate, for a total of 102 tubes per run.
d Solvent is DMSO (4%) in tubes 7-9.
e The highest folpet concentration for the ER assay tube was reported as 1 x 10^{-3} M on page 12 of the study report. Based on the language used in the study protocol (p. 59), the reviewer believes that folpet was not completely soluble in DMSO at 10^{-3} M and therefore the highest concentration used in the competitive binding assay was changed to 10^{-4} M.

Tubes were gently vortexed and incubated for 16-20 hours at 4±2°C. To separate bound from free estradiol, HAP slurry was added to each tube and vortexed once every 5 minutes for 15 minutes. Subsequently, the contents of each tube were washed three times as follows: TEDG+PI buffer was added, vortexed, centrifuged for 10 minutes at 1000 × *g*, and the supernatant decanted and discarded. Ethanol was added to the HAP pellet remaining in each tube to extract the bound [^{3}H]-17β-estradiol, followed by vortexing, and centrifugation for 10 minutes at 1000 × *g*. Aliquots of supernatant were radioassayed by liquid scintillation counting. The temperature was maintained at 4±2 ºC throughout the assay prior to extraction with ethanol.

C. <u>DATA ANALYSIS</u>: For the saturation binding experiment, total binding and non-specific binding data were modeled via non-linear regression by using Graph Pad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA)], incorporating automatic outlier elimination according to the method of Motulsky and Brown (2006)[1] implemented by using the ROUT procedure in Prism v. 5 with a Q value of 1.0. Receptor binding data plots were corrected for ligand depletion with the method of Swillens (1995)[2]. For the competitive binding assay, similar methods of nonlinear regression were used to fit a curve (for 17β-estradiol, the positive control, and the test substance) to the Hill equation formula which incorporated log IC_{50} as a parameter to be estimated. For parameters reported from the saturation binding experiment (K_d and B_{max}) and competitive binding experiment (log IC_{50} and RBA), mean and

1 Motulsky, H.J. and Brown, R.E. (2006) Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics, Vol 7, pp 123-142.
2 Swillens, S. (1995) Interpretation of binding curves obtained with high receptor concentrations: practical aid for computer analysis. *Molec. Pharmacol.* 47(6):1197-1203.

standard deviation were calculated for each run and mean and standard error were calculated for the composite three runs using Microsoft Excel 2007 (v. 12.0.6557.5000; Microsoft Corporation, Redmond, WA), and mean and standard error were calculated for the composite three runs with Microsoft Excel 2010.

1. Definitions

a. Classification of test material: Classification of the test material is based on the average of three runs. Each run was first individually classified as follows:

Interactive = lowest point on the fitted curve within the range of the data is less than 50% (i.e., >50% of the radiolabeled estradiol has been displaced from the ER).

Not interactive = there are usable data points at or above 10^{-6} M and either the lowest point on the fitted response curve within the range of the data is above 75% (i.e., <25% of the radiolabeled estradiol has been displaced from the ER) or a binding curve cannot be fitted and the lowest average percent binding among concentration groups in the data is above 75%.

Equivocal up to the limit of concentrations tested = If there are no data points at or above a test chemical concentration of 10^{-6}M and either a binding curve can be fit but ≤50% of the radiolabeled estradiol has been displaced from the ER or a binding curve cannot be fit and the lowest average percent binding among concentration groups in the data is >50%.

Equivocal = A run is classified as equivocal if it does not fall into any of the categories above.

The categorical classification of each run was assigned a numerical value as follows:

Run Classification	Numerical Value
Interactive	2
Equivocal	1
Not interactive	0
Equivocal up to the limit of concentrations tested	"missing"

The values for each run were then averaged across runs and the chemical classified using the following ranges:

Test Material Classification	Numerical Range
Interactive	average ≥1.5
Equivocal	0.5≥ average <1.5
Not interactive	average <0.5
Equivocal up to the limit of concentrations tested	"missing"

b. Descriptors for receptor binding:

B_{max}: maximum specific binding number (fmol ER/100 µg cytosol protein) measures the concentration of active receptor sites

K_d: dissociation constant (nM), measures the affinity of the receptor for its natural ligand

IC_{50}: concentration of the test substance (M) at which 50% of the radioligand is displaced from the receptor

Relative Binding Affinity (RBA %): (IC_{50} of 17β-estradiol ÷ IC_{50} of test substance) × 100

Log RBA: Log_{10}(IC_{50} of 17β-estradiol ÷ IC_{50} of test substance)

II. RESULTS

A. <u>SATURATION BINDING EXPERIMENT</u>: Figure 1 illustrates the non-specific, specific, and total binding curves for [^{3}H]-17β-estradiol to the ER for the three independent runs. The specific binding reached a plateau in each run, and non-specific binding was less than 20% of total binding. Figure 2 contains the Scatchard plots that illustrate the binding of [^{3}H]-17β-estradiol to the ER.

The parameters determined from the saturation binding experiment are presented in Table 5. The mean K_d for [^{3}H]-17β-estradiol was 0.331 nM (± 0.061), and the estimated B_{max} was 74.55 fmol/100 µg protein (± 3.03) for the prepared rat uterine cytosol. The K_d for each run was within the expected range of 0.03 to 1.5 nM. Although the Scatchard plots fit straight lines to the data, the concavity observed in the data sets may indicate issues with ligand depletion. Confidence in these numbers is high due to the goodness of fit and the small variation among runs.

TABLE 5. Saturation Binding Experiment of [^{3}H]-17β-estradiol with Estrogen Receptor from Rat Uterine Cytosol[a]

Parameter	Run 1	Run 2	Run 3	Mean ± SE[b]
R^2 (unweighted)	0.976	0.982	0.980	0.976-0.982
B_{max} (nM)	0.148	0.102	0.080	0.110±0.035
B_{max} (fmol/100 µg protein)	69.25	79.76	74.65	74.55±3.03
K_d (nM)	0.453	0.286	0.255	0.331±0.061

a Data were obtained from page 3 of the study report (MRID 48843501).
b The range of R^2 is reported and the mean ± SE is reported for the other parameters.
R^2 Goodness of fit for curve calculated for specific binding.

FIGURE 1. Binding of [^{3}H]-17β-estradiol to the Estrogen Receptor during the Saturation Binding Experiment.

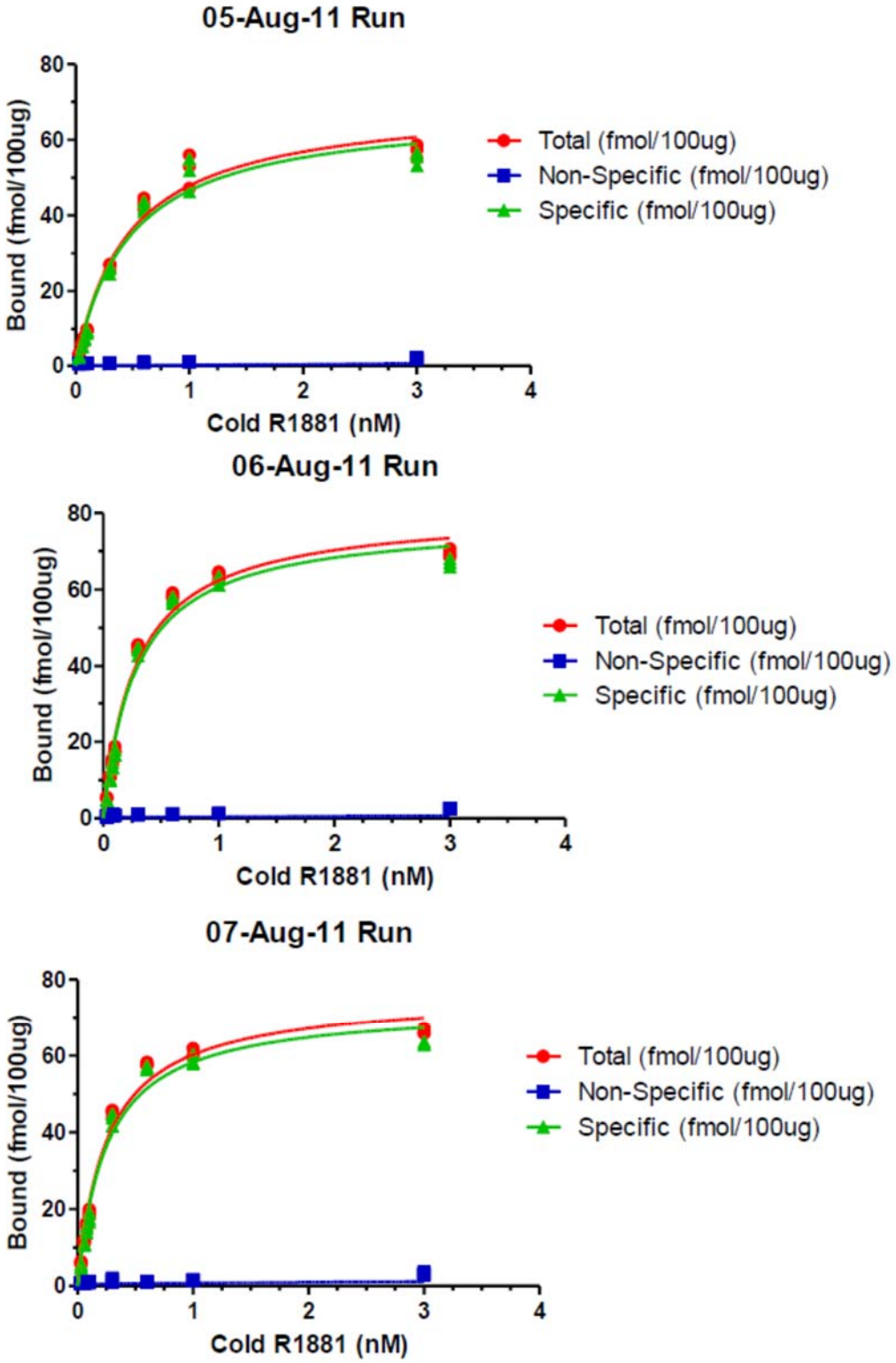
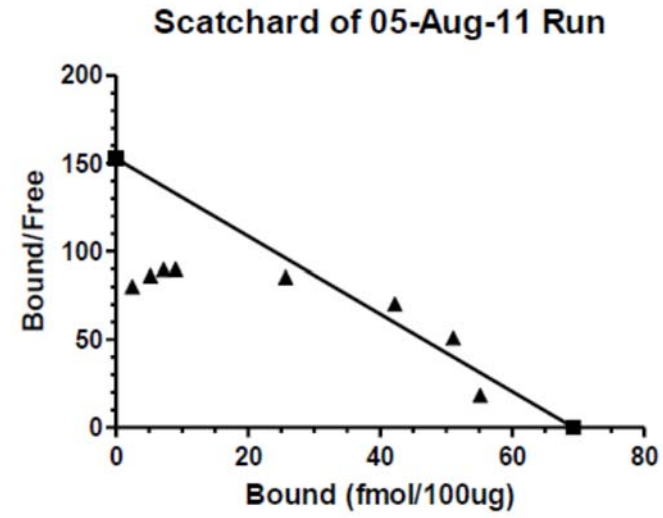
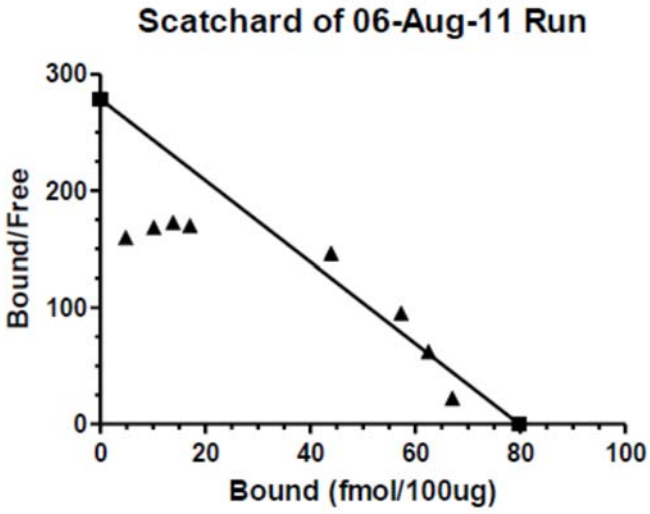
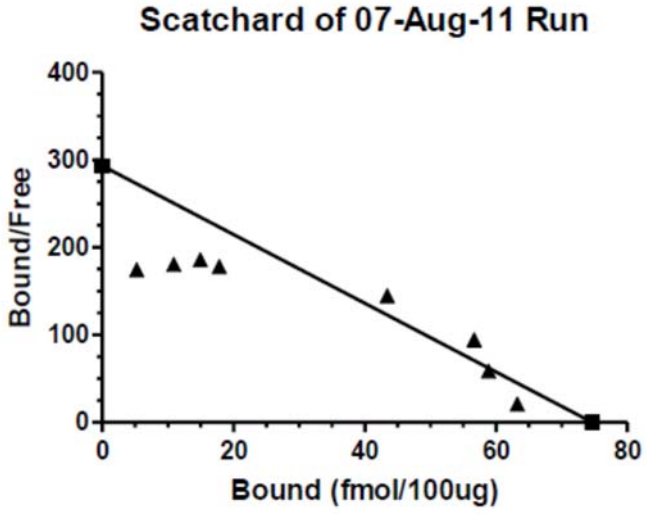


FIGURE 2. Scatchard Plots of the Binding of [^{3}H]-17β-estradiol to the Estrogen Receptor.







B. COMPETITIVE BINDING EXPERIMENT: The results from the competitive binding experiments are presented graphically in Figures 3-5. The parameters determined from the competitive binding curves (IC_{50}, Log IC_{50}, and RBA) are presented in Table 6.

Since the specific [^{3}H]-ligand binding was >75% at all soluble folpet concentrations tested in all three runs, folpet is classified as "not interactive" (0) in this assay (Table 7).

The three reference chemicals generally performed as expected. The estimated mean log IC_{50} was −9.0 M for 17β-estradiol and −5.5 M for the weak positive control. The mean RBA was 0.030% for the weak positive control. Mean specific binding of [^{3}H]-17β-estradiol in the negative control assays were 44.4% in Run 1 and 41.5% in Run 2 at a concentration of 10^{-3} M octyltriethoxysilane, which is lower than the tolerance limit (≥75%) for the negative control. The study author stated that binding of octyltriethoxysilane to [^{3}H]-17β-estradiol at 10^{-3} M has been previously observed in other studies, although typically accompanied by precipitation, which was not observed in this study. Excluding the highest concentration (10^{-3} M) in Run 1 and Run 2, the negative control, octyltriethoxysilane, had no effect on specific binding of the [^{3}H]-ligand (84.8 to 101.3%). Confidence in these numbers is high due to the small variation. One run was unacceptable due to positive and weak positive control data too far outside of the acceptable range, and the run was repeated. Data from the unacceptable run were not reported.

TABLE 6. Competitive Binding Assay of Folpet with Estrogen Receptor from Rat Uterine Cytosol [a]

Parameter		Run 1	Run 2	Run 3	Mean ± SE [b]
R^2 (unweighted)	17β-estradiol	NR	NR	NR	NA
	19-norethindrone	NR	NR	NR	NA
	Folpet	NR	NR	NR	NA
Log IC_{50} (M)	17β-estradiol	−9.1	−9.0	−8.9	−9.0 ± 0.058
	19-norethindrone	−5.5	−5.5	−5.4	−5.5 ± 0.033
	Folpet	NA	NA	NA	NA
IC_{50} (M) [b]	17β-estradiol	7.94×10^{-10}	1.00×10^{-9}	1.26×10^{-9}	1.02×10^{-9} (± 0.13)
	19-norethindrone	3.16×10^{-6}	3.16×10^{-6}	3.98×10^{-6}	3.44×10^{-6} (± 0.27)
	Folpet	NA	NA	NA	NA
Log RBA (%)[b]	19-norethindrone	−3.60	−3.50	−3.50	−3.53 ± 0.03
	Folpet	NA	NA	NA	NA
RBA (%)[b]	19-norethindrone	0.025	0.032	0.032	0.030 ± 0.002
	Folpet	NA	NA	NA	NA

a Data were obtained from pages 21-22 of the study report.

b All values are reviewer-calculated from the log IC_{50} values reported in the study report. For means expressed in scientific notation, the SE values in parentheses are presented in the same order of magnitude as the mean value.

SE = Standard Error
NA = Not applicable.
NR = Not reported
R^2 = Goodness of fit
RBA (%) = relative binding affinity

TABLE 7. Binding Classification of Folpet with Estrogen Receptor[a]					
Run	1	2	3	Mean[c]	Binding Classification[d]
Classification category value[b]	0	0	0	0	Not interactive

a Data were obtained from pages 21-22 of the study report.
b Classification category value: Interactive = 2; Equivocal = 1; Not interactive = 0; Equivocal up to the limit of concentrations tested ("missing", i.e., not included in calculation of mean).
c Mean of three runs expressed to the tenths place.
d Interactive = mean ≥1.5; Equivocal = 0.5≤ mean <1.5; Not interactive = mean <0.5.

FIGURE 3. Percentage [^{3}H]-E2 Bound to the Estrogen Receptor in the Presence of Unlabeled E2, 19-Norethindrone, Octyltriethoxysilane or Folpet, Run 1.

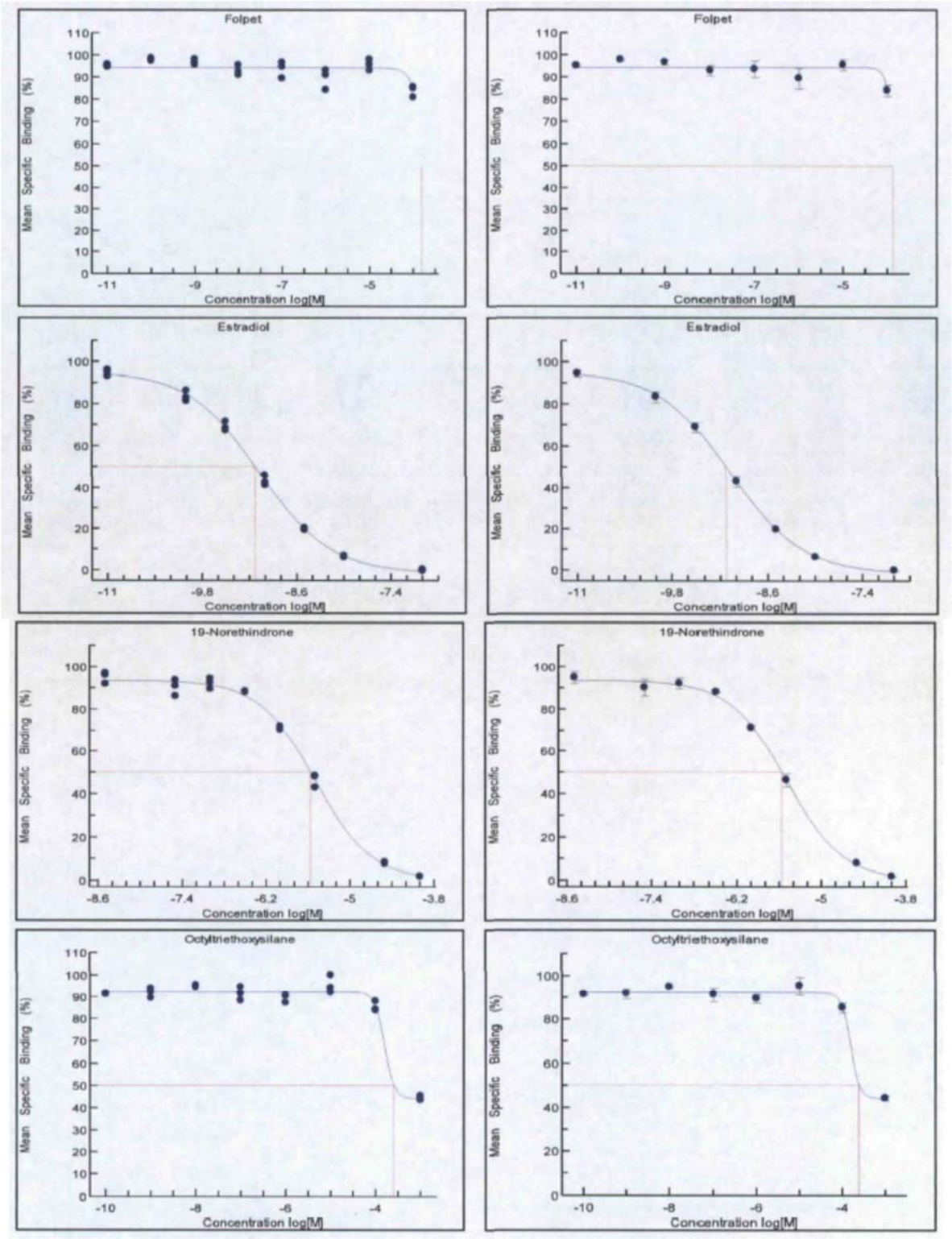


FIGURE 4. Percentage [^{3}H]-E2 Bound to the Estrogen Receptor in the Presence of Unlabeled E2, 19-Norethindrone, Octyltriethoxysilane or Folpet, Run 2.

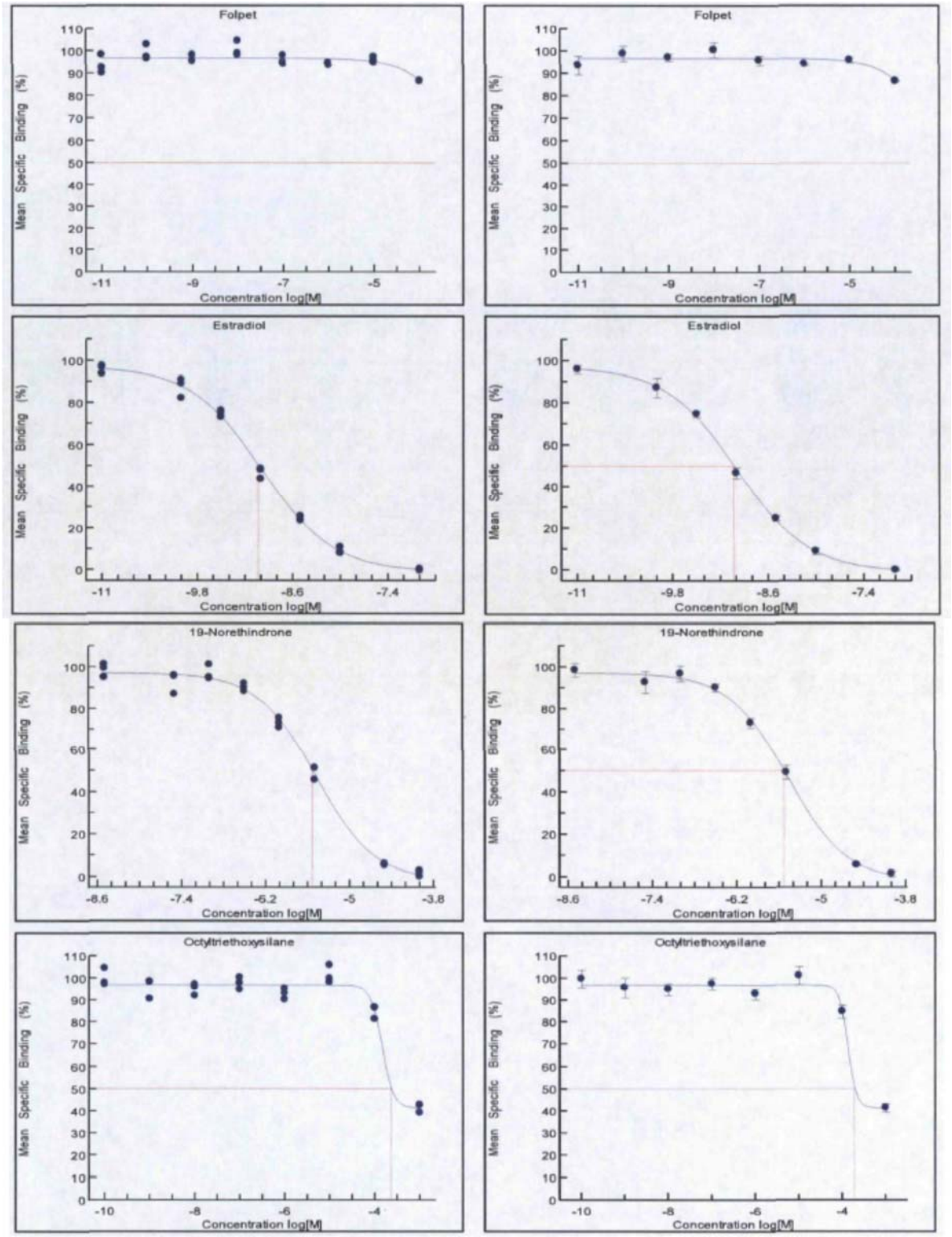
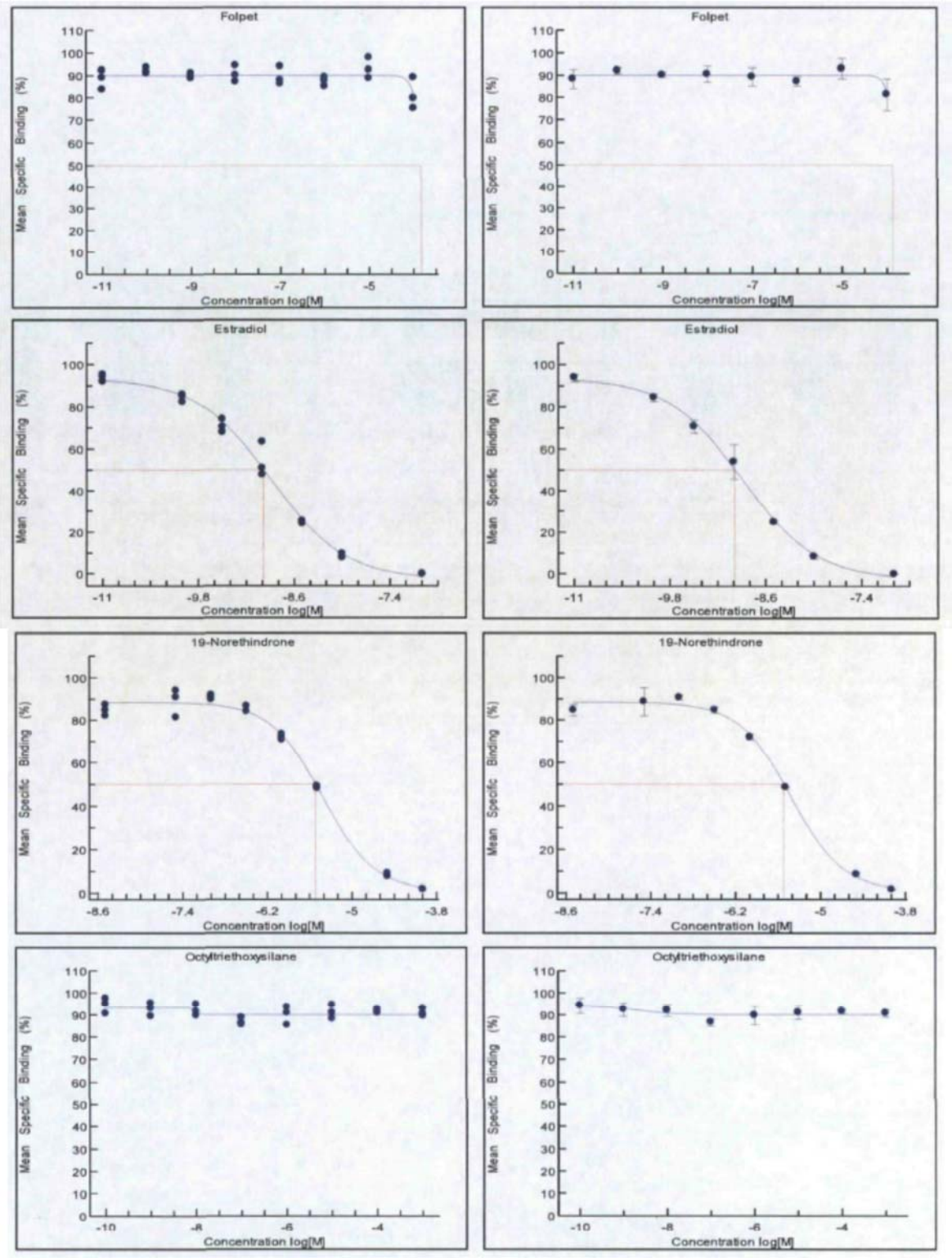


FIGURE 5. Percentage [^{3}H]-E2 Bound to the Estrogen Receptor in the Presence of Unlabeled E2, 19-Norethindrone, Octyltriethoxysilane or Folpet, Run 3.



C. PERFORMANCE CRITERIA: To ensure that the competitive binding assay functioned properly, each run was evaluated using the criteria shown in Table 8.

TABLE 8. Criterion [a]	Tolerance Limit(s)	Value	Yes	No
17β-estradiol fitted curve parameters				
Log_e residual SD	≤2.35	0.5 to 1.31	X	
Top (% binding)	94 to 111	94 to 97	X	
Bottom (% binding)	−4 to 1	-3 to -1	X	
Hill Slope ($Log_{10}(M)^{-1}$)	−1.1 to −0.7	-1.0 to -0.9	X	
Weak Positive control (19-norethindrone) fitted curve parameters[b]				
Log_e residual SD	NA	0.91 to 1.20		
Top (% binding)	NA	89 to 97		
Bottom (% binding)	NA	-2 to 1		
Hill Slope ($Log_{10}(M)^{-1}$)	NA	-1.2 to -1.0		
Solvent concentration				
DMSO	≤10%	4%	X	
Negative control (octyltriethoxysilane) does not displace more than 25% of [^{3}H]-17β-estradiol from the ER on average across all concentrations	≤25%	≤58.5%		X[c]

a Data were obtained from pages 24-29 of the study report.
b The EPA Guideline does not define a set of tolerance limits for 19-norethindrone. Acceptance criteria were only defined for norethynodrel, which cannot be obtained commercially. The values reported were considered acceptable as they show 19-norethindrone to be an acceptable weak positive control.
c Reviewer-calculated. The minimum specific binding of [^{3}H]-17β-estradiol in the negative control assays were 44.4% in Run 1 and 41.5% in Run 2 at 10^{-3} M; however, excluding these two replicates at the highest test concentration, octyltriethoxysilane did not displace more than 25% of [^{3}H]-17β-estradiol from the ER (minimum specific binding of 84.8%).
NA = Not applicable

Additionally, the curve for the reference material showed that increasing concentrations of unlabeled 17β-estradiol displaced [^{3}H]-17β-estradiol in a manner consistent with one-site binding, as indicated by a descent from 83.5-87.3% to 6.4-9.0% binding over approximately an 81-fold increase in concentration of the test chemical (i.e., covering approximately 2 log units).

Folpet was tested over a concentration range that fully defined the top of the curve. The percent binding at this top plateau 88.6-98.9% was within 25 percentage points of the value for the lowest concentration of the estradiol standard 93.9-96.0%.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Folpet was classified as "non-interacting" in all three valid independent runs and thus has a final classification of "non-interacting" for the estrogen receptor.

B. AGENCY COMMENTS: In the saturation binding experiment, the protein concentrations used for the assay varied greatly between each run, and were approximately 3- to 6-fold

greater than recommended. The K_d for [^{3}H]-17β-estradiol was 0.331 nM and the estimated B_{max} was 74.55 fmol/100 µg protein for the prepared rat uterine cytosol. The K_d for each run was within the expected Guideline range of 0.03 to 1.5 nM. Although the Scatchard plots fit straight lines to the data, the concavity observed in all of the data sets may indicate issues with ligand depletion.

For the competitive binding experiment, the estimated mean log IC_{50} was −9.0 M for 17β-estradiol and −5.5 M for the weak positive control. The mean RBA was 0.030% for the weak positive control. All performance criteria were generally met; however, octyltriethoxysilane displaced more than 25% of [^{3}H]-17β-estradiol from the ER at the highest concentration tested (10^{-3} M) in two of the three assay runs. One run was repeated.

Folpet is classified as not interactive in this assay as the mean specific [^{3}H]-17β-estradiol bound to the ER was >75% at folpet concentrations up to 10^{-4} M in all three independent runs.

C. <u>STUDY DEFICIENCIES:</u> The following deficiencies were noted that were not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- The protein concentrations used in the saturation binding runs varied between each run, and were approximately 3- to 6-fold greater than recommended.
- Performance criteria were not met for octyltriethoxysilane in Runs 1 and 2 at 10^{-3} M.
- Curves were not provided showing the average binding of each test substance across all three runs.
- Details of the [^{3}H]-17β-estradiol used in the assay were not reported (i.e. source, Lot number, radiochemical purity).

DATA EVALUATION RECORD

FOLPET

Study Type: OCSPP 890.1300, Estrogen Receptor Transcriptional Activation

EPA Contract No. EP10H001452
Task Assignment No. 3-06-2012

(Revisions to MRID 48616904 to include laboratory proficiency data;
Main study was originally reviewed under TA 2-41-2012)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
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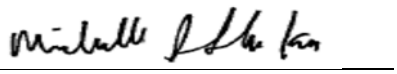
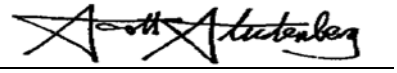
Durham, NC 27713

Primary Reviewer:	Signature:	
Michelle Sharpe-Kass, M.S.	Date:	7/06/2012
Secondary Reviewer:	Signature:	
Scott D. Studenberg, Ph.D., D.A.B.T.	Date:	7/11/2012
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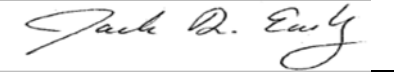
Jack D. Early, M.S.	Date:	7/20/2012
Quality Assurance:	Signature:	
Jack D. Early, M.S.	Date:	7/20/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel.

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Ayaad Assaad, D.V.M., Ph.D. **Signature:** AAssaad
Health Effects Division **Date:** 6/2/2015

Secondary Reviewer: Minerva Mercado-Feliciano, Ph.D. **Signature:**
Health Effects Division **Date:** 6·9·15

DATA EVALUATION RECORD

STUDY TYPE: Estrogen Receptor Transcriptional Activation (Human cell Line, HeLa-9903); OCSPP 890.1300; OECD 455.

PC CODE: 081601 **DP BARCODE:** D398813

TXR#: 0055725 **CAS No.:** 133-07-3

TEST MATERIAL (PURITY): Folpet (94.5% a.i.)

SYNONYMS: 2-[(trichloromethyl)thio]-1H-isoindole-1,3(2H)-dione, Folpan Technical

CITATION: Willoughby, J.A. (2012) Folpet: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903)). CeeTox, Inc., Kalamazoo, MI. Laboratory Report No.: 9141V-100357ERTA, January 5, 2012. MRID 48616904. Unpublished.

SPONSOR: Makhteshim Chemical Works Ltd. c/o Makhteshim Agan of North America, 4515 Falls of Neuse Road, Suite 300, Raleigh NC

TEST ORDER #: EDSP-081601-175

EXECUTIVE SUMMARY: In an estrogen receptor transcriptional activation assay (MRID 48616904), hERα-HeLa-9903 cells cultured *in vitro* were exposed to folpet (94.5% a.i., Lot # 00138518) at logarithmically increasing concentrations from 10^{-12} M to 10^{-4}M in DMSO (0.1%) in two independent runs. Experiments were performed using 96-well plates and each folpet concentration was tested in 6 wells/plate in each run. Cells were exposed to test agents for approximately 24 hrs to induce reporter (luciferase) gene products. Luciferase expression in response to estrogen receptor activation was measured using a proprietary assay.

No cytotoxicity was observed at 10^{-4} M folpet in the range-finding test, however cytotoxicity was observed at that concentration in the main assays. The mean RPC_{Max} was 3.5% for the first run and 3.9% for the second, and the associated PC_{Max} was 10^{-7} M.

In the main assays, the responsiveness of the cells to the very weak positive control 17α-methyltestosterone was lower than the expected values, indicating a decreased sensitivity of the assay to very weak agonists. Furthermore, 17α-methyltestosterone responses reached only 6.3-7.9% PC and were very similar to those of the negative control corticosterone, which averaged 2.6-3.7% PC. Therefore, under the conditions of this study, the reviewer cannot determine if folpet is a very weak estrogen. Nevertheless, the responses of folpet (3.5-3.9% PC)

were not comparable to the response of the weak estrogen control, 17α-estradiol (117-124% PC) and the $RPC_{Max} < PC_{10}$ in both assay runs.

This assay **satisfies** the EDSP Tier 1 Test Order requirement for an Estrogen Receptor Transcriptional Activation assay (OCSPP 890.1300).

COMPLIANCE: Signed and dated GLP Compliance, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

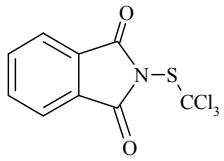
A. MATERIALS

1. Test Facility: CeeTox, Inc.

Location: Kalamazoo, MI
Study Director: J.A. Willoughby
Other Personnel: D. Blakeman (Senior Scientist); C. Haines (Scientist); S. McColley (Scientist); Benjamin Meyer (Scientist), B. Wallace (Lead Cell Culture Scientist); and C. Toole (Director of Project Management)
Study Period: June 06, 2011 to January 05, 2012

2. Test Substance: Folpet

Description: Technical, off-white powder, MW 299.56 g/mol
Source (cat#): Makhteshim Chemical Works Ltd. (Not Reported)
Lot/Batch # (Exp. Date): 00138518 (May 26, 2012)
Purity: 94.5%
Solubility: Soluble in DMSO
Volatility: Not reported
Stability: Two years
Storage conditions: Ambient
CAS #: 133-07-3
Structure:



3. Reference substances

17β-estradiol (strong estrogen; positive control)
Supplier: Sigma-Aldrich, St. Louis, MO
Catalog and Batch #: E8875
Purity: 110M0138V
CAS # : 50-28-2

17α-estradiol (weak estrogen)
Supplier: Sigma-Aldrich, St. Louis, MO
Catalog and Batch #: E8750
Purity: 041M4065V
CAS # : 57-91-0

Corticosterone (negative compound)
Supplier: Sigma-Aldrich, St. Louis, MO
Catalog and Batch #: 27840
Purity: BCBC6322V
CAS # : 50-22-6

17α-methyltestosterone (very weak agonist)
Supplier: Sigma-Aldrich, St. Louis, MO
Catalog and Batch #: M7252
Purity: 060M1543V
CAS # : 58-18-4

4. Vehicle(s)

Solvent: DMSO
Solvent control: 0.1% (final concentration)

B. METHODS

1. **Cell Culture:** Stably-transfected hERα-HeLa-9903 cells obtained from the Japanese Collection of Research Bioresources Cell Bank were verified to be free of mycoplasma infection using DNA Fluorochrome analysis. Cells were maintained in Eagles Minimum Essential Medium without phenol red, supplemented with 60 mg/L kanamycin and 10% dextran-coated charcoal-treated fetal bovine serum (source not reported) in an incubator under 5% CO_2 at 37ºC. Upon reaching 75-90% confluence, cells were subcultured at least twice prior to exposure to the test material. The cells used in this study were Passage 21(rangefinder), and Passage 19 and 20 (test runs) prior to seeding into plates.

2. **Transcriptional Activation Assays:** For each test, cells were plated at a density of $1x10^4$ cells/100 µL medium/well in a 96 well plate and allowed to attach for 3 hrs. Growth medium was replaced with medium containing serial log dilutions in DMSO (0.1% total final concentration). Cells were incubated for 24±2 at 37±2°C. Cytotoxicity was determined by propidium iodide uptake. Runs 1 and 2 for the main test were conducted on August 23 and August 25, 2011. Transcriptional activation of the estrogen receptor was determined as described in CeeTox Standard Operating Protocol 2041. A list of reagents was provided, but the assay reagent was classified as proprietary information.

a. **Preliminary Test:** A preliminary test evaluating concentrations ranging from 10^{-4} to $10^{-7.5}$M was conducted to determine the appropriate concentration range and to determine concentrations resulting in insolubility and/or cytotoxicity.

b. **Proficiency Chemicals:** Responsiveness of the test system was tested on March 5, April 12 and April 28, 2011 (MRID 48843501), using cells at Passage 15, 25 and 28, respectively. Based on passage number and main assay dates, it is unlikely the cells used for proficiency testing were from the same frozen stock as the cells used in the main assay. The cells were tested using the following set of proficiency chemicals in duplicate on separate days:

Compound	CAS No.	Concentration Range (M)	Expected Response [a]	Notes
Diethylstilbestrol (DES)	56-53-1	10^{-14} to 10^{-8}	Positive	---
17α-Ethynyl estradiol (EE)	57-63-6	10^{-14} to 10^{-8}	Positive	---
Hexestrol	84-16-2	10^{-13} to 10^{-7}	Positive	---
Genistein	446-72-0	10^{-12} to 10^{-5}	Positive	Cytotoxic at 0.01[b], 0.1, and 1 mM
Estrone	53-16-7	10^{-12} to 10^{-6}	Positive	---
Butyl paraben	94-26-8	10^{-11} to 10^{-4}	Positive	Cytotoxic at 0.1[b] and 1 mM
1,3,5-Tris(4-hydroxyphenyl)benzene [c]	15797-52-1	10^{-12} to 10^{-5}	Positive	Cytotoxic at 100µM. PC_{max} approx. 50% of PC. Binds to hERα and has ER antagonistic activity
Dibutyl phthalate (DBP)	84-74-2	10^{-11} to 10^{-4}	Negative [d]	Cytotoxic at 1 mM
Atrazine	1912-24-9	10^{-11} to 10^{-4}	Negative	Cytotoxic at 1 mM [b]
Corticosterone	50-22-6	10^{-10} to 10^{-4}	Negative	If not cytotoxic at 1 mM, then that should be the highest tested concentration

a Positive = RPC_{max} ≥10% of the response of the positive control in at least 2 of 2 (or 2 of 3) runs
Negative = RPC_{max} fails to achieve at least 10% of the response of the positive control in 2 of 2 (or 2 of 3) runs
b Cytotoxicity is expected to be close to 80% at this concentration.
c Compound selected to challenge solubility and cytotoxicity.
d DBP is negative for ERα mediated transcriptional activation, but may not be negative for non-ERβ mediated transcriptional activation. A positive result would indicate that the system is detecting activity other than that due to pure ERα, and is therefore unacceptable.

c. Reference Chemicals: To ensure the stability of the response from the cell line, eight concentrations of each of the following reference chemicals were included in each plate in the current assay, along with the test chemical:

Reference Chemical	CAS No.	Concentration Range	Class
17β-estradiol (E2)	50-28-2	10^{-15} to 10^{-8}	Strong estrogen
17α-estradiol	57-91-0	10^{-13} to 10^{-6}	Weak estrogen
Corticosterone	50-22-6	10^{-11} to 10^{-4}	Negative compound
17α-methyltestosterone	58-18-4	10^{-12} to 10^{-5}	Very weak agonist

3. Data analysis: To obtain the relative transcriptional activity to the 1 nM E2 positive control (PC), the luminescence signals from the concurrent plate were analyzed by subtracting the mean value of the vehicle control from each well value to normalize the data; each normalized value was then divided by the mean value of the normalized PC. The resulting value was multiplied by 100 in order to express relative transcriptional activity as a percentage of the PC. The test material was defined as negative for inducing estrogen receptor transcriptional activation if the $RPC_{Max} < PC_{10}$ in at least 2 of 2 runs. Log EC_{50} and Hill slope values are calculated only if a positive response is observed. Coefficients of variation (CV) were calculated for the luminescence data triplicates. Concentrations showing >20% cytotoxicity or evidence of insolubility were excluded from analyses.

4. Definitions

EC_{50} = concentration of agonist that induces a response halfway between the baseline (bottom) and maximum (top) response

PC_{10} = concentration of a test chemical at which the response is 10% of the response induced by the positive control (E2 at 1 nM) in each plate

PC_{50} = concentration of a test chemical at which the response is 50% of the response induced by the positive control (E2 at 1 nM) in each plate

RPC_{Max} = maximum level of response induced by a test chemical, expressed as a percentage of the response induced by the positive control (1 nM E2) on the same plate

PC_{Max} = concentration of a test chemical inducing the RPC_{Max}

II. RESULTS

A. PRELIMINARY TEST: In order to identify a suitable top concentration for use in the transcriptional activation assays, preliminary cytotoxicity and precipitation assays were conducted. Precipitation or cytotoxicity was not observed in any of the folpet solutions up to 10^{-4} M (Table 1). Based on these results, logarithmically increasing concentrations from 10^{-11} to 10^{-4} M were selected for the assay.

TABLE 1. Preliminary Test for Solubility, Cytotoxicity, and Concentration-Selection for Folpet[a]

Concentration (M)	% Viability	Comments
10^{-4}	97	
$10^{-4.5}$	99	
10^{-5}	94	
$10^{-5.5}$	95	
10^{-6}	93	
$10^{-6.5}$	97	
10^{-7}	96	
$10^{-7.5}$	98	

a Data were obtained from page 19 of the study report.

B. POSITIVE AND NEGATIVE REFERENCE CHEMICALS

1. Proficiency Chemicals: The laboratory proficiency assays using the required reference compounds were not included in the original study report, but were provided to the Agency at a later date (MRID 48843501). The responsiveness of cells to the required proficiency chemicals was performed in duplicate on different days for each chemical. The reported responses are in Table 2a. In the proficiency tests, the reference chemicals 17β-estradiol, 17α-estradiol and 17α-methyltestosterone were tested concurrently with each run of the assay (Table 2b). In the first run, the responsiveness of 17β-estradiol indicated decreased sensitivity to strong agonists, and the response to 17α-methyltestosterone showed an increased responsiveness to very weak agonists; despite the minor deviations this run is considered acceptable. Run 2 was inadequate as the PC_{50} could not be calculated for 17α-methyltestosterone indicating a decreased sensitivity to very weak agonists. Run 3 was acceptable as 17β-estradiol and 17α-methyltestosterone performed within the expected range, but the Hill Slope for 17α-estradiol was higher than expected. The PC-induced fold induction for the three reference chemicals was within the Guideline-recommended historical range of 4- to 30-fold in Runs 1 and 3, but fold induction was 75.1- to 84.7-fold in Run 2 with no explanation given for this 3- to 4-four increase. Although reportedly performed, the results of the cytotoxicity assay were not provided for review. Raw data pertaining to the RTA of each chemical were not reported, but the scales of the graphs provided indicate Genistein and Butyl paraben had maximum RTAs well above 400%. The responses do not demonstrate proficiency as the assay cannot be fully evaluated by the reviewer.

TABLE 2a. Proficiency Chemicals				
Compound	**Expected Response**	**Lab Response**		
		Run 1	**Run 2**	**Run 3**
Diethylstilbestrol	Positive	Positive	Positive	NA
17α-Ethynyl estradiol	Positive	Positive	Positive	NA
Hexestrol	Positive	Positive	Positive	NA
Genistein	Positive	Positive	Positive	NA
Estrone	Positive	Positive	Positive	NA
Butyl paraben	Positive	Positive	Positive	NA
1, 3, 5-Tris(4-hydroxyphenyl)benzene	Positive	NA	Positive	Positive
Dibutyl phthalate	Negative	Negative	Negative	NA
Atrazine	Negative	Negative	NA	Negative
Corticosterone	Negative	Negative	NA	Negative

NA Not applicable. The chemical was not tested at this time.

Table 2b. Performance Criteria for Reference Chemicals in the Proficiency test						
Reference Chemical Parameter	**Acceptable Range**	**Values**			**Acceptable**	
		Run 1	**Run 2**	**Run 3**	**Yes**	**No**
17β-estradiol						
Log PC_{50}	−11.4 to −10.1	−9.6	−11.3	−10.6		Run 1
Log PC_{10}	<−11	−11.5	−12.5	−12.1	X	
Log EC_{50}	−11.3 to −10.1	−9.0	−11.3	−10.6		Run 1
Hill Slope	0.7 to 1.5	1.2	0.9	0.8		
Test range (M)	10^{-14} to 10^{-8}	10^{-14} to 10^{-8}	10^{-14} to 10^{-8}	10^{-14} to 10^{-8}	X	
17α-estradiol						
Log PC_{50}	−9.6 to −8.1	−8.3	−9.4	−8.7	X	
Log PC_{10}	−10.7 to −9.3	−9.3	−10.5	−9.9	X	
Log EC_{50}	−9.6 to −8.4	−8.2	−9.3	−8.9		Run 1
Hill Slope	0.9 to 2.0	0.9	0.9	2.9		Run 3
Test range (M)	10^{-12} to 10^{-6}	10^{-12} to 10^{-6}	10^{-12} to 10^{-6}	10^{-12} to 10^{-6}	X	
17α-methyltestosterone						
Log PC_{50}	−6.0 to −5.1	−6.2	NC	−5.2		Run 1, 2
Log PC_{10}	−8.0 to −6.2	−8.1	−6.3	−7.7		Run 1
Test range (M)	10^{-11} to 10^{-5}	10^{-11} to 10^{-5}	10^{-11} to 10^{-5}	10^{-11} to 10^{-5}	X	

2. **Reference Chemicals:** Values derived from the concentration response curve (*e.g.*, Log PC_{50}, Log PC_{10}, Log EC_{50}, Hill slope) for the four concurrently run reference materials are included in Table 3. The acceptance criteria were not met for either run of the assay. In the first run, the cell's responsiveness to 17α- and 17β-estradiol was greater than that of the PC (113-126%). The response of the cells to 17α-methyltestosterone was <10% for any concentration, and cytotoxicity was observed for both corticosterone and 17α-methyltestosterone.

TABLE 3. Performance Criteria for Reference Chemicals[a]					
Reference Chemical Parameter	Acceptable Range	Values		Acceptable	
		Run 1	Run 2	Yes	No
17β-estradiol					
Log PC_{50}	−11.4 to −10.1	-10.6	-10.2	X	
Log PC_{10}	<−11	-11.4	-11.3	X	
Log EC_{50}	−11.3 to −10.1	-10.5	-10.1	X	
Hill Slope	0.7 to 1.5	1.5	1.2	X	
Test range	10^{-14} to 10^{-8} M	10^{-15} to 10^{-8}	10^{-15} to 10^{-8}		X[b]
17α-estradiol					
Log PC_{50}	−9.6 to −8.1	-8.5	-8.4	X	
Log PC_{10}	−10.7 to −9.3	-9.6	-9.4	X	
Log EC_{50}	−9.6 to −8.4	-8.5	-8.4	X	
Hill Slope	0.9 to 2.0	1.2	1.3	X	
Test range	10^{-12} to 10^{-6} M	10^{-13} to 10^{-6}	10^{-13} to 10^{-6}		X[b]
Corticosterone					
Test range	10^{-10} to 10^{-4} M	10^{-11} to 10^{-4}	10^{-11} to 10^{-4}		X[c]
17α-methyltestosterone					
Log PC_{50}	−6.0 to −5.1	NC	NC		X
Log PC_{10}	−8.0 to −6.2	NC	NC		X
Test range	10^{-11} to 10^{-5} M	10^{-12} to 10^{-5}	10^{-12} to 10^{-5}		X[c]

a Data were obtained from pages 20 and 22 of the study report.
b Inappropriately high responses (>100%) were observed in both runs these reference chemicals
c Cytotoxicity was observed in both runs of these reference chemicals
NC Not calculable. The maximum response of the cells to 17α-methyltestosterone was <10% for both runs

C. DEFINITIVE ASSAY

1. **Vehicle and Positive Controls:** Data for the vehicle and positive controls are included in Table 4. The overall mean TA value for the vehicle control was 10333 for the first run and 9196 for the second, and the overall mean TA value for the positive control was 190842 for the first run and 135375 for the second. The induction for the positive control ranged from 15- to 20-fold. The mean normalized value for the positive control was 180509 for the first run and 126179 for the second. The PC_{50} (50% of the maximum response) for E2 in this assay is 90254 for the first run and 63090 for the second and the PC_{10} (10% of the maximum response) is 18051 for the first run and 12618 for the second.

TABLE 4. Transcriptional Activation (TA) Response of Vehicle and Positive Control [a]							
Sample	Vehicle Control		Positive Control [b]			Normalized Positive Control [b]	
Runs	Mean	SD	Mean	SD	Fold Induction [c]	Mean	SD
1	10333	1922	190842	32222	20	180509	32222
2	9196	1179	135375	10725	15	126179	10725

a Data were calculated by reviewers from data obtained on pages 30-31 of the study report.
b Positive control was 17β-estradiol (E2) at 1 nM.
c Fold-induction = (mean TA of PC)/(mean TA of VC)

2. **Test Material:** Relative (to the PC) transcriptional activation at each concentration of the test chemical during the two assay runs is presented in Table 5. The concentration-response curves depicting fold induction of relative transcriptional activation is presented in Figure 1 below. The mean RPC_{Max} was 3.5% for the first run and 3.9% for the second, and the

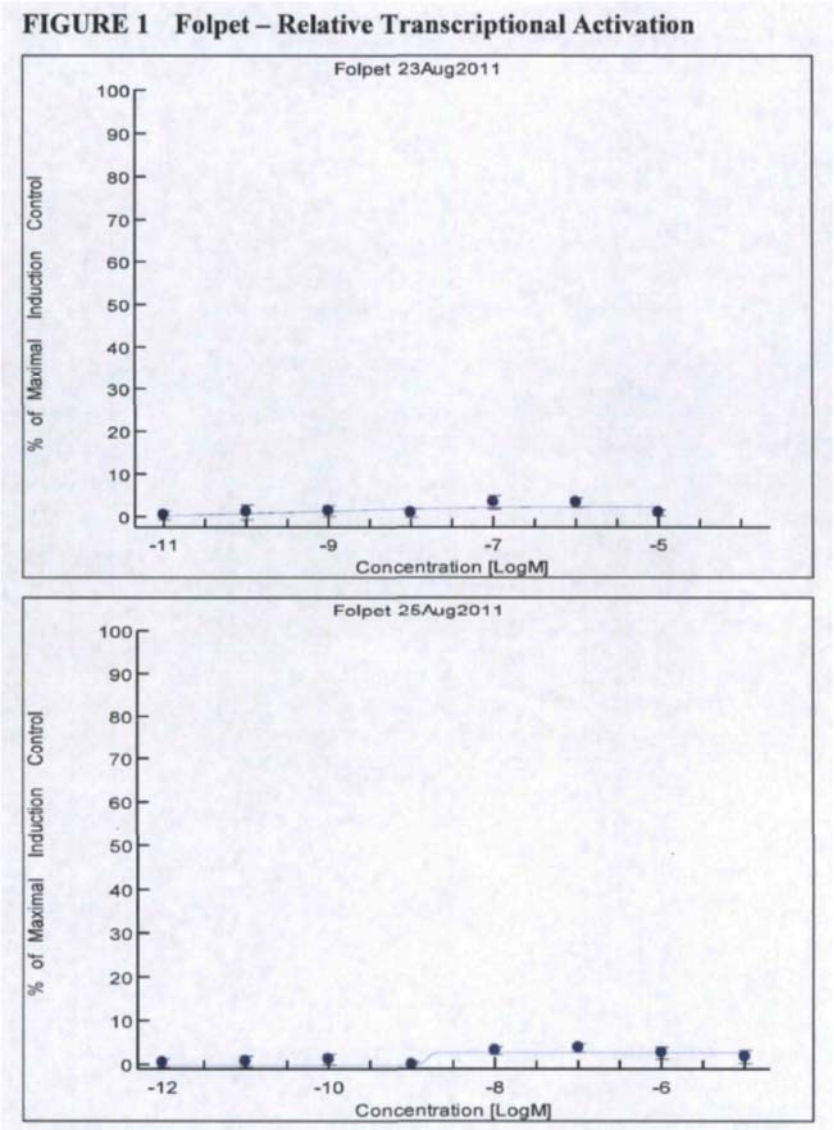
associated PC_{Max} was 10^{-7} M. Because the $RPC_{Max} < PC_{10}$ in both runs, folpet was considered negative for estrogen receptor transcriptional activation in this test system.

TABLE 5. Relative Transcriptional Activation (RTA) of Folpet[a]

Parameter	RTA (mean ± SD); % of Positive Control (PC)			
	Run 1		Run 2	
Conc. (M)	Mean	SD	Mean	SD
10^{-4}	NC[b]	NC[b]	--	--
10^{-5}	1.1	0.7	1.9	1.5
10^{-6}	3.5	1.2	2.8	1.4
10^{-7}	3.5	1.6	3.9	0.9
10^{-8}	1.0	1.0	3.3	0.9
10^{-9}	1.5	1.3	0.0	0.7
10^{-10}	1.2	1.7	1.3	1.2
10^{-11}	0.5	1.0	0.7	1.2
10^{-12}	--	--	0.5	1.0
Log EC_{50}[b]	NA		NA	
Hill Slope[b]	NA		NA	
RPC_{Max}	3.5		3.9	
PC_{Max}	10^{-7}		10^{-7}	
PC_{50}	NA		NA	
PC_{10}	NA		NA	

a Data were obtained from pages 20-21 of the study report
b Not Calculable due to cytotoxicity
NA Not Applicable

FIGURE 1. Fold Induction of Relative Transcription Activation (RTA) of Folpet Compared to the Positive Control.



3. **<u>Performance Criteria</u>:** While the Log PC_{50}, Log PC_{10}, Log EC_{50}, and Hill slope values for the concurrent reference chemicals fell within or near the acceptable ranges (Table 3), the full response of the cells to the reference chemicals was not satisfactory. In the first run, the cell's responsiveness to 17α- and 17β-estradiol was greater than that of the PC (113-126%). The response of the cells to 17α-methyltestosterone was <10% for any concentration, and cytotoxicity was observed for both corticosterone and 17α-methyltestosterone. Cytotoxicity should not be observed at any level for the reference chemicals, and the maximum responsiveness should be observed in the positive control, 1 nM 17-estradiol. Due to this, the ability of this test system to observe transcriptional changes in response to chemical exposure was not considered valid.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATORS' CONCLUSIONS: The suitable top concentration of folpet for use in the transcriptional activation assays was 10^{-5}M, based on excessive cytotoxicity at concentrations ≥ 10^{-4} M identified in the first run. In two independent runs of the transcriptional activation assay, folpet did not result in an increase in luciferase activity <10% at any of the viable concentrations tested. Folpet is not an agonist of the human estrogen receptor alpha hERα in the HeLa-9903 model system.

B. AGENCY COMMENTS: Folpet was tested up to and including the limit of cytotoxicity, 10^{-4}M. In the main assays, the responsiveness of the cells to the very weak positive control 17α methyltestosterone was lower than the expected values, indicating a decreased sensitivity of the assay to very weak agonists. Furthermore, 17α-methyltestosterone responses reached only 6.3-7.9% PC and were very similar to those of the negative control corticosterone, which averaged 2.6-3.7% PC. Therefore, under the conditions of this study, the reviewer cannot determine if folpet is a very weak estrogen. Nevertheless, the responses of folpet (3.5-3.9% PC) were not comparable to the response of the weak estrogen control, 17α-estradiol (117-124% PC) and the RPCMax < PC10 in both assay runs.

C. STUDY DEFICIENCIES: The following deficiencies were noted:

- In both assays, cytotoxicity was observed in cells treated with corticosterone and 17α-methyltestosterone, and the cells responded inappropriately to 17β estradiol and 17α-estradiol.
- Certificates of Analyses were not provided for the Reference Chemicals.
- The source of the fetal bovine serum was not provided.

Data Requirement:	EPA DP Barcode	404648
	OECD Data Point	
	EPA MRID	48684201
	EPA Guideline	890.1350, Fish Short-Term Reproduction Assay

Test material: **Folpet** **Purity:** 94.5%

Common name

Chemical name: IUPAC:

CAS name:

CAS No.: 133-07-3

Synonyms: Folpan Technical

EPA PC Code: 081601

Primary Reviewer: Joan Gaidos **Signature:**

Senior Scientist, CDM Smith **Date:** 06/03/2013

Secondary Reviewer: Teri S. Myers **Signature:**

Project Manager, CDM Smith **Date:** 07/18/2013

Primary Reviewer: Michael Lowit, Ph.D. **Signature:** Digitally signed by MICHAEL LOWIT DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=MICHAEL LOWIT, dnQualifier=0000041144 Date: 2015.06.04 14:34:55 -04'00'

USEPA/OCSPP/OPP/EFED/ERB1 **Date:** 07/29/2013

Additional Reviewer: Justin Housenger **Signature:** Digitally signed by JUSTIN HOUSENGER DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=JUSTIN HOUSENGER, dnQualifier=0000044455 Date: 2015.06.04 14:56:21 -04'00'

USEPA/OCSPP/OPP/EFED/ERB5 **Date:** 12/06/2013

Final Additional Reviewer: Robin Sternberg **Signature:** Digitally signed by ROBIN STERNBERG DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=ROBIN STERNBERG, dnQualifier=0000039126 Date: 2015.06.04 13:56:43 -04'00'

USEPA/OCSPP/OPP/EFED/ERB1 **Date:** 05/27/2015

Date Evaluation Completed: 05/27/2015

CITATION: D.O. York. 2012. Folpet: Short-term Reproduction Assay with the Fathead Minnow (*Pimephales promelas*) Following OPPTS 890.1350 and OECD 229 Guidelines. Performed by Smithers Viscient Wareham, Massachusetts Lab Study No.: 11742.6178; Sponsor Project No.: R-28297. Submitted by Makhteshim Agan of North America, Inc., Raleigh, North Carolina. Completion date July 27, 2012.

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Disclaimer: The guideline recommendations in this DER template are offered as a general reference to aid in preparation of the DER. The purpose of these recommendations is not to serve as substitute for the Test Guidelines, nor to provide any guidance on how the study should be conducted.

EXECUTIVE SUMMARY:

The 21-day short-term reproduction assay of folpet with fathead minnows (*Pimephales promelas)* was conducted under continuous flow-through conditions. Adult fish (20 spawning groups; 2 males and 4 females in each group; 4 groups per treatment; *ca*. 6 months old), were exposed to folpet (94.5% purity) at nominal concentrations of 0 (negative and solvent [0.010 mL/L dimethylformamide, DMF] controls), 0.00054, 0.0036, and 0.024 mg a.i./L; mean-measured concentrations were <0.000031 (<LOQ, controls), 0.00018, 0.0010, and 0.0086 mg a.i./L. The test system was maintained at 24 to 25°C and a pH of 6.8 to 7.4.

There were no significant differences ($p>0.05$) between the negative and solvent controls for any of the endpoints. Unless otherwise indicated in this DER, all effects are reported based on comparison to the negative control.

Adult survival was unaffected by treatment with folpet; survival was 100% for all test levels and controls, except for females in the solvent control which showed 94% survival. No abnormal observations of secondary sexual characteristics (*e.g.*, body color, coloration patterns, body shape, size of dorsal nape pad in males and ovipositor size in females) or clinical signs of toxicity were observed during the exposure period or at study termination. There were no effects on body weight or length at any treatment level compared to the negative control.

In the negative control group, spawning occurred at least every four days in three of the four replicates; mean fecundity was 14 eggs/female/day/replicate; and fertilization success averaged 98%. Replicate B in the negative control averaged 12 eggs/female/reproductive day while another replicate C averaged 7.5 eggs/female/reproductive day and did not spawn at least every four days. In the solvent control group, spawning occurred at least every four days during the exposure period; mean fecundity was 16 eggs/female/day/replicate; and fertilization success averaged 98%. Replicate B in the solvent control averaged 14 eggs/female/reproductive day.

There were no significant differences ($p>0.05$) between the treatment groups and the negative control for any endpoint except male vitellogenin (VTG), which showed a significant increase ($p=0.0049$; Jonckheere-Terpstra test) at the high treatment level. The mid and high treatment groups had only three replicates available for

male VTG analysis because there were no data available for one replicate; no explanation was provided by the study authors for the missing data.

Treatment-related effects were not observed for histopathology severity scores or gonadal stage. However, there was a slight increase in granulomatous inflammation and increased oocyte atresia (females) with increasing folpet concentration. For four, six, and five cases from the low, mid, and high treatment groups, respectively, the oocyte atresia and/or granulomatous inflammation was attributed to *Microsporidia* infection.

All performance and validity criteria were met for this assay with the following two exceptions. Replicate C of the negative control averaged 7.5 eggs/female/reproductive day, which is less than the guideline criterion of 15 eggs/female/reproductive day, and did not spawn at least every four days. The coefficient of variation (CV) for the mean-measured concentration of the low treatment group was 21.8%, exceeding the guideline criterion of <20%. In general, analytical verification of the test material from Days 0, 7, 14, and 21 yielded mean recoveries of 35, 28 and 36% at the low, mid, and high treatment levels, respectively. The test material does not appear to be stable under the test conditions, and recoveries were consistently poor. The study author reported that these low recoveries were within expectations for the test substance, which is known to undergo rapid hydrolysis. These deviations did not impact the interpretation of the study.

This assay **satisfies** the EDSP Tier 1 Test Order requirements for a Fish Short-Term Reproduction Assay (OCSPP Guideline 890.1350).

Results Synopsis

Test Organism age at test initiation: *ca*. 6 months
Mean body weight at test initiation: Male 2.9 g; Female 1.4 g
Mean length at test initiation: Not reported
Test Type: Continuous flow-through

Table 1: Summary of Reproductive and HPG Effects[1,2] in the Fish Short-Term Reproduction Assay (FSTRA) with Folpet.

Treatment (mg a.i./L) [mean-measured]	Fecundity	Fert. Success	Tubercle Score		GSI		Gonadal Histo.		Plasma VTG		Plasma T		Plasma E2	
			M	F	M	F	M	F	M	F	M	F	M	F
0.00018	No	No	No	No	No	No	No	No	No	No	NA	NA	NA	NA
0.0010	No	No	No	No	No	No	No	No	No	No	NA	NA	NA	NA
0.0086	No	No	No	No	No	No	No	No	Yes[3]	No	NA	NA	NA	NA

Abbreviations: Diff. Difference. E2 17β-estradiol. F Female. Fert. Fertilization. GSI Gonado-Somatic Index. Histo. Histopathology. M Male. NA Not applicable. T Testosterone.

VTG Vitellogenin.

1 A "yes" indicates a significant difference based on comparison to the negative (clean water) control, unless otherwise specified.

2 The criteria for significance are described in the Reviewer's Analysis and Statistical Verification sections of the DER. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

3 A significant increase was detected at the high treatment level (p=0.0049; Jonckheere-Terpstra). The results of the analysis of male VTG was based on the mid and high treatment concentrations having data for three replicates available for analysis and the negative control and low treatment level having data for four replicates available for analysis.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: This study was conducted according to the U.S. EPA OCSPP 890.1350: "Fish Short-Term Reproductive Assay" and OECD 229 (2009). The following deviations were noted:

1. Replicate C of the negative control averaged 7.5 eggs/female/reproductive day, which is less than the guideline criterion of 15 eggs/female/reproductive day, and did not spawn at least every four days.

2. The coefficient of variation (CV) for the mean-measured concentration of the low treatment group was 21.8% exceeding the guideline criterion of 20%. Analytical verification of the test material from Days 0, 7, 14, and 21 at nominal concentrations of 0.00054, 0.0036 and 0.024 mg a.i./L yielded mean recoveries of 35, 28 and 36%, respectively; the % CVs were 21.8, 16.7 and 7.8%, respectively. The test material does not appear to be stable under the test conditions and recoveries were consistently poor. The study author reported that these low recoveries were within expectations for the test substance, which is known to undergo rapid hydrolysis. The diluter cycle rate was set at the maximum in an effort to maintain consistent exposure concentration. The study author reported that pre-test (Day -3) samples from two replicates of each treatment level and control were collected, analyzed, and the results used to judge whether the diluter was functioning properly; however, these data were not reported.

3. The unionized ammonia and residual chlorine in the test water were not reported. The OCSPP 890.1350 performance criteria establish maximum levels for these values, and it is unclear if the maximum recommendations were exceeded. The dissolved oxygen decreased to ≤60% of saturation on Day 12 (32%), prior to scraping and siphoning all exposure systems; OCSPP 890.1350 guideline requirements recommend dissolved oxygen (DO) $\geq$4.9 mg/L (>60% air saturation). Additionally, the light intensity was reported to range from 650 to 1100 lux during exposure; OCSPP 890.1350 guideline requirements recommend light intensity 540 – 1080 lux (at water's surface).

4. Individual fish weights at study initiation were ±31% according the study author. Based on the provided information, the reviewer calculated that individual weights were within ±19%. EPA recommends that the subsample of fish weighed before the test be within ±20% of the estimated mean for each sex.

These deviations do not impact the interpretation of the study.

COMPLIANCE: Signed and dated No Data Confidentiality, GLP, and Quality Assurance statements were provided. This study was conducted in compliance with all pertinent U.S. EPA Good Laboratory Practice regulations with the following exceptions: routine food and water screening analyses were conducted at GeoLabs, Inc., Braintree, Massachusetts using standard U.S. EPA procedures and are considered facility records under Smithers Viscient's Standard Operating Procedures. Since the analyses were conducted following standard validated methods, this exception has no impact on the study results.

A. TEST MATERIAL: **Folpet (CAS# 133-07-3)**

Description: Not reported

OECD recommends describing water solubility, melting/boiling point stability in water and light, pKa, Pow or Kow, vapor pressure of test compound, expiration date.

Lot No./Batch No. : 00138518

Purity: 94.5%

Impurities: None reported

Stability of Compound: Analytical verification of the test material from Days 0, 7, 14, and 21 at nominal concentrations of 0.00054, 0.0036 and 0.024 mg a.i./L yielded mean recoveries of 35, 28 and 36%, respectively; the %CV was 21.8, 16.7

and 7.8%, respectively. Quality control samples of folpet in dilution water fortified at concentrations of 0.00024, 0.0035, and 0.024 mg a.i./L yielded recoveries of 80 to 102%. Method validation of folpet fortified at 0.000005, 0.001 and 0.01 mg a.i./L in 0.2% formic acid in FETAX solution yielded recoveries of 102 ± 5.5% (n=9). Analysis of exposure solutions during the pre-test period showed that concentrations of folpet in the exposure system were consistently lower than nominal. The test material does not appear to be stable under the test conditions and recoveries were consistently poor. The study author reported that these low recoveries were within expectations for the test substance, which is known to undergo rapid hydrolysis.

Storage Conditions of Test Chemicals: Stored at room temperature in dark, ventilated cabinet.

B. Test organism:

Table 2: General Information About the Test Species and Acclimation.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Species common name:	Fathead Minnow		*EPA recommends fathead minnow (Pimephales promelas).*
Species scientific name:	*Pimephales promelas*		
Species strain (if stated):	Not reported		
Were fish obtained from a single laboratory stock?	Yes	In-house stock	*EPA recommends that fish be from a single laboratory stock.*
Were acclimation conditions same as definitive test?	Yes		*EPA recommends that fish be acclimated under water quality and illumination conditions that are similar to the definitive test.*
Acclimation period:	2 weeks		*EPA recommends a minimum two-week acclimation period. Note that the acclimation period is different from the subsequent, in situ pre-exposure phase.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Details on health:		There were no mortalities during the 7 days prior to pre-exposure period. Fish did not receive any treatment for disease during acclimation period. Behavioral abnormalities or clinical signs were not reported.	*EPA recommends that mortality during the 7 days prior to the pre-exposure phase be less than 5% of the culture population. If mortality during these 7 days is greater than 10%, EPA recommends that the fish be rejected. If mortality is between 5-10%, EPA recommends that fish be held another 7 days. If mortalities greater than 5% occur during this extended acclimation period, EPA recommends that the fish not be used.*
Type of food:	Live brine shrimp nauplii (*Artemia salina*)		*EPA recommends that fish be fed frozen brine shrimp twice per day to promote active reproduction and maintain body condition.*
Source of food:	Not reported		
Frequency of feeding:	2 times/day		
Details on feeding:	Fish not fed 24 hours prior to test termination.		

Table 3: Fish Selection and Pre-Exposure Performance.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Age at test initiation:	6 months		*EPA recommends reproductively mature (sexually dimorphic) fish, 4.5 - 6 months old.*
Mean weight of males at test initiation (if determined):	2.9 g	Based on 20 males used to stock aquaria for pre-exposure phase.	*EPA recommends that a subsample of fish be weighed before the test to estimate the mean weight for each sex. It is recommended that the individual weight of each fish selected for the test be within ±20% of the estimated mean for each sex.*
Range of individual weights (males) at test initiation (if determined):	2.4 to 3.5 g	Individual weights within ± 31% of the estimated mean according the study author. Based on the provided information the reviewer calculated that individual weights were within ± 19%	
Mean weight of females at test initiation (if determined):	1.4 g	Based on 20 females used to stock aquaria for pre-exposure phase.	
Range of individual weights (females) at test initiation (if determined):	1.1 to 1.6 g	Individual weights within ± 15% of the estimated mean.	
Mean length of males at test initiation (if determined):	Not reported		
Mean length of females at test initiation (if determined):	Not reported		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Duration of pre-exposure phase:	14 days		*EPA recommends a minimum of 14 days.*
Were pre-exposure conditions identical to the definitive test?	Yes		*EPA recommends that pre-exposure conditions, including temperature, photoperiod, feeding, etc., be identical to definitive test conditions.*
Number of pre-exposure tanks:	36	Additional breeding groups were maintained to account for a potential lack of spawning and/or mortality during this phase.	*EPA recommends that additional tanks set up at the beginning of pre-exposure will ensure that sufficient replicates with the correct sex ratio are available for the definitive test.*
Number of males per tank:	2		
Number of females per tank:	4		
Pre-exposure fecundity:	Not reported	The top 20 spawning groups from the pre-exposure period were selected for the definitive test.	*EPA recommends that pre-exposure fecundity in each replicate (tank) selected for use in the definitive test be at least 15 eggs/female/reproductive day/replicate during the 7 days prior to the definitive test.*
Number of spawns during pre-exposure:	$\geq$ two times in 7 days and at least 15 eggs/female/day		*EPA recommends that spawning occur at least twice in the 7 days prior to the definitive test.*
Details on pre-exposure:		None.	

C. Exposure System

Table 4: Summary of Information on the Exposure System and Test Vessel Characteristics.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Type of exposure:	Proportional flow-through		*EPA recommends the use of a flow-through system. As noted in the Corrections and Clarifications document[1], the use of a static renewal system is not recommended for this assay.*
Type of flow-through dilution system:	Flow-through diluters		*Intermittent flow proportional diluters or continuous flow serial diluters are recommended.[2]*
Flow-through rate:	13 volume additions/test vessel/day; 92 mL/min	Not further described	*Recommended flow-through rate is 45 mL/min (2.7 L/hr), or at least 6 total volume exchanges per day.*

[1] U.S. Environmental Protection Agency (EPA). (2011). Corrections and Clarifications on Technical Aspects of the Test Guidelines for the Endocrine Disruptor Screening Program Tier 1 Assays (OCSPP Test Guideline Series 890). March 3, 2011. Office of Chemical Safety and Pollution Prevention (OCSPP), Washington, D.C. (http://www.epa.gov/endo/pubs/assayvalidation/clarificationdoc.pdf).

[2] Additional guidance for aquatic test design is located in OCSPP Guideline 850.1000, Special Considerations for Conducting Aquatic Laboratory Studies.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Details on toxicant mixing for flow-through systems:		A proportional diluter was used to produce nominal concentrations. Flow-splitting chambers were used between diluter cells and the 4 replicate test vessels. Treatment recoveries prior to test initiation were not reported. Flow splitting accuracy was not reported.	*Recommended toxicant mixing for flow-through systems: 1) Mixing chamber is recommended but not required; 2) Aeration is not recommended for mixing; 3) A demonstration that the test solution is completely mixed before introduced into the test system is recommended; 4) The recommended flow splitting accuracy is within 10%.*
Aeration?	No		*EPA recommends aeration if dissolved oxygen reaches ≤4.9 mg/L (≤ 60% saturation).*
Source of dilution water:	Well water		*EPA recommends natural or reconstituted water; it is recommended that natural water be sterilized with UV and tested for pesticides, heavy metals, and other possible contaminants. OECD accepts any water in which the test species show control survival at least as good as indicated in the test guideline.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was dilution water analyzed for pesticides, heavy metals, and other contaminants?	Yes		
Test vessel type/materials:	Glass aquaria		*EPA and OECD recommend that water-contact portions of the system not compromise the study (e.g., all glass vessels or glass vessels with stainless steel frames are acceptable examples).*
Test vessel size:	20L (39 x 20 x 25 cm)		*EPA recommends the use of 18 L test chambers (e.g., 40 x 20 x 20 cm).*
Fill volume:	10 L; 13 cm depth		*EPA recommends 10 L solution per tank.*
Spawning substrate material:	Three 4-inch diameter inverted semi-circular aged PVC pipes.		*EPA recommends that each tank contain three semi-circular spawning substrates, e.g., aged PVC pipe, 10 - 20 cm in length, split lengthwise.*
Spawning substrate size:	*Ca.* 7 cm length		
Additional details on exposure system:		None	

Table 5: Summary of Water Quality Characteristics in the Test System.

Parameter	Minimum	Maximum	Mean[1]	Measurement Interval	Guideline Recommendations
Temperature (°C)	24.0	25.0	24.5	Continuously	*EPA recommends temperature 25±1°C; inter-replicate and inter-treatment differentials should not exceed 1°C.*
pH	6.8	7.4	7.1	Weekly	*EPA recommends pH 6.5 to 9.0.*
Dissolved oxygen (mg/L)	2.6	8.8	5.7	Weekly	*EPA recommends dissolved oxygen (DO) ≥4.9 mg/L (>60% air saturation)*
Total alkalinity (mg/L as $CaCO_3$)	22	26	24	Weekly	*EPA recommends total alkalinity >20 mg/L as $CaCO_3$.*
Hardness (mg/L as $CaCO_3$)	64	80	72	Weekly	
Total organic carbon (mg C/L)	0.62	0.66	0.64	Twice	*EPA recommends that total organic carbon in dilution water be ≤2 mg/L.*
Unionized ammonia (µg/L)	Not reported	Not reported	Not reported	None	*EPA recommends that unionized ammonia in the dilution water be ≤1 µg/L.*
Residual chlorine (µg/L)	Not reported	Not reported	Not reported	None	*EPA recommends that residual chlorine in dilution water be <10 µg/L.*

Parameter	Minimum	Maximum	Mean[1]	Measurement Interval	Guideline Recommendations
Conductivity (µS/cm)	410	540	475	Weekly	*General recommendations for frequency of measurements: EPA recommends that temperature, pH, and dissolved oxygen be measured in all test tanks at least weekly and that hardness and alkalinity be measured in controls and in one tank at the highest test concentration at least weekly. In addition, continuous temperature monitoring of at least one tank is encouraged.*

[1] Means were calculated by the reviewer as the average of the minima and maxima for the ranges provided across control and treated levels.

D. Study Design and Additional Experimental Conditions

Table 6: Range-Finding Study Conditions (if Applicable).

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was a range-finder conducted?	Yes		*EPA recommends conducting a range-finder if 96-hour LC_{50} data for the fathead minnow are unavailable.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
If yes, what was the method for determining the highest test concentration in the range-finder?	The solubility of the test substance and LC_{50} were determined prior to selection of definitive folpet test concentrations.	Recoveries were 22 and 40% of nominal in dilution water and were 34 and 46% of nominal in the solvent after 48 and 72 hours, respectively. The LC_{50} based on measured concentrations was 0.0317 mg a.i./L (0.0703 mg a.i./L based on nominal concentrations) after 96 hours. Based on the predicted LC_{50}, the maximum definitive exposure level was selected as 1/3 of the nominal LC_{50} (0.024 mg a.i./L)	*EPA recommends that the highest test concentration be selected based on toxicity data for other fish studies or species, if available. Otherwise, either the solubility limit of the test compound or 100 mg/L (whichever is lower) is appropriate.*
Species:	*Pimephales promelas*		
Life stage:	Not reported		*EPA recommends that range-finding tests be performed with fish of similar age and size to those that would be utilized in the test.*
Test duration:	48 or 72-hours (solubility) 96-hours (LC_{50})		*EPA recommends a 96-hour exposure.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Additional details:	Test concentrations: Negative control Without solvent: 0.001 and 0.01 mg a.i./L With DMF: 0.01 and 0.1 mg a.i./L	Testing was conducted with and without the solvent dimethylformamide (DMF). The diluter system was maintained at 12 tank turnovers/day (2x's the standard turnover rate). Duplicate test vessels for each treatment were used, each containing 2 males and 2 females.	*EPA recommends conducting a range-finder with five test concentrations plus a control (six total treatment levels), with four females and two males per exposure tank (36 fish total). The number of mortalities that occur may be used to develop a concentration-response curve.* *Based upon the results, the highest concentration that does not result in increased mortality or signs of overt morbidity compared to controls, or 1/3 the derived 96-hr $LC_{50,}$ may be selected as the highest exposure concentration in the 21-day test.*

Table 7: Definitive Study Conditions.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Test duration:	21 days		*EPA recommends that the duration of the definitive test be 21 days.*
Method for selecting the highest test concentration in the definitive test:	Maximum concentration based on 1/3 of the		*EPA recommends that the highest test concentration is either the solubility limit of the test compound, 100 mg/L, or demonstrates*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
	nominal LC_{50} of 0.0703 mg a.i./L.		*adequate evidence of toxicity (e.g., 1/3 the 96-hour LC_{50}), whichever concentration is lowest.*
Reference study citation (if applicable):	Not reported		
Separation of test concentrations:	Six-fold		*EPA suggests that a concentration separation of between 0.33 (or three-fold) and 0.1 (or ten-fold) is scientifically acceptable[1].*
Number of test concentrations:	3		*EPA recommends a minimum of 3 concentrations and a control, plus solvent control if appropriate.*
Are nominal concentrations adjusted for purity?	Yes		
Indicate the type of values presented for measured concentrations:	Mean measured		
Limit of quantification (LOQ):	<0.000031 mg a.i./L		*EPA recommends that for chemical test concentrations below the LOQ, analyses be conducted on the stock solutions.*
Level of detection (LOD):	Not reported		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Frequency of measurement:	0, 7, 14, and 21 days		*It is recommended that test item concentration be measured prior to the addition of fish in all tanks and at least weekly thereafter in two replicates per treatment level.*
Was the randomized complete block design used?	Yes		*EPA recommends that all fish be randomly assigned to tanks during pre-exposure. Tanks are then ranked according to pre-exposure fecundity, and the tanks with the highest fecundity are randomly assigned to a definitive test treatment and block first. Each block contains one replicate of each treatment, including controls.*
Number of replicates in control:	4		*EPA recommends 4 replicates.*
Number of replicates in solvent control (if applicable):	4		*EPA recommends the use of a concurrent solvent control when a solubilizing agent is used. EPA recommends 4 replicates.*
Number of replicates per test item treatment level:	4		*EPA recommends 4 replicates.*
Number of male fish per replicate at test initiation:	2		*EPA recommends 2 males per replicate.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Number of female fish per replicate at test initiation:	4		*EPA recommends 4 females per replicate.*
Was a solvent used?	Yes		
Solvent type (if applicable):	Dimethylformamide (DMF)		
Maximum solvent concentration (if applicable):	0.010 mL/L		*EPA recommends that the solvent not exceed 0.02 ml/L*[3]*. OECD recommends that solvent have no effect on survival nor produce any other adverse effects and that concentration not be greater than 0.1 mL/L*[4]*.*
Was a positive control used?	No		
Positive control (if applicable):	NA		
Positive control concentration(s) (if applicable):	NA		

[3] Hutchinson TH, Shillabeer N, Winter MJ, Pickford DB (2006). Acute and chronic effects of carrier solvents in aquatic organisms: A critical review. Review. Aquatic Toxicology, 76, pp.69–92.

[4] OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. Environmental Health and Safety Publications. Series on Testing and Assessment. No. 23. Paris, France.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Photoperiod:	16 hrs light: 8 hrs dark		*EPA recommends photoperiod 16:8 (light:dark).*
Light intensity at water's surface:	650-1100 lux	Fluorescents	*EPA recommends light intensity 540 – 1080 lux (at water's surface).*
Additional details:	None		

Table 8: Summary of Treatment Concentrations in the Fish Short-Term Reproduction Assay with Folpet.

Treatment ID	Nominal Concentration (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Mean CV (%)	Details or Remarks	Guideline Recommendations
Negative control	0	<LOQ	NA		*EPA recommends that test item concentrations be maintained at a coefficient of variation (CV) ≤20%.*
Solvent control	0	<LOQ	NA		
Treatment 1	0.00054	0.00018	21.8	± 0.00004	
Treatment 2	0.0036	0.0010	16.7	± 0.000166	
Treatment 3	0.024	0.0086	7.8	± 0.000672	

Abbreviations: [CV] Coefficient of variation. [NA] Not applicable.

LOQ=0.000031 mg a.i./L.

E. Observations

Biological Endpoints: Survival, fecundity, fertilization success, and clinical signs were observed daily. At test termination (Day 21), secondary sex characterization (body color, pattern, body shape), body weight, length, tubercle score, gonadal staging and histopathology, and plasma vitellogenin were evaluated.

Were raw (individual) data provided? Yes

EPA recommends that observations of survival, fecundity, fertilization success, secondary sex characteristics, and other clinical signs occur at least daily. At test termination (Day 21), additional observations include body weight and length, nuptial tubercle score, gonadal staging and histopathology, plasma vitellogenin, and plasma sex steroids (testosterone and 17β-estradiol, if measured). Gonado-somatic index (GSI) is calculated using a ratio of gonad weight to body weight (gonad weight to the nearest 0.1 mg / body weight in mg x 100) at test termination.

Clinical signs of overt toxicity may include (but are not limited to) hemorrhage, cessation of feeding, and other abnormal behavior.

II. RESULTS AND DISCUSSION

A. Results

Mean male and female survival was 100% at all treatment levels, with the exception of the female solvent control, which was 94% (Table 9).

Table 9: Adult Fish Survival in Fathead Minnow (*Pimephales promelas*).

Treatment (mg a.i./L) [mean-measured]	Males			Females		
	n[1]	# Surviving	% Survival	n[1]	# Surviving	% Survival
Negative control (<LOQ)	8	8	100	16	16	100
Solvent control (<LOQ)	8	8	100	16	15	94
0.00018	8	8	100	16	16	100
0.0010	8	8	100	16	16	100
0.0086	8	8	100	16	16	100

[1] Total number of fish at test initiation.

LOQ=0.000031 mg a.i./L.

Mean male body weight values were 2.80, 2.94, 3.1942, 2.83 and 3.12 g and mean female body weight values were 1.44, 1.38, 1.40, 1.38 and 1.40 g in the negative control, solvent control, and mean-measured 0.00018, 0.0010 and 0.0086 mg a.i./L treatment levels, respectively (Table 10). Mean male body length values were 51.1, 50.5, 52.8, 50.6 and 53.4 mm and female body length values were 41.8, 40.9, 41.4, 42.0 and 41.1 mm in the negative control, solvent control, and mean-measured 0.00018, 0.0010 and 0.0086 mg a.i./L treatment levels, respectively.

Table 10: Size at Test Termination in Fathead Minnow (*Pimephales promelas*).

Treatment (mg a.i./L) [mean-measured]	Body Weight						Length					
	Males			Females			Males			Females		
	n	Mean (g)	±SD	n	Mean (g)	±SD	n	Mean (mm)	±SD	n	Mean (mm)	±SD
Negative control (<LOQ)	4	2.80	0.22	4	1.44	0.12	4	51.1	1.2	4	41.8	1.5
Solvent control (<LOQ)	4	2.94	0.11	4	1.38	0.10	4	50.5	1.9	4	40.9	1.0
0.00018	4	3.1	0.35	4	1.40	0.12	4	52.8	3.1	4	41.4	1.6
0.0010	4	2.83	0.61	4	1.38	0.04	4	50.6	3.5	4	42.0	0.69
0.0086	4	3.12	0.41	4	1.40	0.15	4	53.4	0.58	4	41.1	1.3

Abbreviations: SD Standard deviation.

LOQ=0.000031 mg a.i./L.

Mean fecundity values were 14, 16, 14, 18 and 11 eggs/female/day and fertilization success was 98, 98, 98, 98, and 99% in the negative control, solvent control, and 0.00018, 0.0010 and 0.0086 mg a.i./L treatment levels, respectively (Table 11). One replicate in the negative control averaged 12 eggs/female/reproductive day while another replicate averaged 7.5 eggs/female/reproductive day. One replicate in the solvent control averaged 14 eggs/female/reproductive day.

Table 11: Fecundity and Fertilization Success in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [mean-measured]	Fecundity[1]	Fertilization Success (%)[2]
Negative control (<LOQ)	14	98
Solvent control (<LOQ)	16	98
0.00018	14	98
0.0010	18	98
0.0086	11	99

LOQ=0.000031 mg a.i./L.

[1] Fecundity is calculated as the number of eggs per surviving female per reproductive day per replicate.

[2] Fertilization success (%) is calculated as the number of embryos divided by the number of eggs, multiplied by 100.

Median male tubercle scores were 39, 34, 28, 32 and 26 in the control, solvent control, and mean-measured 0.00018, 0.0010 and 0.0086 mg a.i./L treatment levels, respectively (Table 12). None of the surviving females were found to have tubercles.

Table 12: Nuptial Tubercle Score in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [mean-measured]	Males		Females	
	n	Median Tubercle Score[1]	n	Median Tubercle Score
Control (<LOQ)	4	39	4	0
Solvent control (<LOQ)	4	34	4	0
0.00018	4	28	4	0
0.0010	4	32	4	0
0.0086	4	26	4	0

LOQ=0.000031 mg a.i./L.

[1] Mean tubercle scores: 36, 32, 29, 34, and 27 for the negative control, solvent control, and mean-measured 0.00018, 0.0010, and 0.0086 mg a.i./L treatment levels, respectively.

Mean male GSI was 1.4, 1.3, 1.2, 1.2 and 1.1% and mean female GSI was 14, 13, 13, 16, and 13% in the negative control, solvent control, and mean-measured 0.00018, 0.0010 and 0.0086 mg a.i./L treatment levels, respectively (Table 13).

Table 13: Gonado-Somatic Index (GSI) in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [mean-measured]	Males			Females		
	n	Mean GSI[1] (%)	±SD	n	Mean GSI[1] (%)	±SD
Negative control (<LOQ)	4	1.4	0.17	4	14	1.2
Solvent control (<LOQ)	4	1.3	0.23	4	13	1.1
0.00018	4	1.2	0.33	4	13	1.3
0.0010	4	1.2	0.16	4	16	5.5
0.0086	4	1.1	0.25	4	13	1.1

LOQ=0.000031 mg a.i./L.

[1] Gonado-somatic index (%) is calculated as gonad weight (to the nearest 0.1 mg) / body weight (mg) x 100.

Median male gonadal stage was three (negative control and 0.0086 mg a.i./L treatment level) or two (solvent control and other treatment levels). Median female gonadal stage was three for all controls and treatment levels (Table 14). Folpet related effects were not observed to have an effect on gonadal stage.

Table 14: Gonadal Staging in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [mean-measured]	Males		Females	
	n	Median Stage[1]	n	Median Stage[2]
Negative control (<LOQ)	4	3	4	3
Solvent control (<LOQ)	4	2	4	3
0.00018	4	2	4	3
0.0010	4	2	4	3
0.0086	4	3	4	3

LOQ=0.000031 mg a.i./L.

[1] The guideline recommends the following gonadal staging scale for male fathead minnow: 0=undeveloped, 1=early spermatogenic, 2=mid-spermatogenic, 3=late spermatogenic, 4=spent.

[2] The guideline recommends the following gonadal staging scale for female fathead minnow: 0=undeveloped, 1=early development, 2=mid-development, 3=late development, 4=late development/hydrated, 5=post-ovulatory.

Folpet related effects were not observed for male or female histopathology severity scores (Tables 15-18). However, there was a slight increase in granulomatous inflammation (females) with increasing folpet concentration. It was reported that in 4 cases from the low treatment concentration, 6 cases from the mid treatment concentration, and 5 cases from the high treatment concentration that the oocyte atresia and/or granulomatous inflammation was attributed to *Microsporidia* infection. Additionally, male fish showed increased incidence of interstitial cell hypertrophy/hyperplasia, increased proportion of spermatogonia, testicular degeneration, and decreased proportion of spermatozoa due to exposure to the co-solvent DMF control (Tables 15-16).

Table 15: Gonadal Histopathology in Male Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [mean-measured]	Diagnostic Observations[1]										
	Severity	Increased Proportion of Spermatogonia		Presence of Testis-Ova		Increased Testicular Degeneration		Duct Mineralization		Interstitial cell (Leydig) hypertrophy/hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative control (<LOQ)	0	8	7	NA	NA	8	8	NA	NA	8	8
	1	8	1	NA	NA	8	0	NA	NA	8	0
	2	8	0	NA	NA	8	0	NA	NA	8	0
	3	8	0	NA	NA	8	0	NA	NA	8	0
	4	8	0	NA	NA	8	0	NA	NA	8	0
Solvent control (<LOQ)	0	8	4	NA	NA	8	5	NA	NA	8	2
	1	8	4	NA	NA	8	1	NA	NA	8	3
	2	8	0	NA	NA	8	2	NA	NA	8	3
	3	8	0	NA	NA	8	0	NA	NA	8	0
	4	8	0	NA	NA	8	0	NA	NA	8	0
0.00018	0	7	3	NA	NA	7	4	NA	NA	7	1
	1	7	3	NA	NA	7	1	NA	NA	7	4
	2	7	1	NA	NA	7	2	NA	NA	7	2
	3	7	0	NA	NA	7	0	NA	NA	7	0
	4	7	0	NA	NA	7	0	NA	NA	7	0
0.001	0	9	4	NA	NA	9	7	NA	NA	9	1
	1	9	4	NA	NA	9	2	NA	NA	9	3
	2	9	0	NA	NA	9	0	NA	NA	9	5
	3	9	0	NA	NA	9	0	NA	NA	9	0
	4	9	1	NA	NA	9	0	NA	NA	9	0

Treatment (mg a.i./L) [mean-measured]	Diagnostic Observations[1]										
	Severity	Increased Proportion of Spermatogonia		Presence of Testis-Ova		Increased Testicular Degeneration		Duct Mineralization		Interstitial cell (Leydig) hypertrophy/hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
0.0086	0	8	5	NA	NA	8	4	NA	NA	8	4
	1	8	2	NA	NA	8	3	NA	NA	8	3
	2	8	1	NA	NA	8	1	NA	NA	8	1
	3	8	0	NA	NA	8	0	NA	NA	8	0
	4	8	0	NA	NA	8	0	NA	NA	8	0

Abbreviation: NA Not applicable.

LOQ=0.000031 mg a.i./L.

[1] Gonadal histopathology diagnostic observations are graded 0 – 4 based on severity: 0=Not remarkable, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe. See Appendix E of the test guideline for reference.

Table 16: Additional Gonadal Histopathology Observations in Male Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]									
	Severity	Decreased Proportion of Spermatogonia		Increased Vascular or Interstitial Proteinaceous Fluid		Asynchronous Gonad Development		Altered Proportions of Spermatocytes or Spermatids		Granulomatous Inflammation	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative control (<LOQ)	0	NA	NA	NA	NA	8	7	8	8	8	8
	1	NA	NA	NA	NA	8	1	8	0	8	0
	2	NA	NA	NA	NA	8	0	8	0	8	0
	3	NA	NA	NA	NA	8	0	8	0	8	0
	4	NA	NA	NA	NA	8	0	8	0	8	0
Solvent control (<LOQ)	0	NA	NA	NA	NA	8	8	8	7	8	8
	1	NA	NA	NA	NA	8	0	8	0	8	0
	2	NA	NA	NA	NA	8	0	8	1	8	0
	3	NA	NA	NA	NA	8	0	8	0	8	0
	4	NA	NA	NA	NA	8	0	8	0	8	0
0.00018	0	NA	NA	NA	NA	7	6	7	5	7	7
	1	NA	NA	NA	NA	7	0	7	1	7	0
	2	NA	NA	NA	NA	7	1	7	1	7	0
	3	NA	NA	NA	NA	7	0	7	0	7	0
	4	NA	NA	NA	NA	7	0	7	0	7	0

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]									
	Severity	Decreased Proportion of Spermatogonia		Increased Vascular or Interstitial Proteinaceous Fluid		Asynchronous Gonad Development		Altered Proportions of Spermatocytes or Spermatids		Granulomatous Inflammation	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
0.0010	0	NA	NA	NA	NA	9	8	9	7	9	9
	1	NA	NA	NA	NA	9	1	9	2	9	0
	2	NA	NA	NA	NA	9	0	9	0	9	0
	3	NA	NA	NA	NA	9	0	9	0	9	0
	4	NA	NA	NA	NA	9	0	9	0	9	0
0.0086	0	NA	NA	NA	NA	8	7	8	6	8	7
	1	NA	NA	NA	NA	8	0	8	1	8	1
	2	NA	NA	NA	NA	8	1	8	1	8	0
	3	NA	NA	NA	NA	8	0	8	0	8	0
	4	NA	NA	NA	NA	8	0	8	0	8	0

Abbreviation: [NA] Not applicable.

LOQ=0.000031 mg a.i./L.

[1] Gonadal histopathology diagnostic observations are graded 0 – 4 based on severity: 0=Not remarkable, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe. See Appendix E of the test guideline for reference.

Table 17: Gonadal Histopathology in Female Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]								
	Severity	Increased Oocyte Atresia		Perifollicular Cell Hyperplasia/ Hypertrophy		Decreased Yolk Formation		Aggregates of macrophages, multifocal	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative control (<LOQ)	0	16	11	NA	NA	NA	NA	NA	NA
	1	16	4	NA	NA	NA	NA	NA	NA
	2	16	1	NA	NA	NA	NA	NA	NA
	3	16	0	NA	NA	NA	NA	NA	NA
	4	16	0	NA	NA	NA	NA	NA	NA
Solvent control (<LOQ)	0	15	8	NA	NA	NA	NA	NA	NA
	1	15	5	NA	NA	NA	NA	NA	NA
	2	15	2	NA	NA	NA	NA	NA	NA
	3	15	0	NA	NA	NA	NA	NA	NA
	4	15	0	NA	NA	NA	NA	NA	NA
0.00018	0	16	10	NA	NA	NA	NA	NA	NA
	1	16	1	NA	NA	NA	NA	NA	NA
	2	16	4	NA	NA	NA	NA	NA	NA
	3	16	1	NA	NA	NA	NA	NA	NA
	4	16	0	NA	NA	NA	NA	NA	NA
0.0010	0	16	10	NA	NA	NA	NA	NA	NA
	1	16	2	NA	NA	NA	NA	NA	NA
	2	16	1	NA	NA	NA	NA	NA	NA
	3	16	2	NA	NA	NA	NA	NA	NA
	4	16	0	NA	NA	NA	NA	NA	NA

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]							
	Severity	Increased Oocyte Atresia		Perifollicular Cell Hyperplasia/ Hypertrophy		Decreased Yolk Formation		Aggregates of macrophages, multifocal	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
0.0086	0	16	9	NA	NA	NA	NA	NA	NA
	1	16	1	NA	NA	NA	NA	NA	NA
	2	16	3	NA	NA	NA	NA	NA	NA
	3	16	2	NA	NA	NA	NA	NA	NA
	4	16	1	NA	NA	NA	NA	NA	NA

Abbreviation: NA Not applicable.

LOQ=0.000031 mg a.i./L.

[1] Gonadal histopathology diagnostic observations are graded 0 – 4 based on severity: 0=Not remarkable, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe. See Appendix E of the test guideline for reference.

Table 18: Additional Gonadal Histopathology Observations in Female Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]								
	Severity	Interstitium/multifocal inflammation		Egg Debris in Oviduct		Granulomatous Inflammation		Decreased Post-Ovulatory Follicles	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative control (<LOQ)	0	NA	NA	16	13	16	15	NA	NA
	1	NA	NA	16	3	16	1	NA	NA
	2	NA	NA	16	0	16	0	NA	NA
	3	NA	NA	16	0	16	0	NA	NA
	4	NA	NA	16	0	16	0	NA	NA

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]								
	Severity	Interstitium/multifocal inflammation		Egg Debris in Oviduct		Granulomatous Inflammation		Decreased Post-Ovulatory Follicles	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Solvent control (<LOQ)	0	NA	NA	15	9	15	14	NA	NA
	1	NA	NA	15	5	15	1	NA	NA
	2	NA	NA	15	1	15	0	NA	NA
	3	NA	NA	15	0	15	0	NA	NA
	4	NA	NA	15	0	15	0	NA	NA
0.00018	0	NA	NA	16	11	16	12	NA	NA
	1	NA	NA	16	3	16	3	NA	NA
	2	NA	NA	16	2	16	1	NA	NA
	3	NA	NA	16	0	16	0	NA	NA
	4	NA	NA	16	0	16	0	NA	NA
0.0010	0	NA	NA	16	13	16	10	NA	NA
	1	NA	NA	16	0	16	4	NA	NA
	2	NA	NA	16	0	16	0	NA	NA
	3	NA	NA	16	2	16	1	NA	NA
	4	NA	NA	16	0	16	0	NA	NA
0.0086	0	NA	NA	16	14	16	9	NA	NA
	1	NA	NA	16	2	16	4	NA	NA
	2	NA	NA	16	0	16	1	NA	NA
	3	NA	NA	16	0	16	2	NA	NA
	4	NA	NA	16	0	16	0	NA	NA

[1] Gonadal histopathology diagnostic observations are graded 0 – 4 based on severity: 0=Not remarkable, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe. See Appendix E of the test guideline for reference.

LOQ=0.000031 mg a.i./L.

Mean male VTG was 1600, 700, 5000, 26,000 and 11,000,000 ng/mL and mean female VTG was 3.8 x 10^6, 1.8 x 10^6 , 4.5 x 10^6 , 5.4 x 10^6 and 2.1 x 10^6 ng/mL in the control, solvent control, and mean-measured 0.00018, 0.0010 and 0.0086 mg a.i./L treatment levels, respectively (Table 19).

Table 19: Plasma Vitellogenin in Fathead Minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [mean-measured]	Plasma Vitellogenin (VTG)					
	Males			Females		
	n	Mean (ng/mL plasma)	±SD	n	Mean (ng/mL plasma)	±SD
Negative control (<LOQ)	4	1600	2100	4	3.8 x 10^6	3.2 x 10^6
Solvent control (<LOQ)	4	700	1100	4	1.8 x 10^6	1.1 x 10^6
0.00018	4	5000	9500	4	4.5 x 10^6	3.1 x 10^6
0.0010	3[1]	26000	43000	4	5.4 x 10^6	3.3 x 10^6
0.0086	3[1]	11000000	3200000	4	2.1 x 10^6	2.1 x 10^6

Abbreviations: SD Standard deviation.

LOQ=0.000031 mg a.i./L.

[1] The results for only 3 replicates were available for mid and high treatment concentrations; the study report listed the fourth replicate as NA without further explanation.

Plasma testosterone and plasma 17β-estradiol in male and females were not measured (Table 20).

Table 20: Plasma Sex Steroids in Fathead Minnow (*Pimephales promelas*).

Treatment (mg a.i./L) [mean-measured]	Plasma Testosterone (T)						Plasma 17β-estradiol (E2)					
	Males			Females			Males			Females		
	n	Mean (ng/mL plasma)	±SD	N	Mean (ng/mL plasma)	±SD	n	Mean (ng/mL plasma)	±SD	n	Mean (ng/mL plasma)	±SD
Negative control (<LOQ)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Solvent control (<LOQ)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.00018	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.0010	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.0086	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviations: NA Not applicable. SD Standard deviation.

LOQ=0.000031 mg a.i./L.

There were no notable observations with regards to behavior or changes in appearance, specifically color/banding, ovipositor appearance, or size of dorsal nape pad in the control or treated groups (Table 21).

Table 21: Secondary Sex Characteristics and Clinical Signs in Fathead minnow (*Pimephales promelas)*.

Treatment (mg a.i./L) [mean-measured]	Secondary Sex Characteristics and Clinical Signs[1]					
	Males			Females		
	Type	n	Incidence	Type	n	Incidence
Negative control (<LOQ)	None	4	0	None	4	0
Solvent control (<LOQ)	None	4	0	None	4	0
0.00018	None	4	0	None	4	0
0.0010	None	4	0	None	4	0
0.0086	None	4	0	None	4	0

LOQ=0.000031 mg a.i./L.

B. Study Author's Analysis and Conclusions

The study author analyzed survival, wet weight, tubercle score, gonadal development, GSI, fertility, fecundity, VTG, and incidence and severity of gonad abnormalities. Data were gender specific and analyzed in comparison to the pooled controls.

Descriptive statistics (mean, standard deviation, etc.) were determined for each endpoint. Significant effects were detected for $p \leq 0.05$ (CETIS, version 1.8.4.2). Survival data were analyzed using Cochran-Amitage Trend Step-Down Test. Prior to analysis, survival and fertilization success were transformed using the arcsine square-root transformation. If the results were consistent with a monotonic concentration-response, Jonckheere-Terpstra test was used (fertilization success, male VTG and male tubercle scores). All other endpoints were analyzed by performing pair-wise comparisons using Dunnett's Multiple Comparison test to determine which treatment groups differed statistically from the control, with the exception of female GSI, which was analyzed by Wilcoxon's Test with Bonferroni-Holms adjustment. Prior to Dunnett's, data were analyzed by Shapiro-Wilk's test and Levene's to test for normality and homogeneity of variances, respectively ($\alpha = 0.01$). If normality and homogeneity tests passed ($p > 0.01$), a parametric analysis was performed. If non-normality or unequal variance were indicated ($p < 0.01$), a non-parametric analysis was performed. These methods appear to be consistent with the methods recommended in the guideline except treatments were compared to a pooled control (comparison of treatments to the negative control is recommended). An equal variance t two-sample test was conducted to statistically compare control to the solvent control data. Length data was not statistically analyzed.

There was a significant differences in male VTG at the 0.0086 mg a.i./L concentration compared to the pooled controls ($p < 0.05$; Jonckheere-Terpstra Step-Down test). There were no other statistically significant differences in treated groups compared to pooled controls for any endpoint ($p > 0.05$). Folpet related effects were not observed for histopathology severity scores or gonadal stage.

C. Reviewer's Analysis and Conclusions

Statistical Methods: The reviewer assessed survival (mortality) data based on visual observation. Female and male body weight, male and female body length, fecundity, fertility, male GSI, and male tubercle score data were not consistent with a monotonic concentration-response, but all data satisfied the parametric assumptions of normality using Shapiro-Wilks test and homogeneity of variances using Levene's test. These endpoints were analyzed using Dunnett's test. Female GSI and were analyzed using the nonparametric Mann-Whitney U test because data did not satisfy parametric assumptions or exhibit a monotonic concentration response. None of the surviving females were found to have tubercles. These analyses were conducted using CETIS version 1.8.7.7 and backend settings approved for use by EFED on 5/29/13. Treated groups were compared to only the negative control; no significant differences ($p>0.05$) were detected between the negative and solvent control groups using a two-sided Student's t-test assuming equal variances. Mean-measured concentrations were used to discuss effects in this study.

Conclusions: Adult male and female survival were unaffected by treatment with folpet; survival was 100% for all test levels and the controls, except for females in the solvent control which showed 94% mortality.

There were no significant differences between the treatment levels and negative control for any endpoint ($p>0.05$) except male VTG, which showed a significant increase at the 0.0086 mg a.i./L treatment level ($p=0.0049$; Jonckheere-Terpstra Step-Down Test). Male VTG showed a monotonic increase with increasing folpet concentration based on mean values. Folpet related effects were not observed for histopathology severity scores or gonadal stage. However, there was a slight increase in granulomatous inflammation (females) with increasing folpet concentration. There were no notable observations with regards to behavior or changes in appearance, specifically color/banding, ovipositor appearance, or size of dorsal nape pad in the control or treated groups or clinical signs of toxicity for any treatment group compared to the controls.

Table 22: Reproductive and HPG Endpoints[1,2] for Male Fathead Minnow (*Pimephales promelas)* in the FSTRA with Folpet.

Treatment (mg a.i./L) [mean-measured]	Tubercle Score		GSI		Gonadal Staging and Histo.	Plasma VTG[3]		Plasma T		Plasma E2	
	Median	p	% Diff.	p	Effect? (Yes/No)	% Diff.	P	% Diff.	p	% Diff.	p
Negative control (<LOQ)	39	NA	0	NA	No	0	NA	NA	NA	NA	NA
Solvent control (<LOQ)	34	0.4014	-10.0	0.4262	No	-128	0.4885	NA	NA	NA	NA
0.00018	28	0.4335	-16.7	0.3840	No	213.3	0.6571	NA	NA	NA	NA
0.0010	32	0.9493	-14.5	0.4881	No	1570	0.1853	NA	NA	NA	NA
0.0086	26	0.2921	-16.9	0.3760	No	681100	0.0049	NA	NA	NA	NA
Statistical Test	Dunnett's[4]		Dunnett's		NA	Jonckheere-Terpstra Step-Down Test		NA		NA	

Abbreviations. Diff. Difference. E2 17β-estradiol. GSI Gonado-Somatic Index. Histo. Histopathology. NA Not applicable. T Testosterone. VTG Vitellogenin. NA Not applicable. LOQ=0.000031 mg a.i./L.

1 Unless otherwise indicated, effects and percent (%) differences are reported based on comparison to the negative (clean water) control. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

2 Unless otherwise specified, effects are considered statistically significant at $p<0.05$.

3 This endpoint could not be statistically evaluated using an $\alpha=0.05$ because of insufficient replication required to run the two-sided exact Mann-Whitney comparison test at this level. To successfully run this test for this endpoint, the α was set at 0.10.

4 Mean tubercle scores: 36, 32, 29, 34, and 27 for the negative control, solvent control, and mean-measured 0.00018, 0.0010, and 0.0086 mg a.i./L treatment levels, respectively.

Table 23: Reproductive and HPG Endpoints[1,2] for Female Fathead Minnow (*Pimephales promelas)* in the FSTRA with Folpet.

Treatment (mg a.i./L) [mean-measured]	Fecundity		Fert. Success		Tubercle Score		GSI		Gonadal Staging and Histo.	Plasma VTG		Plasma T		Plasma E2	
	% Diff.	p	% Diff.	P	Median	p	% Diff.	p	Effect? (Yes/No)	% Diff.	p	% Diff.	p	% Diff.	p
Negative control (<LOQ)	0	NA	0	NA	0	NA	0	NA	No	0	NA	NA	NA	NA	NA
Solvent control (<LOQ)	14.6	0.4566	-0.77	0.5239	0	NA	-5.80	0.3559	No	-119	0.2639	NA	NA	NA	NA
0.00018	5.41	0.9894	-0.51	0.8373	0	NA	-9.09	0.2857	No	17.1	0.9788	NA	NA	NA	NA
0.0010	31.5	0.3800	-1.01	0.4184	0	NA	16.4	0.8857	No	42.0	0.7861	NA	NA	NA	NA
0.0086	-18.9	0.7294	0.0	1.0000	0	NA	-3.64	0.6571	No	-45.5	0.7463	NA	NA	NA	NA
Statistical Test	Dunnett's		Dunnett's		NA		Mann-Whitney		NA	Dunnett's		NA		NA	

Abbreviations. [Diff.] Difference. [E2] 17β-estradiol. [Fert.] Fertilization. [GSI] Gonado-Somatic Index. [Histo.] Histopathology. [NA] Not applicable. [T] Testosterone. [VTG] Vitellogenin.

LOQ=0.000031 mg a.i./L.

[1] Unless otherwise indicated, effects and percent (%) differences are reported based on comparison to the negative (clean water) control. Conclusions regarding histopathology may be heavily weighted by the expert opinion of a board-certified pathologist.

[2] Unless otherwise specified, effects are considered statistically significant at $p<0.05$.

Table 24: Growth Endpoints[1,2] in the Fish Short-Term Reproduction Assay (FSTRA) with Folpet.

Treatment (mg a.i./L) [mean-measured]	Body Weight				Length			
	Males		Females		Males		Females	
	% Diff.	p	% Diff.	p	% Diff.	p	% Diff.	p
Negative control (<LOQ)	0	NA	0	NA	0	NA	0	NA
Solvent control (<LOQ)	4.56	0.3141	-4.45	0.4553	-1.19	0.6089	-2.2	0.3639
0.00018	14.0	0.4413	-3.16	0.8663	3.28	0.6596	-0.78	0.9716
0.0010	1.07	0.9992	-4.44	0.7714	-0.93	0.9854	0.6	0.9865
0.0086	11.5	0.5858	-3.21	0.8922	4.5	0.4359	-1.68	0.7992
Statistical Test	Dunnett's		Dunnett's		Dunnett's		Dunnett's	

Abbreviations: [Diff.] Difference. [NA] Not applicable.

LOQ=0.000031 mg a.i./L.

1 Unless otherwise indicated, percent (%) differences are reported based on comparison to the negative (clean water) control.

2 Unless otherwise specified, effects are considered statistically significant at $p<0.05$.

D. Study Deficiencies

There were deviations from the guideline as noted in Section I. Materials and Methods of the DER. All performance and validity criteria were met with the following two exceptions. Replicate C of the negative control averaged 7.5 eggs/female/reproductive day, which is less than the guideline criterion of 15 eggs/female/reproductive day, and did not spawn at least every four days. The coefficient of variation (CV) for the mean-measured concentration of the low treatment group was 21.8% exceeding the guideline criterion of 20%. In general, analytical verification of the test material from Days 0, 7, 14, and 21 yielded mean recoveries of 35, 28 and 36%, at the low, mid, and high treatment levels, respectively. The test material does not appear to be stable under the test conditions, and recoveries were consistently poor. The study author reported that these low recoveries were within expectations for the test substance, which is known to undergo rapid hydrolysis. These deviations did not impact the interpretation of the study.

E. Reviewer's Comments

The reviewer's and the study authors' results were in general agreement. The study author did not statistically analyze male and female body or length. Both the study author's and reviewer's analysis detected no statistically significant differences for any endpoint except male VTG. Male VTG showed a monotonic increase with increasing folpet concentration based on mean values but not median values. The study author found a statistically significant increase in male VTG at the 0.0086 mg a.i./L treatment level compared to the pooled control ($p<0.05$; Jonckheere-Terpstra Step-Down test) and the reviewer found a significant increase in male VTG at the 0.0086 mg a.i./L compared to the negative control ($p=0.0571$; Mann-Whitney and $p=0.0049$; Jonckheere-Terpstra Step-Down Test). The study author reported a statistically significant difference in male VTG at the 0.0086 mg a.i./L level both with and without outliers included in the analysis. Despite the Jonckheere-Terpstra and Mann-Whitney U tests usage of the median values in their analysis and not the means (where no trend was exhibited), the data were not suitable for parametric analysis (that is based on means) because the data did not satisfy the assumptions of normality and homogeneity of variance. Therefore, while the Jonckheere-Terpstra test would assume a monotonic trend in the medians (which male VTG data do not exhibit), it is considered to be an appropriate test for this data set considering assumptions tests failed and the Mann-Whitney U test had limited power due to the reduced number of replicates.

For male VTG, concentrations were reported in only 3 replicates in the 0.001 and 0.0086 mg a.i./L test groups (VTG in some samples was "above detectable limits"). In CETIS, when the sample size is too small to be able to detect any size difference between the treatment group and the control group, CETIS will not provide an output for the Mann-Whitney U test. Figures 1 and 2 below show the response plot of male VTG in the R Statistical Program. In Figure 1, the response of control and all treatment groups is shown. The scale for all data spans seven orders of magnitude. Figure 2 shows the response of the control and two lower treatment concentrations in scale that allows one to discern differences within an order of magnitude. Despite the highest treatment group having a reduced number of replicates (3 instead of 4 that the control and lowest concentration have), all replicates values are within an order of magnitude and there does not appear to be an outlier.

During the exposure, the original purity value (i.e., 94.5%, which was reported by the manufacturer and later determined by the lab to be 97.6%) was inadvertently used for calculations; using the updated purity, the actual nominal concentrations for the exposure were 0.00054, 0.0036, and 0.024 mg a.i./L.

The diluter system and aquaria were chemically cleaned prior to exposure and were brushed and siphoned 2X/week during the 21-day exposure study. The diluter mixing chamber, chemical cells and splitters and delivery tubing were cleaned as necessary.

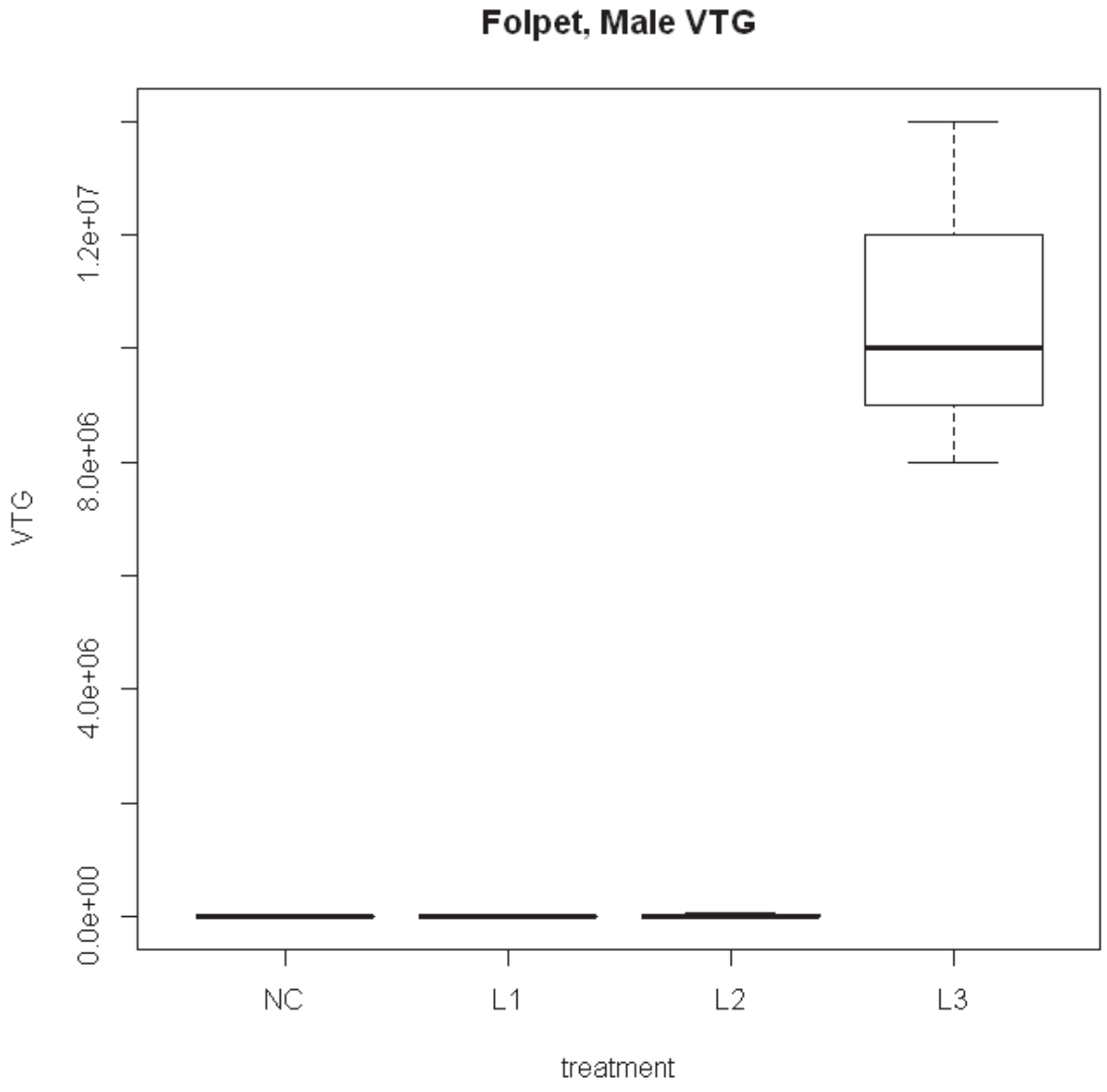


Figure 1. Male VTG response plot in R of negative control and all treatment levels.

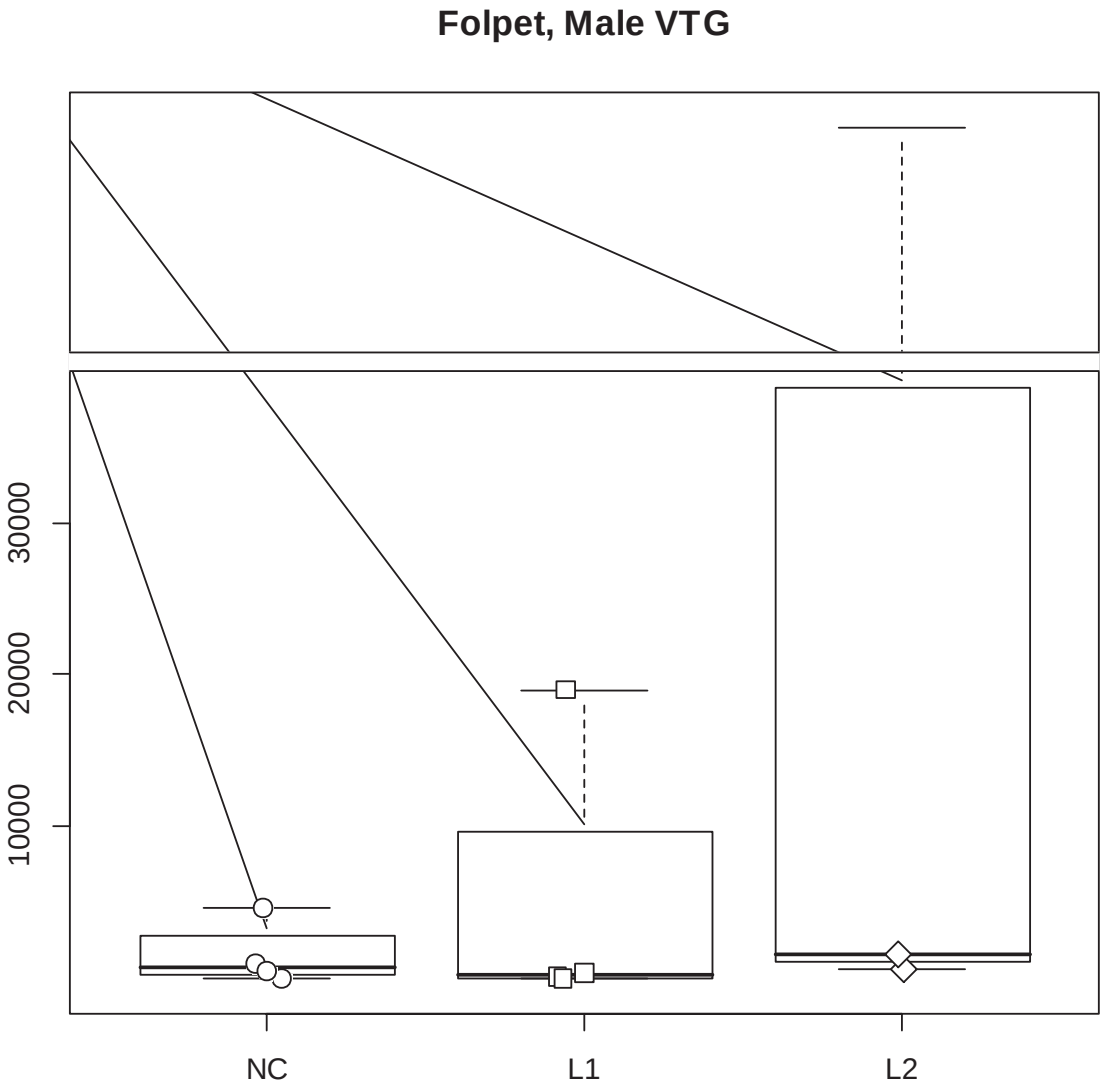


Figure 2. Male VTG response plot in R of negative control, low, and mid treatment levels.

III. REFERENCES

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OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	10-1599-5583	**Endpoint:**	Fecundity	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:49	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	52.6%	Passes fecundity

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.796	2.45	7.3	6	0.4566	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	11.28125	11.28125	1	0.633	0.4566	Non-Significant Effect
Error	106.9375	17.82292	6			
Total	118.2188		7			

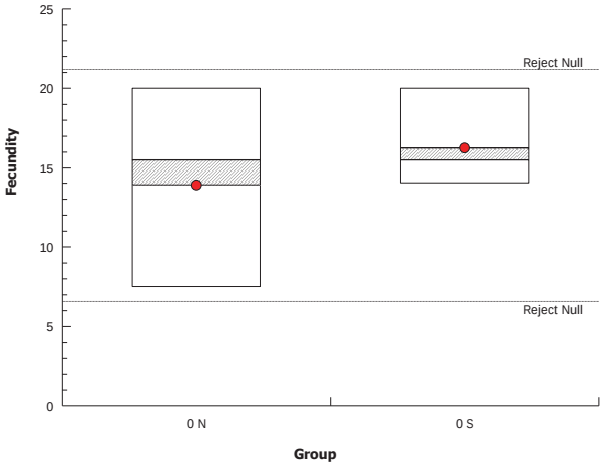
Distributional Tests

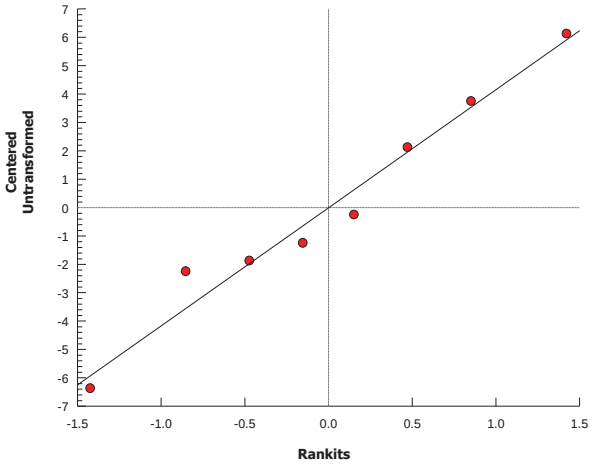
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	4.15	47.5	0.2727	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.976	0.645	0.9426	Normal Distribution

Fecundity Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	16.3	12.1	20.4	15.5	14	20	1.31	16.2%	0.0%
0	Negative Control	4	13.9	5.35	22.4	15.5	7.5	20	2.68	38.6%	14.6%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	10-5374-3739	**Endpoint:**	Fecundity	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:51	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	59.1%	0.0086	>0.0086	NA	

Dunnett Multiple Comparison Test

Control	vs	Group	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	0.245	2.68	8.2	6	0.9894	CDF	Non-Significant Effect
		0.001	1.43	2.68	8.2	6	0.3800	CDF	Non-Significant Effect
		0.0086	0.859	2.68	8.2	6	0.7294	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	100.125	33.375	3	1.78	0.2035	Non-Significant Effect
Error	224.375	18.69792	12			
Total	324.5		15			

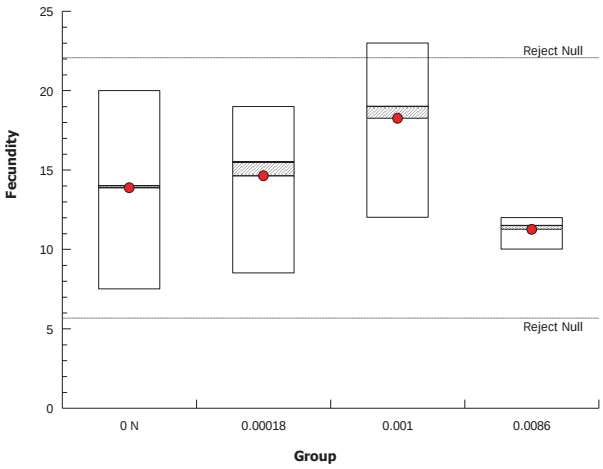
Distributional Tests

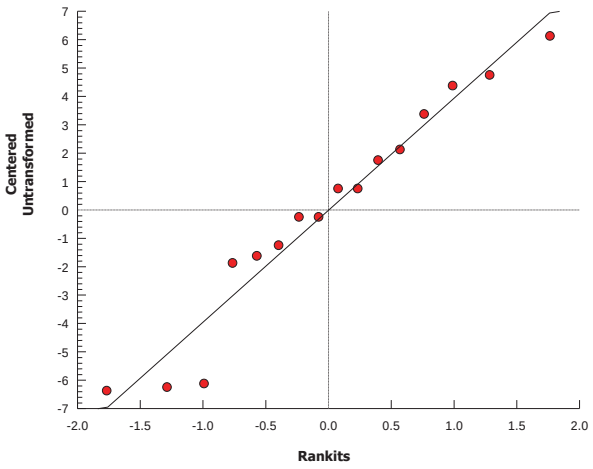
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	5.82	11.3	0.1205	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.94	0.841	0.3543	Normal Distribution

Fecundity Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	13.9	5.35	22.4	14	7.5	20	2.68	38.6%	0.0%
0.00018		4	14.6	6.9	22.3	15.5	8.5	19	2.43	33.2%	-5.41%
0.001		4	18.3	10.9	25.6	19	12	23	2.32	25.5%	-31.5%
0.0086		4	11.3	9.73	12.8	11.5	10	12	0.479	8.51%	18.9%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	14-0828-6857	**Endpoint:**	FemaleBodyWt	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:49	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	13.1%	Passes femalebodywt

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.798	2.45	0.188	6	0.4553	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.007552206	0.007552206	1	0.637	0.4553	Non-Significant Effect
Error	0.0711549	0.01185915	6			
Total	0.07870711		7			

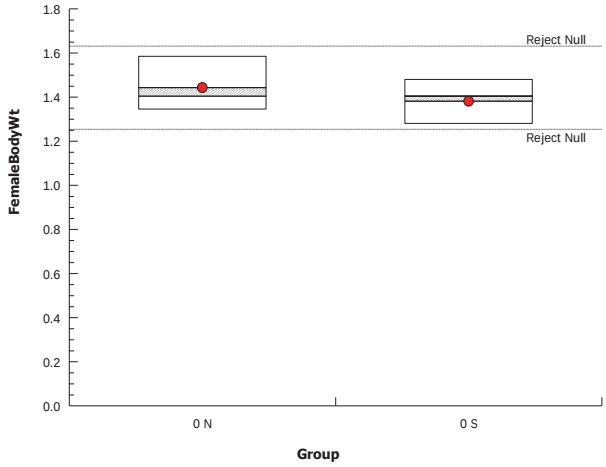
Distributional Tests

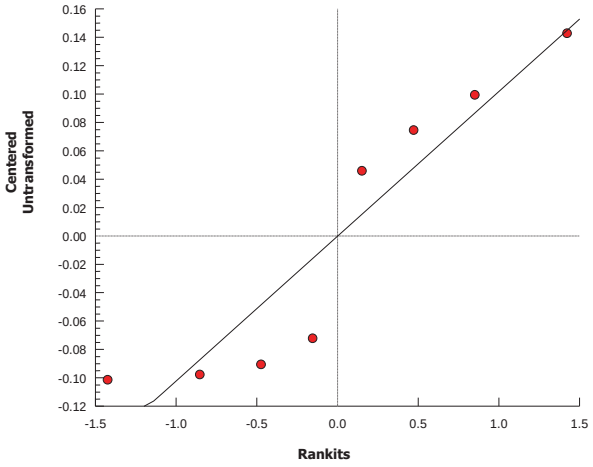
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	1.3	47.5	0.8345	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.848	0.645	0.0909	Normal Distribution

FemaleBodyWt Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	1.38	1.22	1.54	1.4	1.28	1.48	0.0508	7.35%	0.0%
0	Negative Control	4	1.44	1.26	1.63	1.4	1.34	1.59	0.0579	8.03%	-4.45%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	00-0451-0957	**Endpoint:**	FemaleBodyWt	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:51	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	15.1%	0.0086	>0.0086	NA	

Dunnett Multiple Comparison Test

Control	vs	Group	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	0.564	2.68	0.217	6	0.8963	CDF	Non-Significant Effect
		0.001	0.792	2.68	0.217	6	0.7714	CDF	Non-Significant Effect
		0.0086	0.572	2.68	0.217	6	0.8922	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.008981412	0.002993804	3	0.229	0.8747	Non-Significant Effect
Error	0.157122	0.0130935	12			
Total	0.1661035		15			

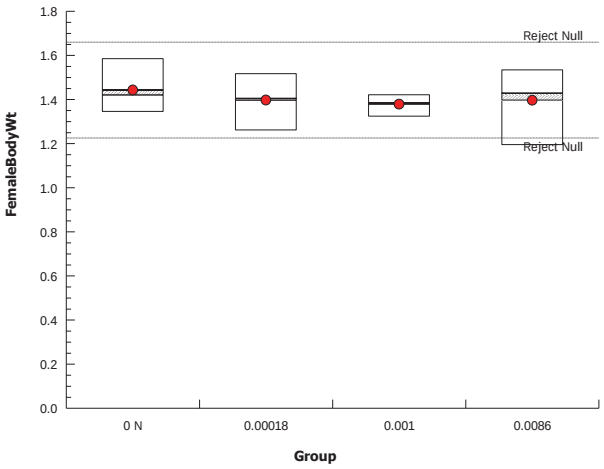
Distributional Tests

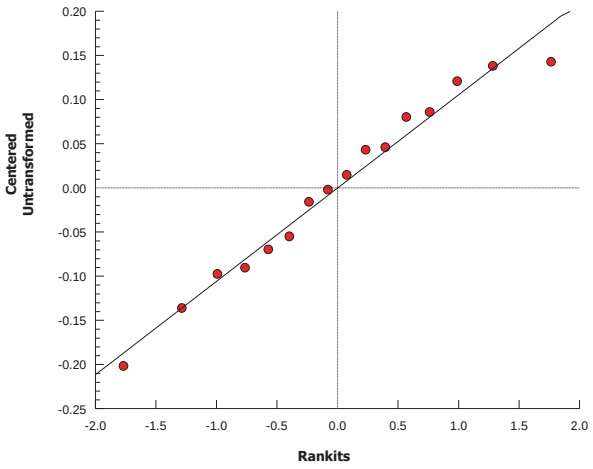
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	3.55	11.3	0.3142	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.965	0.841	0.7494	Normal Distribution

FemaleBodyWt Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	1.44	1.26	1.63	1.42	1.34	1.59	0.0579	8.03%	0.0%
0.00018		4	1.4	1.2	1.59	1.4	1.26	1.52	0.0615	8.8%	3.16%
0.001		4	1.38	1.31	1.44	1.38	1.32	1.42	0.0206	2.99%	4.44%
0.0086		4	1.4	1.16	1.63	1.43	1.19	1.53	0.0744	10.7%	3.21%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	15-6106-0373	**Endpoint:**	FemaleGSI	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:49	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	13.3%	Passes femalegsi

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	1	2.45	1.84	6	0.3559	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	1.125	1.125	1	1	0.3559	Non-Significant Effect
Error	6.75	1.125	6			
Total	7.875		7			

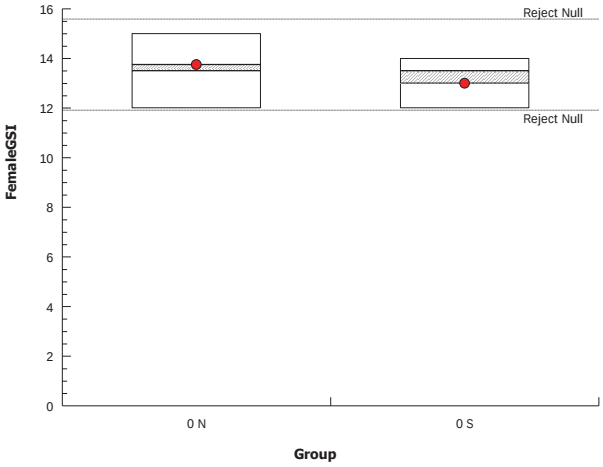
Distributional Tests

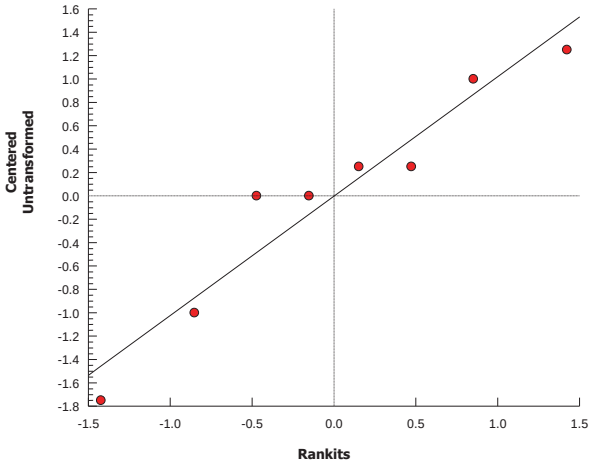
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	2.38	47.5	0.4960	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.93	0.645	0.5174	Normal Distribution

FemaleGSI Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	13	11.7	14.3	13.5	12	14	0.408	6.28%	0.0%
0	Negative Control	4	13.8	11.7	15.8	13.5	12	15	0.629	9.15%	-5.77%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	14-6466-9345	**Endpoint:**	FemaleGSI	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:52	**Analysis:**	Nonparametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	36.8%	0.0086	>0.0086	NA	

Mann-Whitney U Two-Sample Test

Control	vs	Group	Test Stat	Critical	Ties	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	12.5	NA	2	6	0.2857	Exact	Non-Significant Effect
		0.001	9	NA	2	6	0.8857	Exact	Non-Significant Effect
		0.0086	10.5	NA	2	6	0.6571	Exact	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	27.25	9.083333	3	1.06	0.4009	Non-Significant Effect
Error	102.5	8.541667	12			
Total	129.75		15			

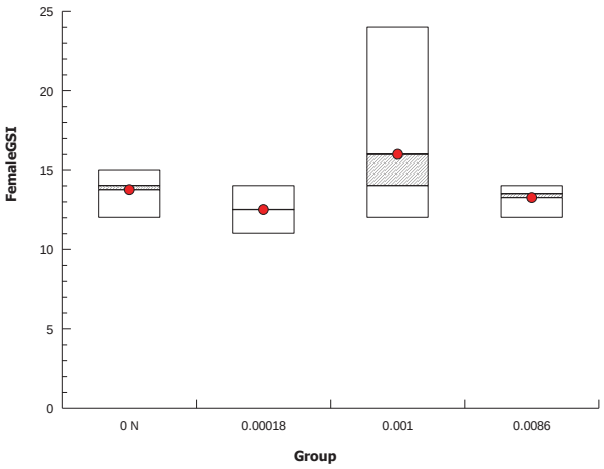
Distributional Tests

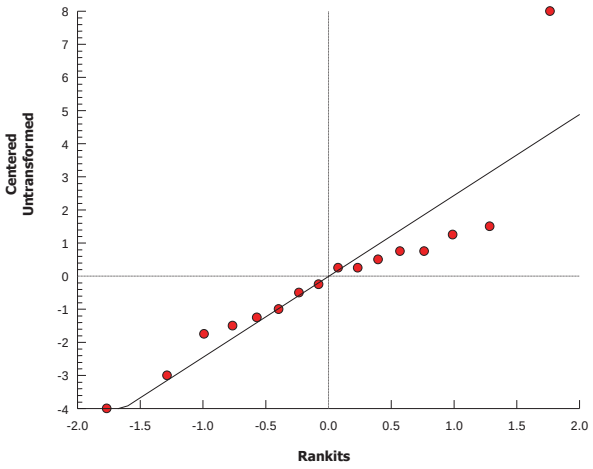
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	11.3	11.3	0.0101	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.832	0.841	0.0075	Non-normal Distribution

FemaleGSI Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	13.8	11.7	15.8	14	12	15	0.629	9.15%	0.0%
0.00018		4	12.5	10.4	14.6	12.5	11	14	0.645	10.3%	9.09%
0.001		4	16	7.28	24.7	14	12	24	2.74	34.2%	-16.4%
0.0086		4	13.3	11.7	14.8	13.5	12	14	0.479	7.23%	3.64%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	03-2908-6428	**Endpoint:**	FemaleLength	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:49	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	5.37%	Passes femalelength

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.982	2.45	2.24	6	0.3639	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	1.619999	1.619999	1	0.965	0.3639	Non-Significant Effect
Error	10.075	1.679167	6			
Total	11.695		7			

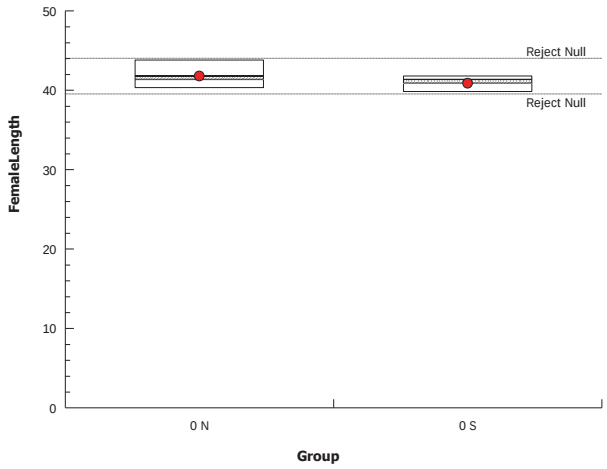
Distributional Tests

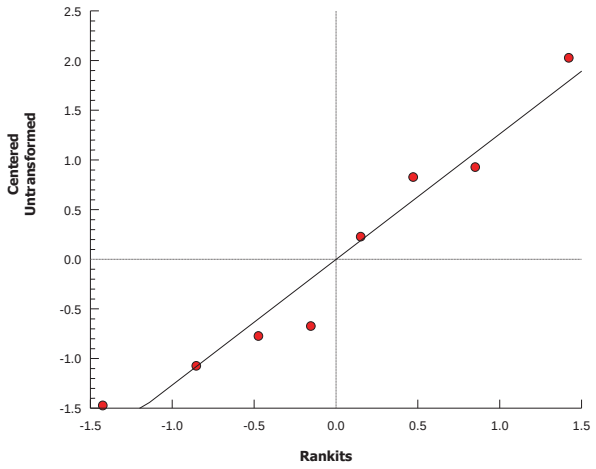
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	2.2	47.5	0.5338	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.942	0.645	0.6348	Normal Distribution

FemaleLength Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	40.9	39.2	42.5	41.3	39.8	41.8	0.512	2.51%	0.0%
0	Negative Control	4	41.8	39.4	44.2	41.3	40.3	43.8	0.76	3.64%	-2.2%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	11-5885-0922	**Endpoint:**	FemaleLength	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:52	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	6.03%	0.0086	>0.0086	NA	

Dunnett Multiple Comparison Test

Control	vs	Group	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	0.346	2.68	2.52	6	0.9716	CDF	Non-Significant Effect
		0.001	0.266	2.68	2.52	6	0.9865	CDF	Non-Significant Effect
		0.0086	0.746	2.68	2.52	6	0.7992	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	2.031878	0.6772925	3	0.384	0.7663	Non-Significant Effect
Error	21.1525	1.762708	12			
Total	23.18437		15			

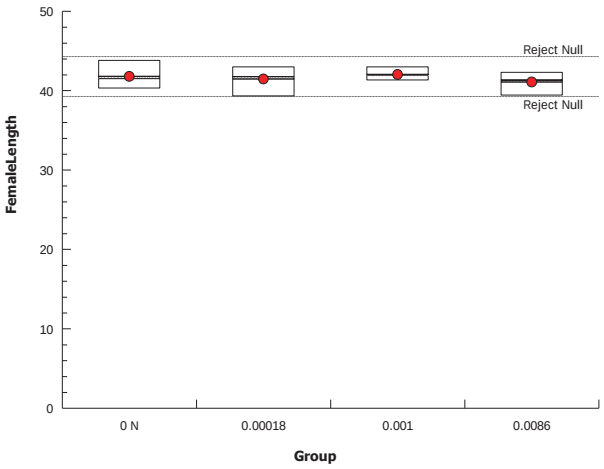
Distributional Tests

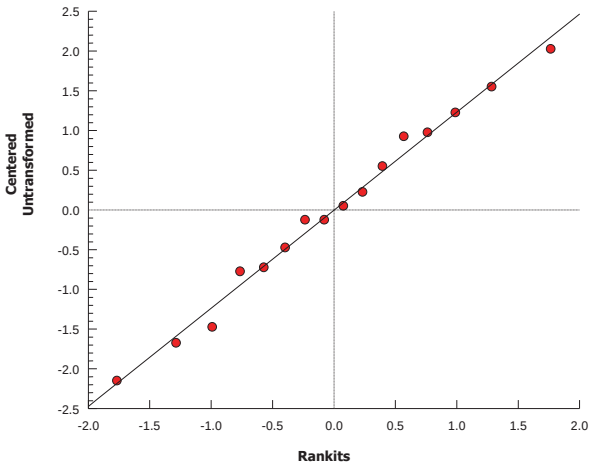
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	1.69	11.3	0.6402	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.982	0.841	0.9782	Normal Distribution

FemaleLength Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	41.8	39.4	44.2	41.5	40.3	43.8	0.76	3.64%	0.0%
0.00018		4	41.4	39	43.9	41.8	39.3	43	0.782	3.77%	0.78%
0.001		4	42	40.9	43.2	41.9	41.3	43	0.354	1.69%	-0.6%
0.0086		4	41.1	38.9	43.2	41.3	39.4	42.3	0.67	3.26%	1.68%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	17-7079-7830	**Endpoint:**	FemaleMedianTubercleScore	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:49	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	Test Result
Untransformed	NA	C <> T	NA	NA	Passes femalemediantuberclescore

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0	2.45		6	1.0000	CDF	Non-Significant Effect

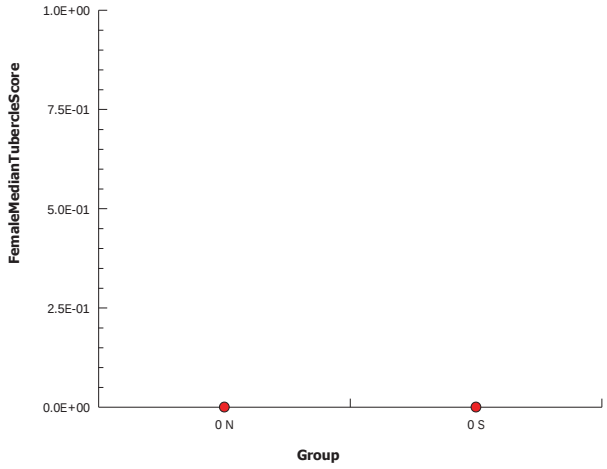
ANOVA Table

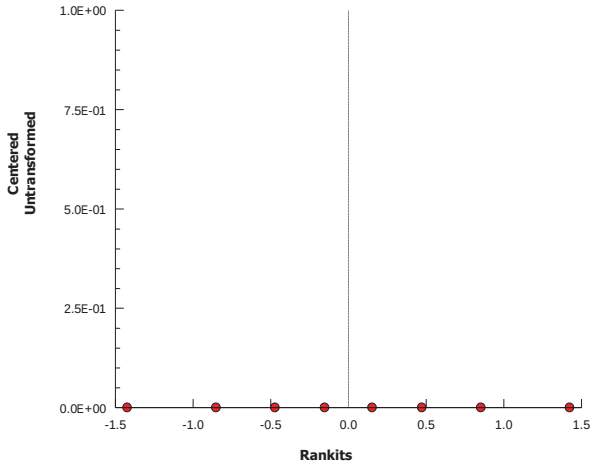
Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0	0	1	65500	<0.0001	Significant Effect
Error	0	0	6			
Total	0		7			

FemaleMedianTubercleScore Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	0	0	0	0	0	0	0		
0	Negative Control	4	0	0	0	0	0	0	0		

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Smithers Viscient**

Analysis ID:	14-3204-5353	**Endpoint:**	FemaleMedianTubercleScore	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:52	**Analysis:**	Nonparametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	0.0086	>0.0086	NA	

Mann-Whitney U Two-Sample Test

Control	vs	Group	Test Stat	Critical	Ties	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	8	NA	1	6	1.0000	Exact	Non-Significant Effect
		0.001	8	NA	1	6	1.0000	Exact	Non-Significant Effect
		0.0086	8	NA	1	6	1.0000	Exact	Non-Significant Effect

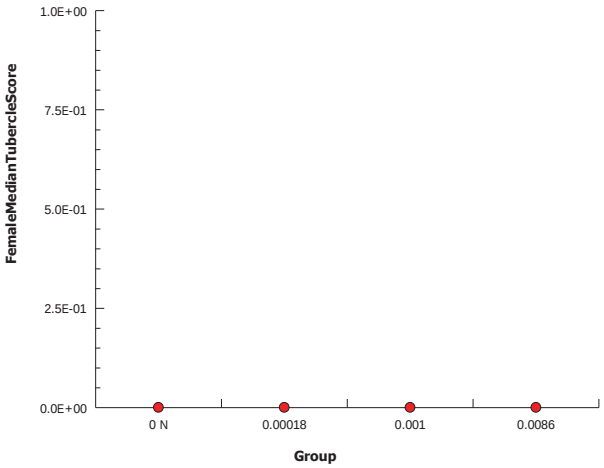
ANOVA Table

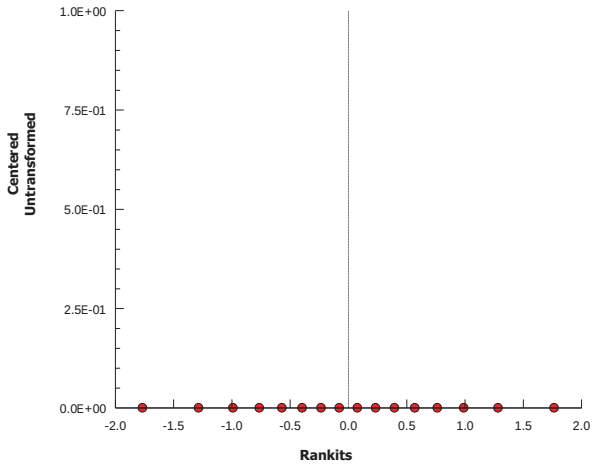
Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0	0	3	65500	<0.0001	Significant Effect
Error	0	0	12			
Total	0		15			

FemaleMedianTubercleScore Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	0	0	0	0	0	0	0		
0.00018		4	0	0	0	0	0	0	0		
0.001		4	0	0	0	0	0	0	0		
0.0086		4	0	0	0	0	0	0	0		

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	03-4342-2354	**Endpoint:**	FemaleVTG	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:50	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	108.0%	Passes femalevtg

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	1.23	2.45	4E+06	6	0.2639	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	8.61125E+12	8.61125E+12	1	1.52	0.2639	Non-Significant Effect
Error	3.40175E+13	5.669583E+12	6			
Total	4.262875E+13		7			

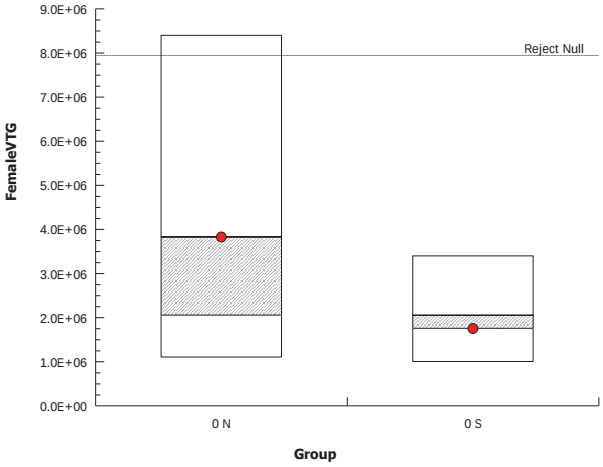
Distributional Tests

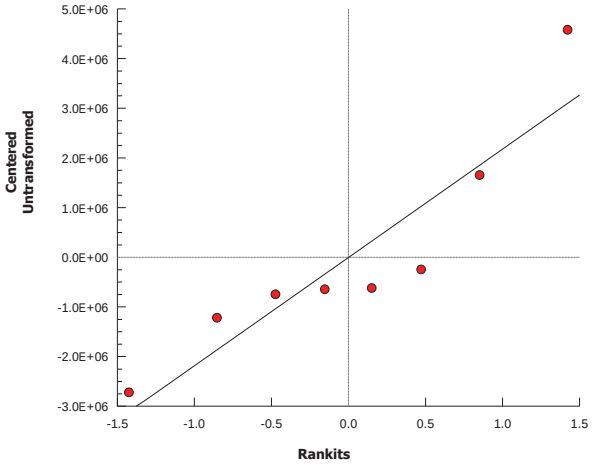
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	8.02	47.5	0.1210	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.862	0.645	0.1259	Normal Distribution

FemaleVTG Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	1.75E+6	-3.38E+4	3.53E+6	2050000	1.00E+6	3.40E+6	5.61E+5	64.1%	0.0%
0	Negative Control	4	3.83E+6	-1.23E+6	8.88E+6	2050000	1.10E+6	8.40E+6	1.59E+6	83.0%	-119.0%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	04-8557-9163	**Endpoint:**	FemaleVTG	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:52	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	147.0%	0.0086	>0.0086	NA	

Dunnett Multiple Comparison Test

Control	vs	Group	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	0.312	2.68	6E+06	6	0.9788	CDF	Non-Significant Effect
		0.001	0.767	2.68	6E+06	6	0.7861	CDF	Non-Significant Effect
		0.0086	0.832	2.68	6E+06	6	0.7463	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	2.384677E+13	7.948922E+12	3	0.909	0.4656	Non-Significant Effect
Error	1.049698E+14	8.747482E+12	12			
Total	1.288165E+14		15			

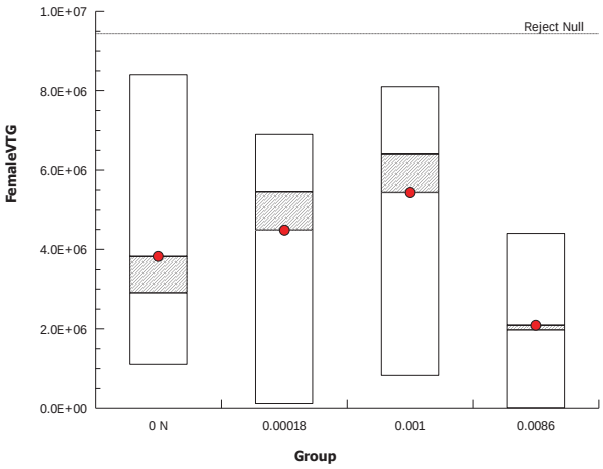
Distributional Tests

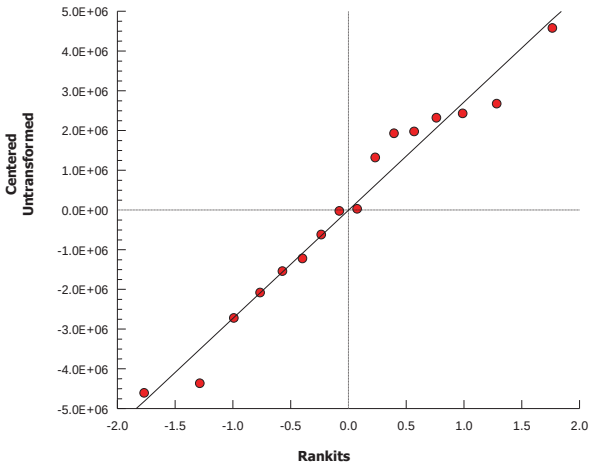
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	0.541	11.3	0.9098	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.963	0.841	0.7085	Normal Distribution

FemaleVTG Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	3.83E+6	-1.23E+6	8.88E+6	2900000	1.10E+6	8.40E+6	1.59E+6	83.0%	0.0%
0.00018		4	4.48E+6	-4.39E+5	9.39E+6	5450000	1.10E+5	6.90E+6	1.54E+6	69.0%	-17.1%
0.001		4	5.43E+6	2.12E+5	1.06E+7	6400000	8.20E+5	8.10E+6	1.64E+6	60.4%	-42.0%
0.0086		4	2.09E+6	-1.33E+6	5.50E+6	1970000	7.90E+2	4.40E+6	1.07E+6	103.0%	45.5%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	15-5973-5531	**Endpoint:**	Fertility	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:50	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	2.75%	Passes fertility

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.676	2.45	2.71	6	0.5239	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	1.125	1.125	1	0.458	0.5239	Non-Significant Effect
Error	14.75	2.458333	6			
Total	15.875		7			

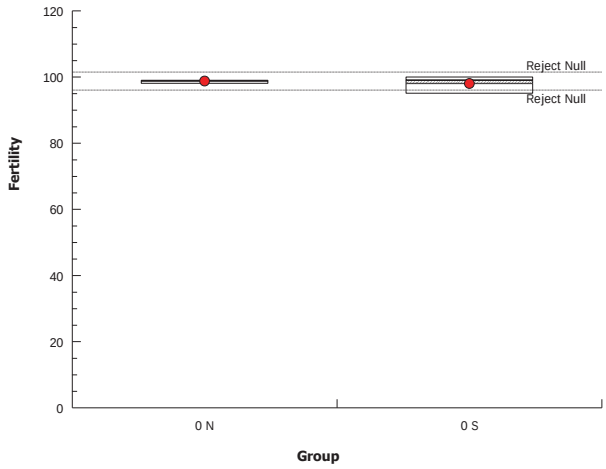
Distributional Tests

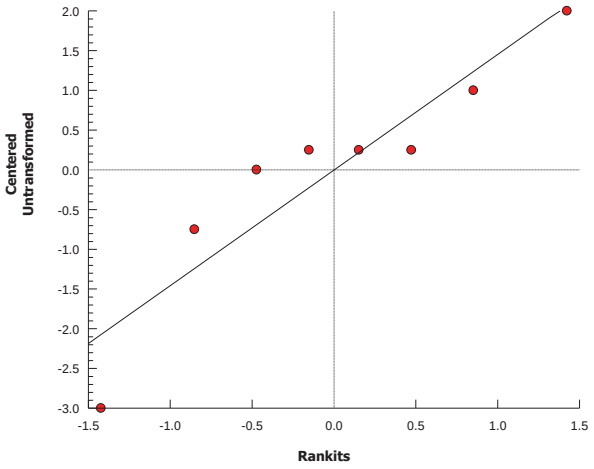
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	18.7	47.5	0.0383	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.89	0.645	0.2326	Normal Distribution

Fertility Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	98	94.6	101	99	95	100	1.08	2.2%	0.0%
0	Negative Control	4	98.8	98	99.5	99	98	99	0.25	0.51%	-0.77%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	08-9163-6327	**Endpoint:**	Fertility	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:53	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	2.0%	0.0086	>0.0086	NA	

Dunnett Multiple Comparison Test

Control	vs	Group	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	0.679	2.68	1.97	6	0.8373	CDF	Non-Significant Effect
		0.001	1.36	2.68	1.97	6	0.4184	CDF	Non-Significant Effect
		0.0086	0	2.68	1.97	6	1.0000	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	2.75	0.9166667	3	0.846	0.4948	Non-Significant Effect
Error	13	1.083333	12			
Total	15.75		15			

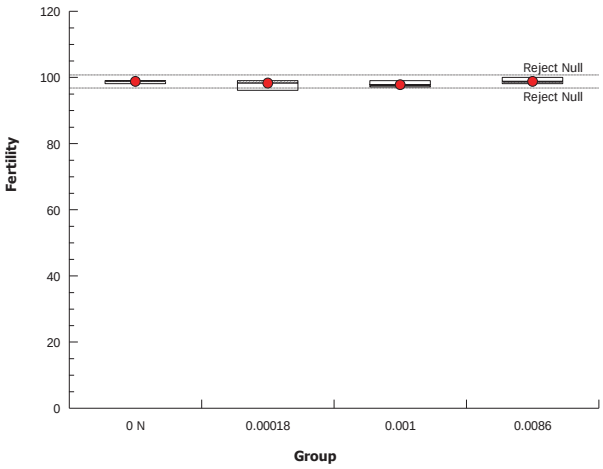
Distributional Tests

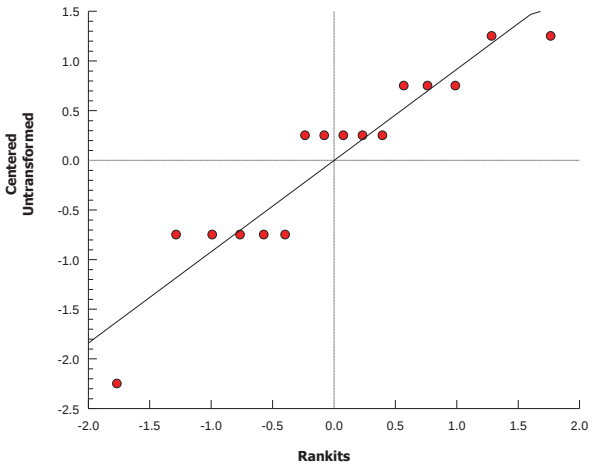
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	2.82	11.3	0.4206	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.897	0.841	0.0714	Normal Distribution

Fertility Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	98.8	98	99.5	99	98	99	0.25	0.51%	0.0%
0.00018		4	98.3	95.9	101	99	96	99	0.75	1.53%	0.51%
0.001		4	97.8	96.2	99.3	97.5	97	99	0.479	0.98%	1.01%
0.0086		4	98.8	97.2	100	98.5	98	100	0.479	0.97%	0.0%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	05-8005-8761	**Endpoint:**	MaleBodyWt	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:50	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	10.6%	Passes malebodywt

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	1.1	2.45	0.298	6	0.3141	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.03577811	0.03577811	1	1.21	0.3141	Non-Significant Effect
Error	0.1779235	0.02965392	6			
Total	0.2137016		7			

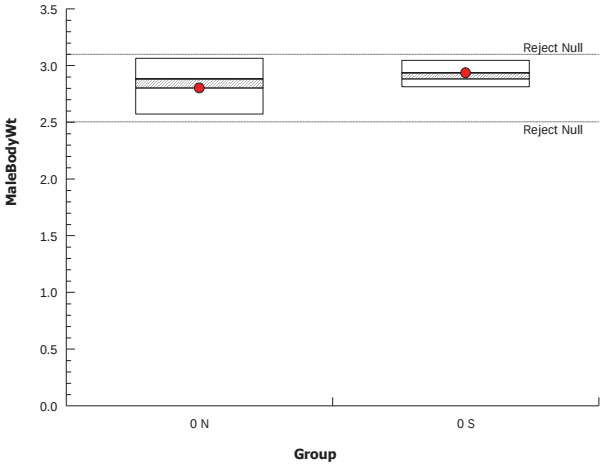
Distributional Tests

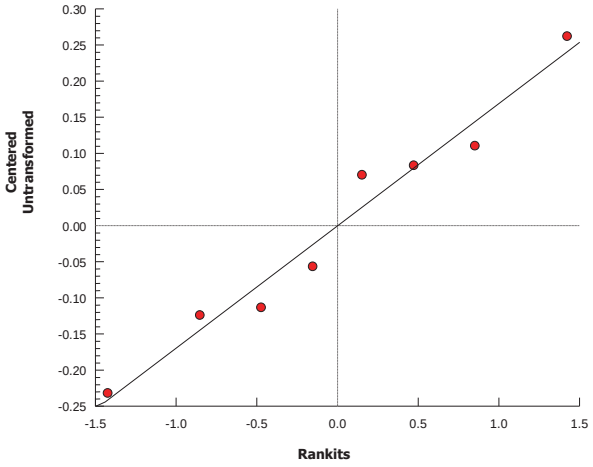
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	3.99	47.5	0.2857	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.966	0.645	0.8666	Normal Distribution

MaleBodyWt Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	2.94	2.76	3.11	2.88	2.81	3.05	0.0545	3.71%	0.0%
0	Negative Control	4	2.8	2.45	3.15	2.88	2.57	3.06	0.109	7.77%	4.56%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	06-4220-5817	**Endpoint:**	MaleBodyWt	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:53	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	28.6%	0.0086	>0.0086	NA	

Dunnett Multiple Comparison Test

Control	vs	Group	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	1.32	2.68	0.8	6	0.4413	CDF	Non-Significant Effect
		0.001	0.1	2.68	0.8	6	0.9992	CDF	Non-Significant Effect
		0.0086	1.08	2.68	0.8	6	0.5858	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.4813336	0.1604445	3	0.902	0.4686	Non-Significant Effect
Error	2.133838	0.1778199	12			
Total	2.615172		15			

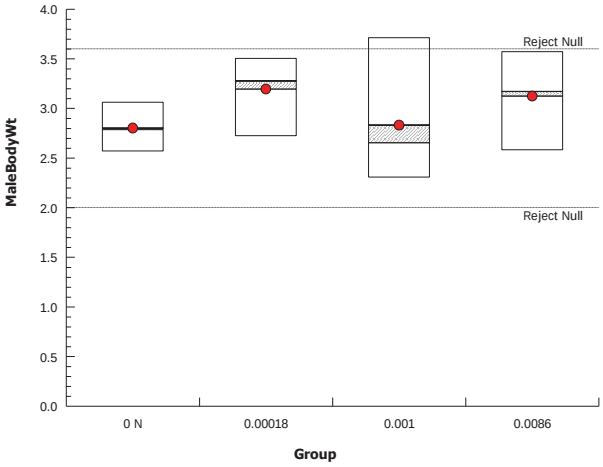
Distributional Tests

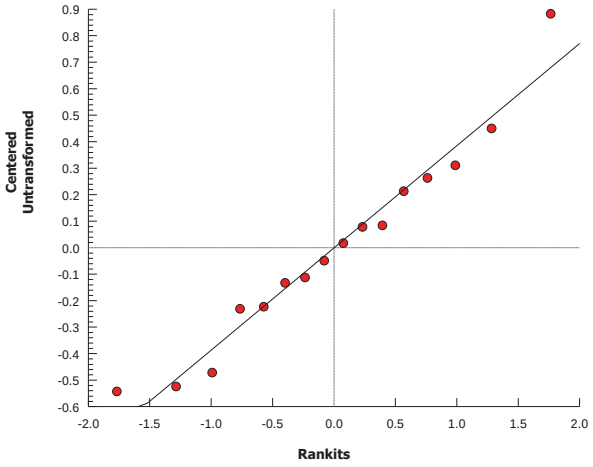
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	2.66	11.3	0.4462	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.961	0.841	0.6735	Normal Distribution

MaleBodyWt Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	2.8	2.45	3.15	2.79	2.57	3.06	0.109	7.77%	0.0%
0.00018		4	3.19	2.64	3.75	3.28	2.72	3.5	0.175	10.9%	-14.0%
0.001		4	2.83	1.86	3.8	2.65	2.31	3.71	0.306	21.6%	-1.07%
0.0086		4	3.12	2.47	3.78	3.17	2.58	3.57	0.205	13.1%	-11.5%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	04-6399-4979	**Endpoint:**	MaleGSI	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:50	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	26.1%	Passes malegsi

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.853	2.45	0.358	6	0.4262	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.03124999	0.03124999	1	0.728	0.4262	Non-Significant Effect
Error	0.2575	0.04291667	6			
Total	0.28875		7			

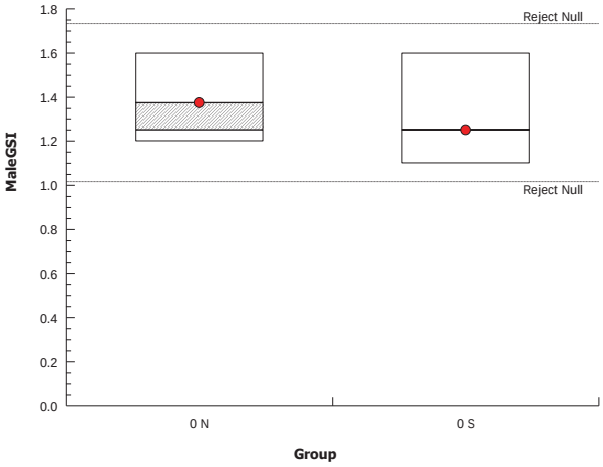
Distributional Tests

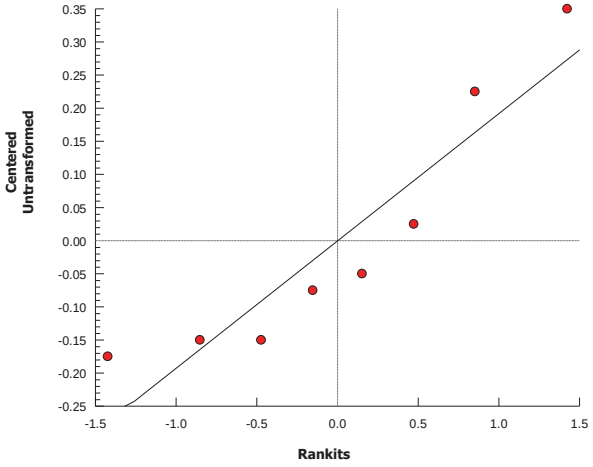
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	1.94	47.5	0.5992	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.852	0.645	0.0993	Normal Distribution

MaleGSI Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	1.25	0.871	1.63	1.25	1.1	1.6	0.119	19.0%	0.0%
0	Negative Control	4	1.38	1.1	1.65	1.25	1.2	1.6	0.0854	12.4%	-10.0%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Smithers Viscient**

Analysis ID:	10-8109-6034	**Endpoint:**	MaleGSI	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:53	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	31.5%	0.0086	>0.0086	NA	

Dunnett Multiple Comparison Test

Control	vs	Group	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	1.42	2.68	0.434	6	0.3840	CDF	Non-Significant Effect
		0.001	1.24	2.68	0.434	6	0.4881	CDF	Non-Significant Effect
		0.0086	1.44	2.68	0.434	6	0.3760	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.1489188	0.04963959	3	0.95	0.4473	Non-Significant Effect
Error	0.6269749	0.05224791	12			
Total	0.7758937		15			

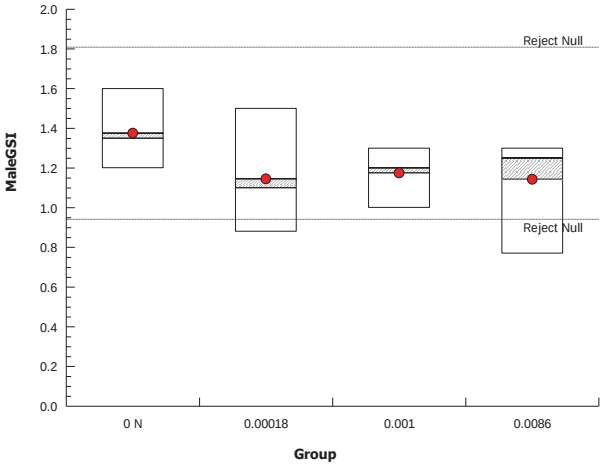
Distributional Tests

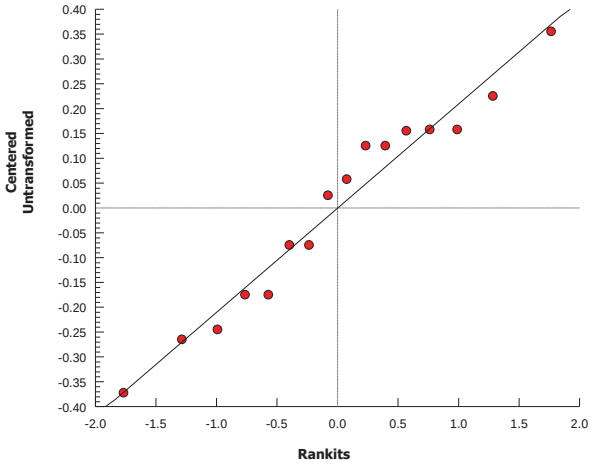
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	1.69	11.3	0.6383	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.959	0.841	0.6417	Normal Distribution

MaleGSI Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	1.38	1.1	1.65	1.35	1.2	1.6	0.0854	12.4%	0.0%
0.00018		4	1.14	0.659	1.63	1.1	0.88	1.5	0.153	26.7%	16.7%
0.001		4	1.17	0.936	1.41	1.2	1	1.3	0.075	12.8%	14.5%
0.0086		4	1.14	0.74	1.54	1.25	0.77	1.3	0.126	22.1%	16.9%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	03-2415-2707	**Endpoint:**	MaleLength	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:50	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	5.33%	Passes malelength

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.54	2.45	2.72	6	0.6089	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	0.7200009	0.7200009	1	0.291	0.6089	Non-Significant Effect
Error	14.83998	2.47333	6			
Total	15.55998		7			

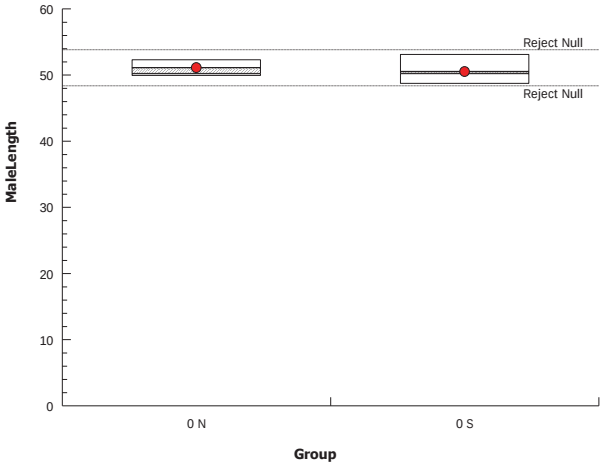
Distributional Tests

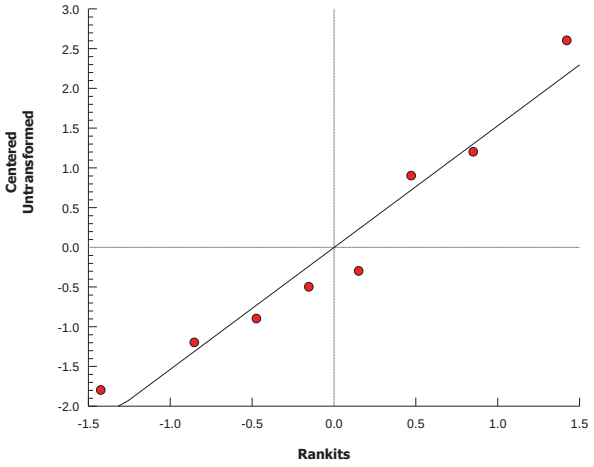
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	2.3	47.5	0.5122	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.947	0.645	0.6763	Normal Distribution

MaleLength Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	50.5	47.5	53.5	50.2	48.7	53.1	0.928	3.68%	0.0%
0	Negative Control	4	51.1	49.2	53	50.2	49.9	52.3	0.612	2.4%	-1.19%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	17-1497-3512	**Endpoint:**	MaleLength	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:54	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	9.1%	0.0086	>0.0086	NA	

Dunnett Multiple Comparison Test

Control	vs	Group	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	0.967	2.68	4.65	6	0.6596	CDF	Non-Significant Effect
		0.001	0.274	2.68	4.65	6	0.9854	CDF	Non-Significant Effect
		0.0086	1.33	2.68	4.65	6	0.4359	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	21.035	7.011665	3	1.17	0.3627	Non-Significant Effect
Error	72.07499	6.006249	12			
Total	93.10999		15			

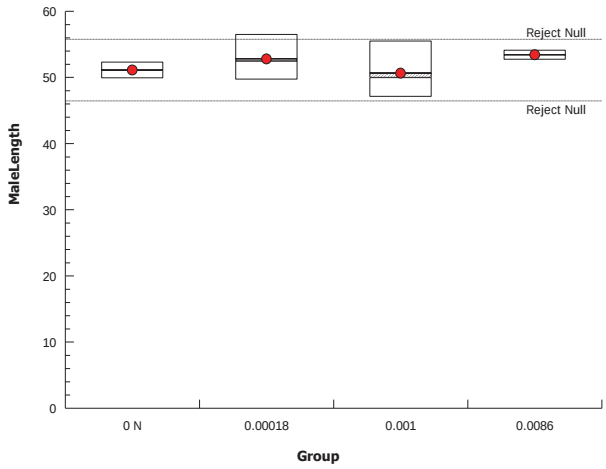
Distributional Tests

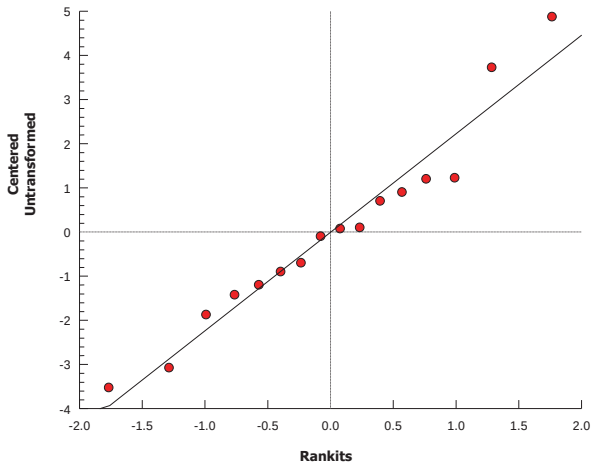
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	8.1	11.3	0.0441	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.954	0.841	0.5544	Normal Distribution

MaleLength Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	51.1	49.2	53	51.1	49.9	52.3	0.612	2.4%	0.0%
0.00018		4	52.8	47.9	57.7	52.5	49.7	56.5	1.54	5.82%	-3.28%
0.001		4	50.6	44.9	56.3	50	47.1	55.5	1.78	7.05%	0.93%
0.0086		4	53.4	52.5	54.3	53.4	52.7	54.1	0.289	1.08%	-4.5%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	00-4478-6645	**Endpoint:**	MaleMedianTubercleScore	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:50	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	30.3%	Passes malemediantuberclescore

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.903	2.45	10.8	6	0.4014	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	32	32	1	0.815	0.4014	Non-Significant Effect
Error	235.5	39.25	6			
Total	267.5		7			

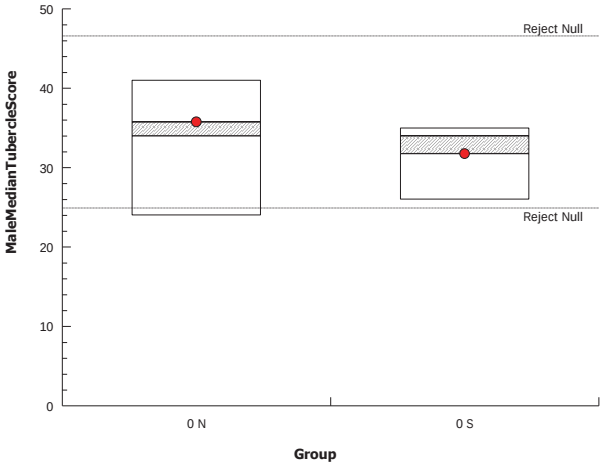
Distributional Tests

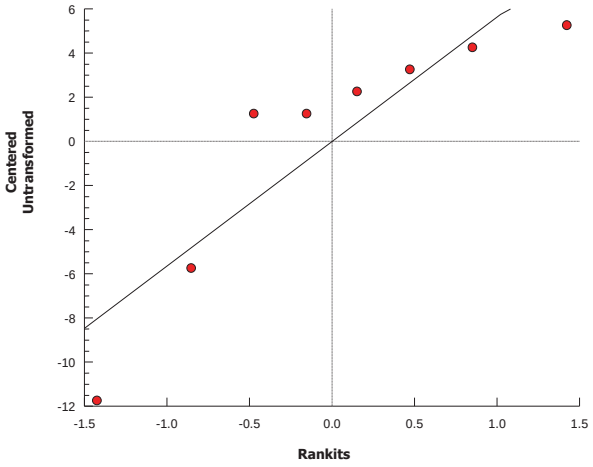
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	4.04	47.5	0.2817	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.815	0.645	0.0415	Normal Distribution

MaleMedianTubercleScore Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	31.8	25.5	38	34	26	35	1.97	12.4%	0.0%
0	Negative Control	4	35.8	23.1	48.4	34	24	41	3.97	22.2%	-12.6%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	18-7794-9561	**Endpoint:**	MaleMedianTubercleScore	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:54	**Analysis:**	Parametric-Control vs Treatments	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	39.5%	0.0086	>0.0086	NA	

Dunnett Multiple Comparison Test

Control	vs	Group	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	1.33	2.68	14.1	6	0.4335	CDF	Non-Significant Effect
		0.001	0.428	2.68	14.1	6	0.9493	CDF	Non-Significant Effect
		0.0086	1.62	2.68	14.1	6	0.2921	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	190.1875	63.39583	3	1.15	0.3699	Non-Significant Effect
Error	663.25	55.27083	12			
Total	853.4375		15			

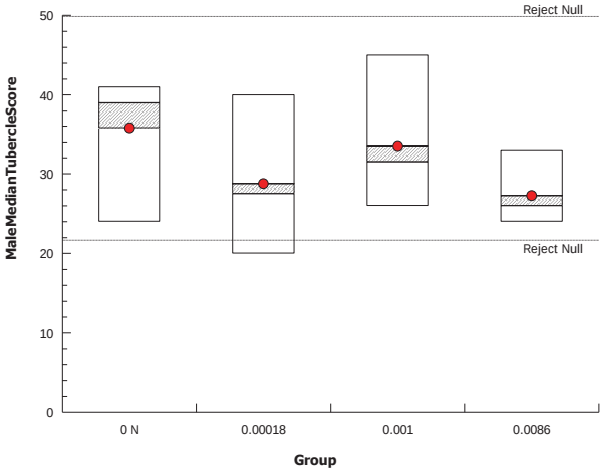
Distributional Tests

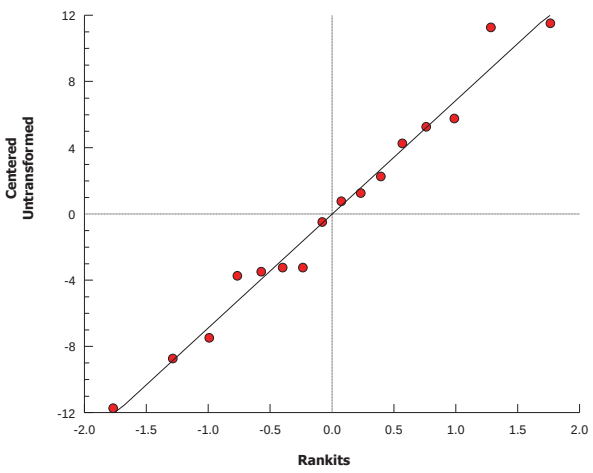
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	1.34	11.3	0.7195	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.972	0.841	0.8677	Normal Distribution

MaleMedianTubercleScore Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	35.8	23.1	48.4	39	24	41	3.97	22.2%	0.0%
0.00018		4	28.8	15.2	42.3	27.5	20	40	4.27	29.7%	19.6%
0.001		4	33.5	20.5	46.5	31.5	26	45	4.09	24.4%	6.29%
0.0086		4	27.3	20.5	34	26	24	33	2.14	15.7%	23.8%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	04-8950-4739	**Endpoint:**	MaleVTG	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:50	**Analysis:**	Parametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	Test Result
Untransformed	NA	C <> T	NA	NA	186.0%	Passes malevtg

Equal Variance t Two-Sample Test

Control	vs	Control	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		Solvent Blank	0.738	2.45	2920	6	0.4885	CDF	Non-Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	1549680	1549680	1	0.544	0.4885	Non-Significant Effect
Error	17082180	2847030	6			
Total	18631860		7			

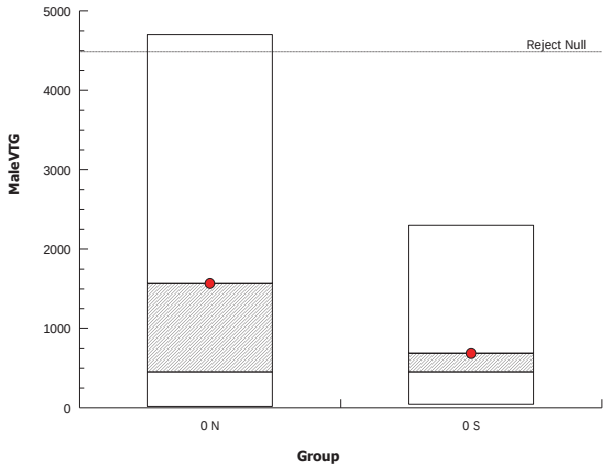
Distributional Tests

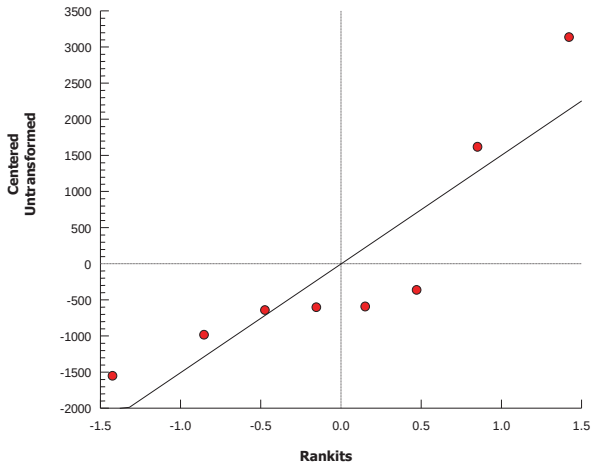
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Variance Ratio F	3.85	47.5	0.2973	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.805	0.645	0.0322	Normal Distribution

MaleVTG Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Solvent Blank	4	686	-1040	2410	450	41	2300	542	158.0%	0.0%
0	Negative Control	4	1570	-1820	4950	450	13	4700	1060	136.0%	-128.0%

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) — **Smithers Viscient**

Analysis ID:	19-6136-6510	**Endpoint:**	MaleVTG	**CETIS Version:**	CETISv1.8.7
Analyzed:	18 Jul-13 17:57	**Analysis:**	Nonparametric-Two Sample	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	

Data Transform	Zeta	Alt Hyp	Trials	Seed	PMSD	NOEL	LOEL	TOEL	TU
Untransformed	NA	C <> T	NA	NA	134000.0	0.001	0.0086	0.002933	

Mann-Whitney U Two-Sample Test

Control	vs	Group	Test Stat	Critical	Ties	DF	P-Value	P-Type	Decision(α:10%)
Negative Control		0.00018	9	NA	0	6	0.8857	Exact	Non-Significant Effect
		0.001	9	NA	0	5	0.4000	Exact	Non-Significant Effect
		0.0086*	12	NA	0	5	0.0571	Exact	Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	2.677158E+14	8.923862E+13	3	47.8	<0.0001	Significant Effect
Error	1.867068E+13	1.867068E+12	10			
Total	2.863865E+14		13			

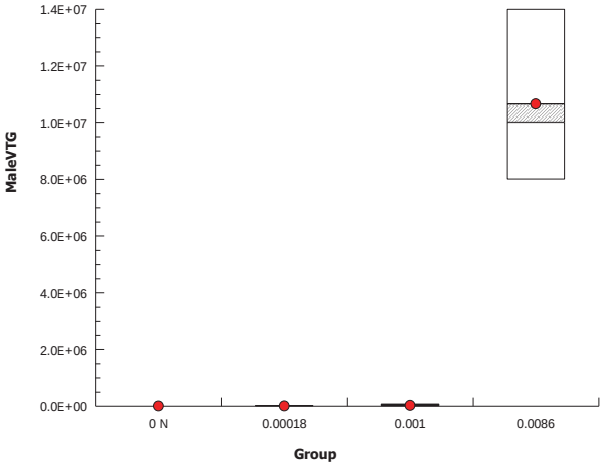
Distributional Tests

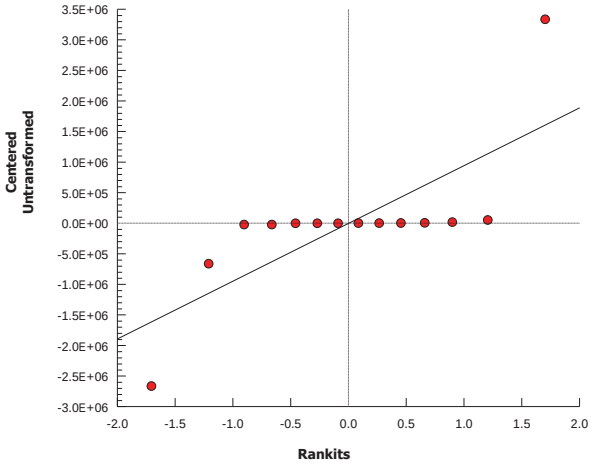
Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	67.5	11.3	<0.0001	Unequal Variances
Distribution	Shapiro-Wilk W Normality	0.62	0.824	<0.0001	Non-normal Distribution

MaleVTG Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	1.57E+3	-1.82E+3	4.95E+3	775	1.30E+1	4.70E+3	1.06E+3	136.0%	0.0%
0.00018		4	4.91E+3	-1.00E+4	1.99E+4	280	6.10E+1	1.90E+4	4.70E+3	192.0%	-213.0%
0.001		3	2.61E+4	-8.13E+4	1.33E+5	1600	6.40E+2	7.60E+4	2.50E+4	166.0%	-1570.0%
0.0086		3	1.07E+7	3.08E+6	1.83E+7	10000000	8.00E+6	1.40E+7	1.76E+6	28.6%	-681000.0

Graphics





OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Smithers Viscient**

Analysis ID:	00-7449-6611	**Endpoint:**	MaleVTG	**CETIS Version:**	CETISv1.8.7
Analyzed:	24 Jul-13 14:22	**Analysis:**	Nonparametric-Control vs Ord. Treatments	**Official Results:**	Yes
Batch ID:	06-4967-6257	**Test Type:**	EDSP FSTRA Tier 1	**Analyst:**	
Start Date:	26 Apr-12	**Protocol:**	OCSPP 890.1350 Tier I FSTRA	**Diluent:**	
Ending Date:		**Species:**	Pimephales promelas	**Brine:**	
Duration:	NA	**Source:**	Lab In-House Culture	**Age:**	
Sample ID:	18-4938-3000	**Code:**	081601 48684201	**Client:**	CDM Smith
Sample Date:	26 Apr-12	**Material:**	Folpet	**Project:**	
Receive Date:		**Source:**	Makhteshim-Agan (MAKHTEAGAN)		
Sample Age:	NA	**Station:**			

Data Transform	Zeta	Alt Hyp	Trials	Seed	NOEL	LOEL	TOEL	TU
Untransformed	NA	C < T	NA	NA	0.001	0.0086	0.002933	

Jonckheere-Terpstra Step-Down Test

Control	vs	Group	Test Stat	Critical	MSD	DF	P-Value	P-Type	Decision(α:5%)
Negative Control		0.00018	7	NA		-2	0.6571	Exact	Non-Significant Effect
		0.001	26	NA		-2	0.1853	Exact	Non-Significant Effect
		0.0086*	59	NA		-2	0.0049	Exact	Significant Effect

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Between	2.677158E+14	8.923862E+13	3	47.8	<0.0001	Significant Effect
Error	1.867068E+13	1.867068E+12	10			
Total	2.863865E+14		13			

Distributional Tests

Attribute	Test	Test Stat	Critical	P-Value	Decision(α:1%)
Variances	Bartlett Equality of Variance	67.51	11.34	<0.0001	Unequal Variances
Distribution	Shapiro-Wilk W Normality	0.6205	0.8239	<0.0001	Non-normal Distribution

MaleVTG Summary

Group	Control Type	Count	Mean	95% LCL	95% UCL	Median	Min	Max	Std Err	CV%	%Effect
0	Negative Control	4	1.566E+3	-1.817E+3	4.949E+3	775	1.300E+1	4.700E+3	1.063E+3	135.8%	0.0%
0.00018		4	4.905E+3	-1.005E+4	1.986E+4	280	6.100E+1	1.900E+4	4.699E+3	191.6%	-213.3%
0.001		3	2.608E+4	-8.132E+4	1.335E+5	1600	6.400E+2	7.600E+4	2.496E+4	165.8%	-1566.0%
0.0086		3	1.067E+7	3.078E+6	1.826E+7	10000000	8.000E+6	1.400E+7	1.764E+6	28.64%	-681100.0

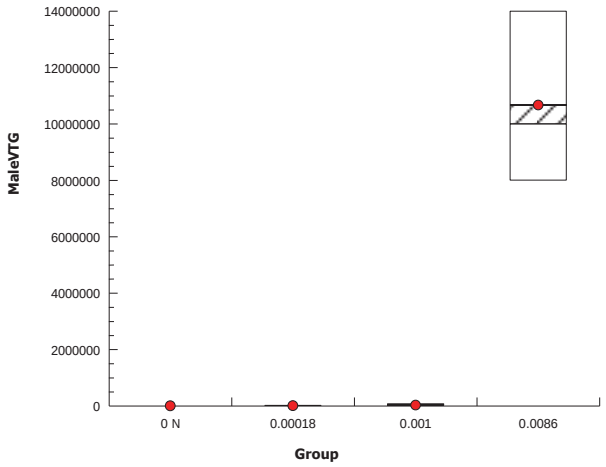
MaleVTG Detail

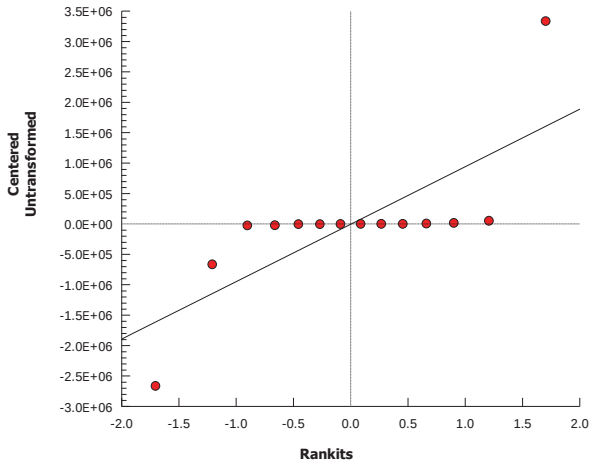
Group	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Negative Control	9.700E+2	1.300E+1	4.700E+3	5.800E+2
0.00018		1.900E+4	1.400E+2	6.100E+1	4.200E+2
0.001		6.400E+2	7.600E+4	1.600E+3	
0.0086		1.400E+7	8.000E+6	1.000E+7	

OPPTS 890.1350 EDSP Fish Short-Term Reproduction Assay (FSTRA) **Smithers Viscient**

Analysis ID:	00-7449-6611	**Endpoint:**	MaleVTG	**CETIS Version:**	CETISv1.8.7
Analyzed:	24 Jul-13 14:22	**Analysis:**	Nonparametric-Control vs Ord. Treatments	**Official Results:**	Yes

Graphics





DATA EVALUATION RECORD

FOLPET

Study Type: OCSPP 890.1400, *In vivo* Hershberger Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-41-2012 (MRID 48616905)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer:	Signature:	Ronnie J. Bever Jr.
Ronnie J. Bever Jr., Ph.D., D.A.B.T.	Date:	4/02/2012
Secondary Reviewer	Signature:	
Kelly Luck, M.S.	Date:	5/10/2012
Program Manager:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	5/21/2012
Quality Assurance:	Signature:	
Steven Brecher, Ph.D., D.A.B.T.	Date:	5/21/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Ayaad Assaad, D.V.M., Ph.D. **Signature:**
Health Effects Division **Date:** 6/12/2015
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:**
Health Effects Division **Date:** 6/16/15

Template version 10/2011

DATA EVALUATION RECORD

STUDY TYPE: *In Vivo* Hershberger Assay (Rat); OCSPP 890.1400; OECD 441

PC CODE: 081601 **DP BARCODE:** D398813

TXR#: 0055725 **CAS No.:** 133-07-3

TEST MATERIAL (PURITY): Folpet (97.6%)

SYNONYMS: Folpan®, 2-[(trichloromethyl)thio]-1*H*-isoindole-1,3(2*H*)-dione

CITATION: Davis, J. (2012). The Hershberger Bioassay (OPPTS 890.1400); Folpet. Integrated Laboratory Systems, Inc., Durham, NC. Laboratory Study No.: C200-200, January 4, 2012. MRID 48616905. Unpublished.

SPONSOR: Makhteshim Chemical Works, Ltd., c/o Makhteshim Agan of North America, Inc., 4515 Falls of Neuse Road, Suite 300, Raleigh, NC 27609

TEST ORDER #: EDSP-081601-175

EXECUTIVE SUMMARY: In a Hershberger assay (MRID 48616905) screening for androgenic activity, folpet (97.6%, batch no. 00138518) in aqueous 1% carboxymethylcellulose was administered daily via oral gavage to groups of eight 61/62-day old castrated male Sprague Dawley rats at dose levels of 0 (vehicle), 250, or 800 mg/kg/day for 10 consecutive days. An androgenic positive control group consisted of eight castrated male rats exposed to carboxymethylcellulose by gavage plus 0.4 mg/kg/day of testosterone propionate (TP) in corn oil by subcutaneous (s.c.) injection.

To screen for potential anti-androgenic activity, folpet in aqueous 1% carboxymethylcellulose was administered daily via oral gavage to groups of eight 61/62-day old castrated male Sprague Dawley rats at dose levels of 0 (vehicle), 100, 250, or 800 mg/kg/day in conjunction with a daily dose of reference androgen TP in corn oil at 0.4 mg/kg/day by s.c. injection. The anti-androgenic positive control group consisted of eight castrated rats exposed to 0.4 mg/kg/day TP and 3 mg/kg/day flutamide (FT) in corn oil. The negative control group consisted of the same animals that served as positive control for the androgenic portion of the assay.

For both components of the assay, body weights were determined daily. The animals were terminated approximately 24 hours after the final dose administration. At necropsy, the five androgen-dependent tissues [seminal vesicles with coagulating glands, ventral prostate, levator ani-bulbocavernosus (LABC), Cowper's glands, and glans penis] were excised, examined macroscopically, and weighed.

Measurements of food consumption, serum hormone concentrations, and liver, kidney, and adrenal weights are optional according to the Guideline and were not performed in this study. No treatment-related effect was noted on mortality due to the chemical (rather than the administration route) or on clinical signs. No gross effect was reported on the sex-related tissues; although, gastrointestinal dilatation was common in the treated groups.

At 800 mg/kg/day, 5/16 rats (3 were co-administered TP) were euthanized before study termination due to moribundity; no signs of dosing error were observed, but animals exhibited loss of body weight, abnormal breathing, and/or soft feces. Additionally, 2/8 rats treated at 800 mg/kg/day folpet (without TP) and 1/8 rats treated with 250 mg/kg/day folpet with TP died before study termination due to gavage error. All other animals survived to the scheduled sacrifice. No explanation was provided why similar moribundity was not observed in the dose range finding study. The oral LD_{50} in rat is >2000 mg/kg and the administration of approximately 1000 mg/kg/day in the diet for three weeks is tolerated. The study author stated that the increase in moribundity may have been due to gavage-related reflux.

In the androgen agonist assay at 800 mg/kg/day, body weights were decreased (not statistically significant [NS]) by 10% at termination, and a body weight loss of 3.3 g ($p \leq 0.05$) was noted over Days 1–11 (compared to a gain of 38.2 g in the vehicle control). Terminal body weights and body weight gains at 250 mg/kg/day were similar to controls. In the anti-androgen assay, terminal body weights and body weight gains in the treated groups were similar to the TP negative control group.

Organ weights in the folpet treated groups were similar to the controls in both the androgen agonist and anti-androgen assays.

There was no effect of flutamide on body weights or body weight gains. TP administration produced a 49% increase in overall (Days 1–11) body weight gain. These body weight gain increases were consistent with an androgenic response in the test animals. For the androgenic portion of the study, the TP positive control caused increases ($p \leq 0.05$) in the weights of the seminal vesicles with coagulating glands (↑1007%), ventral prostate (↑923%), LABC (↑168%), Cowper's glands (↑593%), and glans penis (↑40%), indicating that the test system was sensitive to an androgenic response. For the anti-androgenic portion of the study, the flutamide positive control caused decreases ($p \leq 0.05$) in weights of the seminal vesicles with coagulating glands (↓79%), ventral prostate (↓72%), LABC (↓49%), Cowper's glands (↓64%), and glans penis (↓18%) compared to weights in the negative control group. These data indicate that the test system was sensitive to an anti-androgenic response.

The dose levels for this study were considered adequate based on the moribundity, clinical signs of toxicity, and body weight decreases observed. The Guideline criteria for %CV were met in all cases for the negative control and high dose groups.

Statistically significant changes were not seen in two or more of the five androgen sensitive tissue weights. Folpet was negative for androgenicity and anti-androgenicity in the Hershberger assay.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Hershberger assay (OCSPP 890.1400) in rats.

COMPLIANCE: Signed and dated GLP Compliance, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

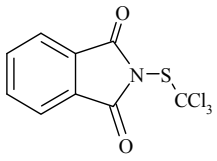
A. MATERIALS

1. Test Facility: Integrated Laboratory Systems, Inc.

Location:	Durham, NC
Study Director:	J. Davis
Other Personnel:	S. Borghoff, Study Toxicologist K. Taylor, Facility Veterinarian
Study Period:	August 31, 2011 through January 4, 2012

2. Test Substance: Folpet

Description:	Fine white powder
Source:	Makhteshim Chemical Works, Ltd.
Batch # (expiration):	00138518 (July 18, 2012)
Purity:	97.6%
Stability:	The report stated that dose formulations in 1% CMC held at 1–10°C for 8 days were stable (data not presented)
CAS #:	133-07-3
Structure:	



3. Reference Androgen: Testosterone propionate (TP)

Supplier	Sigma-Aldrich (St. Louis, MO)
Lot # (expiration):	048K1328 (March 17, 2012)
Purity:	100%
CAS # :	57-85-2

4. Reference Anti-androgen: Flutamide (FT)

Supplier	Sigma-Aldrich (St. Louis, MO)
Lot # (expiration):	107K1293 (February 15, 2012)
Purity:	>99%
CAS # :	13311-84-7

5. Solvent/Vehicle Control: Aqueous 1% carboxymethlcellulose (CMC) for folpet

Supplier:	Sigma-Aldrich (St. Louis, MO)
Lot/Batch #:	100M0113V
Rationale (if other than water):	CMC was selected based on its use in previous studies with Sprague-Dawley rats and the need to maintain folpet stability in the dose formulation from the time of preparation through confirmation of concentration to administration to the animals

Solvent/Vehicle Control: Corn oil for TP and FT

Supplier:	MP Biomedicals, LLC (Solon, OH)
Lot/Batch #:	7862K
Rationale (if other than water):	Not provided; corn oil is an acceptable Guideline solvent

6. Test Animals:

Species:	Rat (castrated males only)	
Strain:	Sprague Dawley (Crl:CD® [SD] IGS)	
Age/weight at dose initiation:	Post-natal day (PND) 61-62/ 268.5 – 349.2 g	
Source:	Charles River Laboratories, Inc. (Raleigh, NC)	
Housing:	Rats were housed 2 per polycarbonate cage with micro-isolator top and absorbent, heat-treated hardwood bedding	
Diet:	Teklad Global 16% Protein Rodent Diet (Teklad Diets, Madison, WI), *ad libitum* (total genistein equivalents = 8.6 µg/g).	
Water:	Reverse osmosis treated tap water, *ad libitum*	
Environmental conditions:	**Temperature:**	21-22ºC
	Humidity:	35–64%
	Air changes:	Not reported
	Photoperiod:	12 hours light/12 hours dark
Acclimation period:	10–11 days at facility prior to initiation of dosing dosing was initiated 16-17 days post-castration	

B. STUDY DESIGN

1. In-Life Dates: Start: September 10, 2011 End: September 21, 2011

2. Study Design: In a Hershberger Assay conducted to screen for potential anti-androgenic and androgenic activity, the test substance was administered daily at two (androgen agonist assay) or three (androgen antagonist assay) dose levels via oral gavage to groups of eight castrated male rats with or without a daily subcutaneous injection of TP (0.4 mg/kg/day). Additionally, a similar group of rats were treated with 0.4 mg/kg/day TP by injection and 3 mg/kg/day FT by daily oral gavage in order to compare the known anti-androgenic effect of FT with the test compound. Anti-androgenic activity is indicated by a statistically significant decrease in two or more target organ weights of the treated groups (test substance + TP) compared to the TP-only control group. Positive androgenic activity is defined as a significant increase in two or more organ weights compared to the vehicle control. For both assays, the animals were treated once daily for 10 consecutive days. Animals were euthanized approximately 24 hours after the final dose administration.

3. Study Schedule: Rats were castrated at post-natal day (PND) 45 at Charles River Laboratories, were received from the animal supplier on PND 52, and the first dose was administered on PND 61 or 62 (>10 days after castration, 10–11 days of acclimation). Doses were administered from PND 61/62 through PND 70/71. Rats were euthanized approximately 24 hours after the last dose, and subjected to necropsy and organ weight measurement.

4. Animal Assignment: Animals were randomly assigned, stratified by body weight, to the test groups noted in Table 1. Statistical analysis indicated that there were no significant differences in group means at study initiation.

TABLE 1. Study Design [a]			
Group Number	**Animal Identification**	**Test Substance/Control**	**Dose Level (mg/kg/day)**
1	1-8	1% CMC	0
2	9-16	Folpet	250
3	17-24	Folpet	800
4	25-32	1% CMC + TP [b]	0 + 0.4
5	33-40	Folpet + TP	100 + 0.4
6	41-48	Folpet + TP	250 + 0.4
7	49-56	Folpet + TP	800 + 0.4
8	57-64	FT + TP	3.0 + 0.4

a Table 1 was copied from Table 3 on pages 18–19 of the study report.
b This dose group served as the positive control for the androgen agonist assay and the negative control for the anti-androgen assay.

5. **Dose-Selection Rationale:** A dose range finding study[1] was conducted at ILS to select a dose to meet the study requirements. Four male Sprague-Dawley rats (PND 36) per dose level were orally administered 1% CMC or folpet at dose levels of 200, 400, 600, 800, and 1000 mg/kg for 14 days. All male rats survived until study termination, except for one rat administered 800 mg/kg that was euthanized due to body weight loss after 10 days on study. No signs of dosing error were observed during the necropsy of this animal. As this animal was not in the high dose group and a treatment-related effect was not observed on body weight, this finding was considered incidental. No treatment-related effect was observed on body weights (in first 10 days of dosing) or food consumption. With the possible exception of soft feces and/or abnormal breathing observed in 2/4 rats at 1000 mg/kg/day, 1/4 rats at 800 mg/kg/day, and 1/4 rats at 600 mg/kg/day compared to 0/4 controls, no clinical signs were considered treatment-related. No changes in absolute or relative liver or kidneys weights were seen in any dose group, and no gross lesions within the stomach or the jejunum (small intestine) were observed at necropsy. The report stated that based on this study, the current test guideline dosing regimen (10 days of dosing), animal model (castrated adult male), and an oral LD_{50} in rat of >2000 mg/kg body weight (folpet material data safety sheet), a high dose of 800 mg/kg was selected to meet the study requirements.

6. **(a) Dose Preparation:** At ILS, appropriate amounts of the folpet (adjusted for compound purity) were added to 1% CMC in distilled water and appropriate amounts of positive controls were added to corn oil such that a dose volume of 5 mL/kg (0.5 mL/kg for testosterone propionate) yielded the targeted dose. Formulations were prepared twice during the assay.

(b) Dose Analysis: Three samples (top, middle, and bottom) of the test substance formulations were collected and shipped (temperature during transit not reported) to Smithers Viscient, LLC (Wareham, MA). Samples were analyzed in duplicate for concentration and homogeneity. Stability was evaluated previously[2]. The report stated that dose formulations in 1% CMC held at 1–10°C for 8 days were stable (data not presented).

[1] Davis, J. (2011). Range Finder Study for *In Vivo* Mammalian Assays for Folpet. Unpublished draft study report prepared by ILS Inc. Study No. C200-500.
[2] Dix, M. (2011). Storage Stability of Folpet in 1% Carboxymethylcellulose Solutions. Unpublished study report prepared by Smithers Viscient Inc. Study No. 11742.6182.

Results

Stability (% of Time 0): Not reported

Concentration (% of nominal): 88.1–100.4%

Homogeneity (%RSD): 0.485–10.0%

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable. Stability data should be submitted for independent verification.

7. **Dosage administration:** Test formulations of folpet and FT were administered to the animals once daily via oral gavage (dose volume 5 mL/kg/day) for 10 consecutive days. TP was given via subcutaneous injection at 0.5 mL/kg/day. The first four animals from each group were dosed beginning on PND 61 and the second four from each group on PND 62. Dosing occurred 24 hours (± 2 hours) from the previous dose. Dose volume was determined on individual animal daily body weight. The dosing sequence was stratified across dose groups; one animal from each group and then repeated until all animals were dosed.

8. **Statistics:** Descriptive statistics (mean, standard deviation and count) were calculated using MS Excel. Final body weight, body weight gain, and tissue weights were analyzed using SAS version 9.2 (SAS Institute, Cary, NC). Studentized residual plots were used to detect possible outliers and Levene's test was used to assess homogeneity of variance. Final body weight, body weight gain, and tissue weights were analyzed by one-way ANOVA followed by pair-wise comparisons using a Dunnett's one-tailed t-test (tissue weights) and Dunnett's two-tailed t-test (final body weight and body weight gain). Statistically significant effects were reported when $p<0.05$. The statistical analyses were considered adequate.

C. METHODS

1. **Clinical Examinations:** Rats were observed for mortality and moribundity twice daily on weekdays and once daily on weekends. Clinical observations were performed within 2 days of rat arrival, at randomization, daily prior to dose administration, and prior to euthanasia. Cage-side observations were conducted 1 hour (± 30 minutes) following dose administration.

2. **Body Weight:** Animals were weighed within 2 days of arrival, at randomization, daily prior to dose administration, and prior to euthanasia. Body weights were reported for each day and at termination. Overall (Days 1-11) body weight gains were also reported.

3. **Food Consumption (Optional):** Food consumption was not measured.

4. **Serum Hormone Measurements (Optional):** Serum hormone levels were not measured.

5. **Dissection and Measurement of Tissue and Organ Weights:** Twenty four hours (± 2 hours) after the final dose administration, animals were humanely euthanized by carbon dioxide (CO_2) asphyxiation with death confirmed by cervical dislocation in the same order

as they were dosed. Gross observations of the tissues that were excised for tissue weights were recorded. The following tissues were excised, trimmed of excess adhering tissue and fat, and weighed: ventral prostate, seminal vesicles with coagulating glands and fluid, LABC, glans penis, and Cowper's (bulbourethral) glands.

II. RESULTS

A. OBSERVATIONS

1. Mortality: At 800 mg/kg/day, 5/16 rats (3 were co-administered TP) were euthanized before study termination due to moribundity; no signs of dosing error were observed, but animals exhibited loss of body weight, abnormal breathing, and/or soft feces. Additionally, 2/8 rats treated at 800 mg/kg/day folpet (without TP) and 1/8 rats treated with 250 mg/kg/day folpet with TP died before study termination due to gavage error. All other animals survived to the scheduled sacrifice.

2. Clinical signs of toxicity: Abnormal breathing (rales, wheezing, or gasping) was noted for 1–3 days in each animal that was euthanized moribund. In the case of the animals sacrificed moribund from the 800 mg/kg/day group with TP, soft feces or loose stools were also observed. In the animals that survived to the scheduled sacrifice, there were no treatment-related clinical signs; abnormal findings were observed on a single day (transient), except for one rat from the 250 mg/kg/day with TP group which was hunched on Days 9–11 (not dose-dependent).

B. BODY WEIGHT AND WEIGHT GAIN: Selected body weight and body weight gain data are presented in Table 2 for the androgen agonist assay and in Table 3 for the anti-androgen assay. In the androgen agonist assay at 800 mg/kg/day, body weights were decreased (NS) by 10% at termination, and a body weight loss of 3.3 g ($p \leq 0.05$) was noted over Days 1–11 (compared to a gain of 38.2 g in the vehicle control). Terminal body weights and body weight gains at 250 mg/kg/day were similar to controls. In the anti-androgen assay, terminal body weights and body weight gains in the treated groups were similar to the TP negative control group.

There was no effect of flutamide on body weights or body weight gains. TP administration produced a 49% increase in overall (Days 1–11) body weight gain. These body weight gain increases were consistent with an androgenic response in the test animals.

TABLE 2. Selected Group Mean (±SD) Body Weights and Cumulative Body Weight Gains (g) in the Androgen Agonist Assay [a]

Study Day #	Dose (mg/kg/day)											
	Vehicle Control			Positive Control Vehicle + TP (0.4)			Folpet (250)			Folpet (800)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1	8	304.7	23.5	8	311.2	21.2	8	309.5	21.6	8	313.6	16.8
5	8	326.1	21.6	8	333.9	17.8	8	315.4	17.9	8	317.2	17.8
10	8	339.4	20.2	8	360.3	23.5	8	326.2	20.7	4	314.1	33.0
11 [b]	8	343.0	19.5	8	368.0	27.4	8	325.3	25.8	4	309.1 (↓10)	32.2
BWG (Days 1-11)	8	38.2	15.4	8	56.8 (↑49)	22.7	8	15.8	16.0	4	-3.3*	28.5

a Data were obtained from Table 9 on page 26 and Appendix 6 on page 88 of the study report. Percent difference of the treated groups from the vehicle control was calculated by the reviewers and included in parentheses.
b Terminal body weight
* Significantly different from control at p≤0.05.
N Number of animals in the group
SD Standard Deviation
BWG Body weight gain

TABLE 3. Selected Group Mean (±SD) Body Weights and Cumulative Body Weight Gains (g) in the Anti-Androgen Assay [a]

Study Day #	Dose (mg/kg/day)														
	Negative Control Vehicle + TP (0.4)			Positive Control FT/TP (3/0.4)			Folpet/TP (100/0.4)			Folpet/TP (250/0.4)			Folpet/TP (800/0.4)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1	8	311.2	21.2	8	318.3	17.6	8	311.2	16.2	8	311.2	16.5	8	312.0	19.3
5	8	333.9	17.8	8	342.0	19.2	8	331.1	19.5	7	324.4	16.6	7	335.2	23.8
10	8	360.3	23.5	8	367.6	28.5	8	344.4	24.1	7	344.4	19.0	5	361.7	24.9
11 [b]	8	368.0	27.4	8	374.7	31.5	8	350.7	26.9	7	348.7	23.2	5	365.2	18.7
BWG (Days 1-11)	8	56.8	22.7	8	56.4	18.2	8	39.5	22.7	7	40.8	18.7	5	50.0	7.7

a Data were obtained from Table 10 on page 27 and Appendix 6 on pages 88–89 of the study report. No significant differences were noted.
b Terminal body weight
N Number of animals in the group
SD Standard Deviation
BWG Body weight gain

C. FOOD CONSUMPTION (Optional): Food consumption was not measured.

D. SERUM HORMONE CONCENTRATIONS (Optional): Serum hormone concentrations were not measured.

E. ORGAN WEIGHTS: Accessory sex organ weights are presented in Tables 4 (androgen agonist assay) and 5 (anti-androgen assay). Organ weights in the folpet treated groups were similar to the controls in both the androgen agonist and anti-androgen assays.

The positive control groups responded as expected. For the androgenic portion of the study, the TP positive control caused increases (p≤0.05) in the weights of the seminal vesicles with coagulating glands (↑1007%), ventral prostate (↑923%), LABC (↑168%), Cowper's glands (↑593%), and glans penis (↑40%), indicating that the test system was sensitive to an androgenic response. For the anti-androgenic portion of the study, the flutamide positive

control caused decreases ($p \leq 0.05$) in weights of the seminal vesicles with coagulating glands (↓79%), ventral prostate (↓72%), LABC (↓49%), Cowper's glands (↓64%), and glans penis (↓18%) compared to weights in the negative control group. These data indicate that the test system was sensitive to an anti-androgenic response.

The Guideline criteria for %CV were met in all cases for the negative control and high dose groups.

TABLE 4. Accessory Sex Organ Weights (mg) from Androgen Agonist Assay in Sprague-Dawley Rats [a]

Organ	Dose (mg/kg/day)															
	Vehicle control				Folpet (250)				Folpet (800)				Positive Control Vehicle + TP (0.4)			
	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Seminal vesicles [b]	8	78.5	10.2	13	8	80.4	21.4	27	4	73.2	15.7	22	8	869.2* (↑1007)	162.4	19
Ventral prostate	8	23.2	2.4	10	8	23.6	4.7	20	4	23.4	5.0	22	8	237.3* (↑923)	45.8	19
LABC	8	193.9	34.6	18	8	187.1	34.1	18	4	163.4	42.2	26	8	518.7* (↑168)	53.8	10
Cowper's glands	8	8.0	2.1	26	8	9.6	2.1	22	4	9.4	1.7	18	8	55.4* (↑593)	11.6	21
Glans penis	8	68.8	4.4	6	8	69.9	8.2	12	4	66.4	3.7	6	8	96.4* (↑40)	12.4	13

a Data were obtained from Table 11 on page 29 of the study report.
b Seminal vesicles and coagulating gland
N Number of animals in the group
SD Standard Deviation
CV Coefficient of Variation (%)
* Significantly different from vehicle control at $p \leq 0.05$. Percent differences of the treated groups from the vehicle control were calculated by the reviewers and included in parentheses.

TABLE 5. Accessory Sex Organ Weights (mg) from Anti-Androgen Assay in Sprague-Dawley Rats [a]

Organ	Dose (mg/kg/day)																			
	Negative Control Vehicle + TP (0.4)				Folpet/TP (100/0.4)				Folpet/TP (250/0.4)				Folpet/TP (800/0.4)				Positive Control FT+TP (3/0.4)			
	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Seminal vesicles [b]	8	869.2	162.4	19	8	804.4	151.5	19	7	871.7	230.7	27	5	808.7	72.7	9	8	183.5* (↓79)	47.6	26
Ventral prostate	8	237.3	45.8	19	8	232.0	52.4	23	7	225.5	48.9	22	5	232.5	26.5	11	8	67.6* (↓72)	23.0	34
LABC	8	518.7	53.8	10	8	518.7	86.6	17	7	563.1	107.2	19	5	515.3	39.2	8	8	263.7* (↓49)	62.9	24
Cowper's glands	8	55.4	11.6	21	8	49.7	10.7	22	7	49.7	9.9	20	5	49.4	7.3	15	8	19.7* (↓64)	3.4	17
Glans penis	8	96.4	12.4	13	8	101.7	6.9	7	7	109.2	11.4	10	5	98.6	10.0	10	8	79.0* (↓18)	12.1	15

a Data were obtained from Table 12 on page 30 of the study report.
b Seminal vesicles and coagulating gland
N Number of animals in the group
SD Standard Deviation
CV Coefficient of Variation (%)
* Significantly different from the TP group at $p \leq 0.05$. Percent differences of the treated groups from the TP control were calculated by the reviewers and included in parentheses.

F. GROSS PATHOLOGY: No gross effect was reported on the sex-related tissues. Gastrointestinal dilatation was common in the treated groups.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Dose levels of 250 and 800 mg/kg folpet did not increase androgen dependent tissue weights compared to the vehicle control group. Folpet co-administered with TP at dose levels of 100, 250, and 800 mg/kg did not decrease androgen dependent tissue weights compared to TP alone. Based on these findings using the castrated rat model Hershberger Bioassay (OCSPP 890.1400), the oral administration of folpet up to a dose level of 800 mg/kg showed no evidence of any androgen agonist or antagonist activity.

B. AGENCY COMMENTS: Measurements of food consumption, serum hormone concentrations, and liver, kidney, and adrenal weights are optional according to the Guideline and were not performed in this study. No gross effect was reported on the sex-related tissues; although, gastrointestinal dilatation was common in the treated groups.

At 800 mg/kg/day, 5/16 rats (3 were co-administered TP) were euthanized before study termination; no signs of dosing error were observed, but animals exhibited loss of body weight, abnormal breathing, and/or soft feces. Additionally, 2/8 rats treated at 800 mg/kg/day folpet (without TP) and 1/8 rats treated with 250 mg/kg/day folpet with TP died before study termination due to gavage error. All other animals survived to the scheduled sacrifice. No explanation was provided why similar moribundity was not observed in the dose range finding study. The oral LD_{50} in rat is >2000 mg/kg and the administration of approximately 1000 mg/kg/day in the diet for three weeks is tolerated[3]. The study author stated that the increase in moribundity may have been due to gavage-related reflux[4].

In the animals that survived to the scheduled sacrifice, there were no treatment-related clinical signs; abnormal findings were observed on a single day (transient), except for one rat from the 250 mg/kg/day with TP group which was hunched on Days 9–11 (not dose-dependent). With the exception of clinical signs observed as part of frank toxicity (moribund leading to unscheduled termination), a treatment-related effect on clinical signs was not observed.

In the androgen agonist assay at 800 mg/kg/day, body weights were decreased (NS) by 10% at termination, and a body weight loss of 3.3 g ($p \leq 0.05$) was noted over Days 1–11 (compared to a gain of 38.2 g in the vehicle control). Terminal body weights and body weight gains at 250 mg/kg/day were similar to controls. In the anti-androgen assay, terminal body weights and body weight gains in the treated groups were similar to the TP negative control group.

[3] Bullock, C.H. (1979) A 21-Day Feeding Study of Technical Phaltan in Rats. Unpublished study report prepared by Chevron Environmental Health Center Study No. S-1407.

[4] Damsch, S., et al (2011). Gavage-Related Reflux in Rats: Identification, Pathogenesis, and Toxicological Implications (Review). Toxicol Pathol, 39: 348-360.

Organ weights in the folpet treated groups were similar to the controls in both the androgen agonist and anti-androgen assays. Folpet showed no evidence of androgen agonist or antagonist activity under the conditions of this assay.

There was no effect of flutamide on body weights or body weight gains. TP administration produced a 49% increase in overall (Days 1–11) body weight gain. These body weight gain increases were consistent with an androgenic response in the test animals. The positive control groups responded as expected. For the androgenic portion of the study, the TP positive control caused the expected increases ($p \leq 0.05$) in the weights of the seminal vesicles with coagulating glands (↑1007%), ventral prostate (↑923%), LABC (↑168%), Cowper's glands (↑593%), and glans penis (↑40%), indicating that the test system was sensitive to an androgenic response. For the anti-androgenic portion of the study, the flutamide positive control caused the expected decreases ($p \leq 0.05$) in weights of the seminal vesicles with coagulating glands (↓79%), ventral prostate (↓72%), LABC (↓49%), Cowper's glands (↓64%), and glans penis (↓18%) compared to weights in the TP-treated group. These data indicate that the test system was sensitive to an anti-androgenic response.

The dose levels for this study were considered adequate based on the moribundity, clinical signs of toxicity, and body weight decreases observed.

Statistically significant changes were not seen in two or more of the five androgen sensitive tissue weights. Folpet was negative for androgenicity and anti-androgenicity in the Hershberger assay.

C. STUDY DEFICIENCIES: None

DATA EVALUATION RECORD

FOLPET

Study Type: OCSPP 890.1450, Female Pubertal Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-68-2012 (MRID 48671201)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
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Building 100, Suite B
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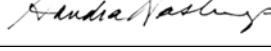
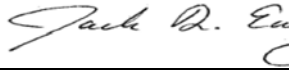
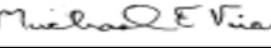
Primary Reviewer: Kelly Luck, M.S.	Signature: Date:	 6/18/2012
Secondary Reviewer: Sandra Hastings	Signature: Date:	 6/21/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	 6/29/2012
Quality Assurance: Michael E. Viana, Ph.D., D.A.B.T.	Signature: Date:	 06/29/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Ayaad Assaad, D.V.M., Ph.D. **Signature:**
Health Effects Division **Date:**
Secondary Reviewer: Elizabeth Mendez, Ph.D. **Signature:**
Health Effects Division **Date:**

Template version 08/2011

DATA EVALUATION RECORD

STUDY TYPE: Female Pubertal Assay; OCSPP 890.1450; OECD None.

PC CODE: 081601 **DP BARCODE:** D401689

TXR#: 0055725 **CAS No:** 133-07-3

TEST MATERIAL (PURITY): Folpet (97.6% a.i.)

SYNONYMS: Folpan; 2-[(trichloromethyl)thio]-1*H*-isoindole-1,3(2*H*)-dione

CITATION: Davis, J. P. (2012) Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Female Rats (OPPTS 890.1450); Folpet. Integrated Laboratory Systems, Inc., Durham, NC. Laboratory Project Study No.: C200-300, April 13, 2012. MRID 48671201. Unpublished.

SPONSOR: Makhteshim Chemical Works, Ltd., c/o Makhteshim Agan of North America, Inc., 4515 Falls of Neuse Road, Suite 300, Raleigh, NC

TEST ORDER #: EDSP-081601-175

EXECUTIVE SUMMARY: In a Female Pubertal Assay (MRID 48671201), 16 Sprague Dawley rats/dose group were treated daily via oral gavage with folpet (97.6% a.i., Batch/lot # 00138518) in aqueous 1% carboxymethylcellulose (CMC) at doses of 0 (vehicle), 400, or 800 mg/kg/day from post-natal day (PND) 22 to 42 or 43. Animals were examined for vaginal opening (VO) daily beginning on PND 22, and age and weight at day of attainment were recorded. Following sacrifice on PND 42 or 43, blood was collected for clinical chemistry analyses; total thyroxine (T_4) and thyroid stimulating hormone (TSH) levels were determined using radioimmunoassays. Adrenal, liver, pituitary, thyroid, and urogenital organs were weighed, and microscopic examinations were performed on the ovaries, uterus, thyroid, and kidneys.

There were no treatment-related clinical signs of toxicity, and no dose-related effects were noted on final body weights, body weight gain, age and body weight at VO, mean age at first vaginal estrus, mean cycle length, percent cycling, or percent regularly cycling.

Two rats in the 400 mg/kg/day group and three rats in the 800 mg/kg/day group were euthanized prior to study termination due to body weight loss and abnormal breathing. No signs of dosing error were observed at necropsy; however, gastrointestinal dilatation was observed in all five animals. All other rats survived until scheduled sacrifice.

At 400 mg/kg/day, relative liver weights were decreased ($p<0.05$) by 5% compared to the vehicle controls; no changes in liver weights were observed in rats in the 800 mg/kg/day group. There were no dose-related effects on the weights of kidneys, pituitary, adrenals, ovaries, uterus (wet and blotted), or thyroid.

Serum T_4 levels were decreased ($p<0.01$) by 33-34% in rats dosed at 400 and 800 mg/kg/day. Sodium and chloride were increased ($p<0.01$) by 2% and 4%, respectively, in rats dosed at 800 mg/kg/day; chloride was also increased ($p<0.05$) in rats dosed at 400 mg/kg/day (↑2%). Alanine aminotransferase and alkaline phosphatase were decreased ($p<0.05$) in rats dosed at 400 and 800 mg/kg/day (↓30% and ↓52%, respectively, for alanine aminotransferase, and ↓20% and ↓18%, respectively, for alkaline phosphatase). There were no dose-related effects on levels of serum TSH, potassium, calcium, phosphorus, aspartate aminotransferase, gamma glutamyl transferase, blood urea nitrogen, creatinine, total bilirubin, sorbitol dehydrogenase, total protein, or albumin.

There were no dose-related histopathological changes in the uterus, thyroid glands, or kidneys. At 800 mg/kg/day, the number of antral ovarian follicles was increased ($p<0.05$) by 58% compared to the controls, which the study author concluded was likely due to the stage of the estrous cycle. The number of other types of ovarian follicles (small, medium, and atretic), follicular cysts, and corpora lutea were not significantly changed in rats from the 400 or 800 mg/kg/day folpet groups compared to vehicle control.

While signs of toxicity (body weight loss, abnormal breathing, and gastrointestinal dilation) were seen 2 and 3 (out of 16) rats at the low and high dose respectively, the remaining animals survived with no treatment-related clinical signs of toxicity.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Female Pubertal Assay (OCSPP 890.1450).

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, and Quality Assurance statements were provided.

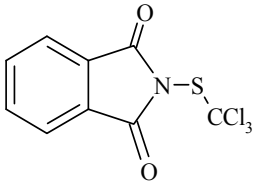
I. MATERIALS AND METHODS

A. MATERIALS

1. Test Facility: Integrated Laboratory Systems, Inc. (ILS)

Location:	Durham, NC
Study Director:	J. P. Davis
Other Personnel:	S. Borghoff (Study Toxicologist); P. Sproul (Toxicology Study Manager); A. Glasscock (Animal Facility Operations Manager); J. Pope (Necropsy Manager); T. Hackett (Histology Manager); D. Giri (Study Pathologist); K. Cummings (QA Manager); K. Taylor (Facility Veterinarian); C. Cachafeiro (Health and Safety Manager)
Study Period:	September 2, 2011 to April 13, 2012

2. Test Substance: Folpet

Description:	Technical; fine white powder
Source:	Makhteshim Chemical Works, Ltd
Lot/Batch #:	00138518 (expiration date 5/26/2012)
Purity:	97.6% a.i.
Stability:	Dose formulations in 1% CMC stable for 8 days at 1-10 °C
CAS #:	133-07-3
Structure:	

3. Vehicle: 1% Carboxymethylcellulose (CMC) in deionized water

4. Test Animals:

Species:	Rat (females only)	
Strain:	Sprague Dawley [Crl:CD®(SD) IGS]	
Age/Weight at Study Initiation:	PND 22/ 50.9-67.7 g	
Source:	Charles River Laboratories (Raleigh, NC)	
Housing:	Animals were housed in polycarbonate cages with absorbent heat-treated hardwood bedding; dams were housed one per cage with litter and F_1 rats were housed two per cage.	
Diet:	Teklad Global 16% Protein Rodent Diet (Teklad Diets, Madison WI) *ad libitum* Total genistein equivalents (genistein plus daidzein) of 8.6 µg/g feed.	
Water:	Reverse-osmosis treated tap water, *ad libitum*	
Environmental Conditions:	**Temperature:**	17-25 °C
	Humidity:	31-66%
	Air changes:	Not reported
	Photoperiod:	14 hrs light/ 10 hrs dark

B. STUDY DESIGN

1. In-Life Dates: Start: September 30, 2011 End: October 21, 2011

2. Mating: Time-mated pregnant dams were received from the supplier on gestation day 8. Between PND 3 and 5, litters with the same date of birth were standardized to 8 pups with equal numbers of males and females.

3. Animal Assignment: Animals were assigned to the test groups noted in Table 1 using a procedure that stratified animals across groups by body weight such that mean body weight

of each group was not statistically different from any other group. Littermates were not assigned to the same treatment group.

TABLE 1. Study Design[a]		
Test group	**Dose (mg/kg/day)**	**# of Females**
Control	0	16
Low	400	16
High	800	16

a Data were obtained from Table 1 on page 15 of the study report.

4. **Dose Selection Rationale:** The dose levels were selected based on the results from a dose range finding study[1] and a previously reviewed uterotrophic study (MRID 48616907). In the range finding study, four female rats (PND 29) per group were dosed with folpet in 1% CMC by oral gavage at 0, 200, 400, 600, 800, or 1000 mg/kg/day for 14 consecutive days. All rats survived until scheduled termination. Body weights of rats dosed at 1000 mg/kg/day were 89% of controls. There were no abnormal cage-side or clinical observations in animals administered up to 400 mg/kg/day. One 600 mg/kg/day female was observed to have abnormal breathing beginning on Day 10, which continued to termination (coinciding with body weight loss). Two 800 mg/kg/day females were observed with abnormal breathing/wheezing beginning on Day 12 and continuing until termination (coinciding with body weight loss). Wheezing was observed in one 1000 mg/kg/day female beginning on Day 13 (coinciding with body weight loss). These observations, however, were considered to be due to gavage error and not compound related. There was no difference in weekly food consumption between the controls and folpet-treated groups, and no changes in relative liver or kidney weights. At necropsy, no gross lesions were observed in the stomach or the jejunum.

   In the uterotrophic study, adult ovariectomized female rats administered 313 or 1000 mg/kg/day for 3 consecutive days survived to the scheduled termination. Body weights of rats administered folpet were not significantly changed compared to the vehicle control group. There were no abnormal clinical observations in all rats administered 313 mg/kg/day folpet. Rales were observed in one rat administered 1000 mg/kg/day folpet on Days 3 and 4, but no abnormal clinical observations were noted in any other animals in this dose group.

   In consideration of the length of the dosing period for the pubertal assay (21/22 days compared to 14 for the range finding study), a dose of 800 mg/kg/day was selected as the high dose, based on the body weight loss following dose administration at higher dose levels. The low dose was one half the high dose (400 mg/kg/day).

5. **Dose Preparation and Analysis:** Dose formulations at 80 and 160 mg/mL were prepared five times during the study by mixing appropriate amounts of test substance with 1% CMC in deionized water. Dose concentrations and homogeneity were tested by Smithers Viscient LLC (Wareham, MA) for each preparation of each formulation prepared by ILS. Three samples (top, middle, and bottom) were analyzed for each concentration level. Analyses to

[1] Davis, J. (2012). Range Finder Study for *In Vivo* Mammalian Assays for Folpet. Unpublished study report prepared by ILS Inc. Study No. C200-500.

demonstrate stability of the test substance in 1% CMC were conducted previously.[2] It was stated that dose formulations in 1% CMC stored at 1-10 °C were stable for up to 8 days.

Results of Dose Analysis

Homogeneity (%CV): 0.388-4.22%

Stability: Not provided

Concentration (% of nominal): 95.8-103%

The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable. The study referenced above should be submitted for verification of the stability findings.

6. **Dosage Administration:** All doses were administered once daily by oral gavage from PND 22 through PND 42 (half the animals in each treatment group) or 43 (remaining animals in each group) in a volume of 5 mL/kg. Dose volume was based on individual animal daily body weight. According to the protocol, dosing was performed between 0700 and 0900 hours daily.

7. **Statistics:** Descriptive statistics (mean, standard deviation, coefficient of variance, and sample size) were calculated using Microsoft Excel 2003/2007. Data sets were statistically analyzed using SAS version 9.2. Studentized residual plots were used to detect possible outliers in the data and Levene's test was used to assess homogeneity of variance. Heterogeneous data were transformed (logarithm, multiplicative inverse, or square root) and if still heterogeneous, analyzed using the non-parametric Kruskal-Wallis and Dunn's test. Homogenous data sets [initial body weights, final body weights (using last day all body weights collected), final body weight gains (using last day all body weights collected), age and body weight at VO, age at first estrus, and cycle length] were analyzed using a one-way analysis of variance (ANOVA) followed by pair-wise comparisons performed using Dunnett's two-tailed t tests. For tissue weights, relative tissue weights (liver, kidneys, pituitary, and adrenals), hormone levels, and clinical chemistry levels, data were analyzed using a two-way ANOVA with treatment and necropsy day (if >1 day) as main effects. Pair-wise comparisons were performed using Dunnett's two-tailed t tests. For initial body weights, final body weight, final body weight gain, age and body weight at VO, and tissue weights, the data were analyzed using a two-way analysis of covariance (ANCOVA) with PND 21 body weight (allocation body weight) as the covariable. Pair-wise comparisons were performed using Dunnett's two-tailed t test. If data sets were not homogenous, ANCOVA analyses were not performed. Trend tests were performed on body weight and tissue weight data sets and reported when significant ($p<0.05$) for endpoints that did not show any significant pair-wise comparisons.

In the instances where VO had not occurred prior to necropsy, the last day of examination plus 1 was used as the age at VO and terminal body weight was used for body weight at VO. In instances when at least one animal in any group exhibited incomplete VO, including

[2] Dix, M. (2011). Storage Stability of Folpet in 1% Carboxymethylcellulose Solutions. Unpublished study report prepared by Smithers Viscient Inc. Study No. 11742.6182.

partial threads for >3 days, the day partial separation was first recorded was used in the analyses.

If VO, body weights, and tissue weight data were not significant, dose-dependent changes were evaluated using a linear regression model for both adjusted and unadjusted values. Chi-square analyses were used to determine significant differences between the cycling status and percent of animals cycling regularly for treated groups compared to the vehicle control group. Statistical analyses of thyroid scoring (colloid area and follicular cell height) were performed by Fisher's Exact test, and when statistically significant, followed by Kruskal-Wallis and Dunn's test.

Statistically significant effects were reported when $p<0.05$. The statistical analyses were considered adequate.

C. METHODS

1. **Mortality and Clinical Examinations:** All animals were examined twice daily (once daily on weekends and holidays) for mortality and moribundity. Clinical examinations were conducted at study allocation, daily prior to dose administration, and at termination. In addition, cage-side observations were performed one hour (± 30 minutes) following dosing each day.

2. **Body Weight:** Animals were weighed at study allocation, daily prior to dosing, and prior to termination.

3. **Vaginal Opening:** Beginning on PND 22, all animals were examined daily for onset of VO. Age and weight on the day of completion of VO were recorded.

4. **Estrous Cyclicity:** Beginning on the day of VO, up to and including the day of necropsy, daily vaginal smears were obtained, evaluated, and classified as diestrus, proestrus, or estrus. The age at first vaginal estrus was recorded. The overall cycling pattern for each female was characterized as regular, irregular, or not cycling. Regular cycling was defined as having recurring 4 to 5 day cycles, while irregular cycling was defined as having cycles with diestrus for a period >3 days or estrus ≥3 days. Animals were not considered to be cycling if there was ≥3 consecutive days of estrus or ≥5 consecutive days of diestrus. Mean cycle length was defined as the number of days from one diestrus to the next diestrus. Incomplete cycles were not used in calculating mean cycle length. In instances where the time between VO and termination did not allow observation of more than one complete cycle, classification was based on the available data and the assumption that animals were regularly cycling if the partial data fit the definition, and cycling irregularly if unable to distinguish between irregular and not cycling at study end.

5. **Sacrifice and Pathology:** Beginning at the initiation of dosing, any rats found moribund or dead were necropsied and the cause of death determined, if possible. Moribund rats were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. On the day of termination, rats were removed to a holding room at least 2 hours before termination. All surviving animals were sacrificed by decapitation on PND 42 or 43 approximately 2 hours after dosing; according to the protocol, sacrifices were completed by 1300 hours. Blood

from the trunk of the animals was collected immediately into serum separation tubes, processed by centrifugation, and the serum was stored at ≤−70°C for subsequent hormone and clinical chemistry evaluations.

a. Hormone Analysis: Total serum T_4 and TSH levels were determined using radioimmunoassays by a commercial laboratory (AniLytics Inc., Gaithersburg, MD).

b. Clinical Chemistry: The following CHECKED (X) parameters were examined by a commercial laboratory (AniLytics Inc., Gaithersburg, MD).

	ELECTROLYTES		OTHER
X	Calcium	X	Albumin
X	Chloride	X	Creatinine*
	Magnesium	X	Urea nitrogen*
X	Phosphorus		Total cholesterol
X	Potassium		Globulins
X	Sodium		Glucose
	ENZYMES	X	Total bilirubin
X	Alkaline phosphatase	X	Total protein
	Cholinesterase		Triglycerides
	Creatine phosphokinase		Serum protein electrophoresis
	Lactic acid dehydrogenase		
X	Alanine aminotransferase		
X	Aspartate aminotransferase		
X	Sorbitol dehydrogenase		
X	Gamma glutamyl transferase		
	Glutamate dehydrogenase		

* Recommended for the pubertal assay in female rats based on Guideline 890.1450.

c. Organ Weights and Histopathology: The following CHECKED (X) tissues were collected and weighed. The (XX) organs, in addition, were subjected to histological examination.

	UROGENITAL		OTHER
XX	Ovaries (paired, without oviducts)*+	XX	Thyroid*+
XX	Uterus*+	X	Liver*
XX	Kidneys (paired)*+	X	Adrenals (paired)*
		X	Pituitary*

* Weights required based on Guideline 890.1450
\+ Histopathological examination required based on Guideline 890.1450

All organs collected, except the thyroid/trachea and uterus, were weighed prior to fixation. Paired organs (kidneys, adrenals, and ovaries) were weighed together. The uterus and cervix were separated from the vagina and weighed. The uterus was weighed again following removal of the fluid in the lumen (blotted weight).
The ovaries (left), kidney (left), uterus (without fluid), and thyroid were fixed in 10% neutral buffered formalin for at least 24 hours and stored in 70% histology grade alcohol prior to embedding; following fixation, the thyroid was dissected from the trachea and weighed before transfer to alcohol storage. All collected tissues were routinely processed, sectioned, stained with hematoxylin and eosin, and examined microscopically.

Two serial sections from each of the two lobes of the thyroid were evaluated for follicular cell height and colloid area using a five point grading scale (1 = shortest/smallest; 5 = tallest/largest). Five random sections of the left ovary were evaluated for any abnormalities/lesions (such as ovarian atrophy) and follicular development, including presence/absence of tertiary/antral follicles, presence/absence of corpora lutea, changes in corpus luteum development, and changes in number of both primary and atretic follicles.

Uterine evaluations included hypertrophy or hyperplasia evaluation as characterized by changes in uterine horn diameter and myometrial, stromal or endometrial gland development. The estrous cycle of the female at the time of necropsy was taken into account during the assessment.

II. RESULTS

A. MORTALITY: Two 400 mg/kg/day females (#18 and #20) were euthanized prior to study termination (Days 18 and 16, respectively) due to loss of body weight and abnormal breathing. Three 800 mg/kg/day females (#33, #40, and #43) were also euthanized prior to study termination (Days 11, 19, and 18, respectively) due to body weight loss and abnormal breathing. No signs of dosing error were observed at necropsy; however, gastrointestinal dilatation was observed in all five animals. All other rats survived until scheduled sacrifice.

B. CLINICAL SIGNS OF TOXICITY: On Day 3, red ocular discharge and a rough coat were observed in two separate controls. One 400 mg/kg/day rat was observed with rales on Days 14-16, but was observed to be clinically normal on all other days. Rales were observed in one 800 mg/kg/day rat on Day 17. No adverse clinical signs were observed in any other animals surviving until the scheduled termination.

C. GENERAL GROWTH AND VAGINAL OPENING: Body weights, body weight gains, and age and weight at day of attainment of VO are presented in Table 2. There were no dose-related effects on final body weights, body weight gain, or age and body weight at VO.

The mean age at VO, body weight at VO, and final body weight in the control group were within the acceptable range of the performance criteria provided in the Guideline (890.1450).

TABLE 2. General Growth and Vaginal Opening (VO)[a]

Parameter Evaluated		Vehicle Control N	Vehicle Control Mean	Vehicle Control SD	Vehicle Control CV (%)	Folpet (400 mg/kg/day) N	Folpet (400 mg/kg/day) Mean	Folpet (400 mg/kg/day) SD	Folpet (400 mg/kg/day) CV (%)	Folpet (800 mg/kg/day) N	Folpet (800 mg/kg/day) Mean	Folpet (800 mg/kg/day) SD	Folpet (800 mg/kg/day) CV (%)
Initial body weight (PND 22; g)	U	16	60.3	3.6	5.9	16	60.3	3.6	6.0	16	60.8	4.3	7.1
	A	16	60.3	NA	NA	16	60.3	NA	NA	16	60.9	NA	NA
Body weight at vaginal opening (g)	U	16	120.9	11.5	9.5	16	125.9	13.3	10.6	15	125.1	14.5	11.6
	A	16	120.8	NA	NA	16	125.9	NA	NA	15	125.2	NA	NA
Final body weight (g)	U	16	171.5	11.7	6.8	14	168.4	11.5	6.8	13	166.5	9.8	5.9
	A	16	171.6	NA	NA	14	168.0	NA	NA	13	166.7	NA	NA
Final body weight (% of control)	U	NA	NA	NA	NA	14	98.2	NA	NA	13	97.1	NA	NA
	A	NA	NA	NA	NA	14	97.2	NA	NA	13	95.6	NA	NA
Body weight gain (final – initial; g)	U	16	111.2	10.4	9.4	14	107.7	11.0	10.2	13	105.8	6.8	6.5
	A	16	111.1	NA	NA	14	107.6	NA	NA	13	105.9	NA	NA
Age at vaginal opening (PND)	U	16	32.9	1.6	5.0	16	34.3	2.3	6.8	15	34.3	2.3	6.6
	A	16	32.9	NA	NA	16	34.3	NA	NA	15	34.3	NA	NA
Proportion unopened (#/N)		0/16				0/16				0/15			

a Data were obtained from Table 7 on page 28 of the study report
U Unadjusted for body weight on PND 21
A Adjusted for body weight on PND 21
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
NA Not applicable

D. <u>ORGAN WEIGHTS</u>: Organ weights at necropsy are presented in Table 3. At 400 mg/kg/day, relative liver weights were decreased ($p<0.05$) by 5% compared to the vehicle controls; no changes in liver weights were observed in rats in the 800 mg/kg/day group. There were no dose-related effects on the weights of kidneys, pituitary, adrenals, ovaries, uterus (wet and blotted), or thyroid.

The unadjusted values for all organ weights in the control group were within the acceptable range of the performance criteria provided in the Guideline (890.1450), with the exception of mean adrenal glands weight (35.6 mg; acceptable range 38.34-48.84 mg).

TABLE 3. Organ Weights at Necropsy[a]

Organ		Vehicle Control				Folpet (400 mg/kg/day)				Folpet (800 mg/kg/day)			
		N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Liver (g)	U	16	7.84	0.59	7.5	14	7.40	0.95	12.8	13	7.44	0.61	8.3
	A	16	7.84	NA	NA	14	7.40	NA	NA	13	7.4	NA	NA
	R	16	4.54	0.18	4.1	14	4.30* (↓5)	0.25	5.7	13	4.38	0.30	6.8
Kidneys (g)	U	16	1.34	0.12	8.7	14	1.39	0.12	8.6	13	1.36	0.08	6.0
	A	16	1.34	NA	NA	14	1.39	NA	NA	13	1.36	NA	NA
	R	16	0.78	0.04	5.0	14	0.81	0.06	7.4	13	0.80	0.06	7.0
Pituitary (mg)	U	16	8.9	1.1	12.1	14	9.1	2.6	28.4	13	7.7	1.7	21.9
	A	16	8.9	NA	NA	14	9.1	NA	NA	13	7.7	NA	NA
	R	16	5.1	0.5	10.6	14	5.3	1.5	28.5	13	4.5	1.0	21.0
Adrenals (mg)	U	16	35.6	7.5	21.1	14	38.1	6.4	16.8	13	37.4	4.2	11.3
	A	16	35.6	NA	NA	14	38.1	NA	NA	13	37.4	NA	NA
	R	16	20.6	4.3	20.8	14	22.1	2.5	11.4	13	22.0	1.6	7.5
Ovaries (mg)	U	16	83.6	12.9	15.4	14	79.3	12.3	15.4	13	78.0	9.2	11.8
	A	16	83.6	NA	NA	14	79.3	NA	NA	13	78.0	NA	NA
Uterus, wet (mg)	U	15[b]	352.0	173.1	49.2	14	300.3	134.8	44.9	13	281.4	85.6	30.4
	A	15[b]	351.9	NA	NA	14	300.2	NA	NA	13	281.6	NA	NA
Uterus, blotted (mg)	U	16	300.7	74.1	24.6	14	256.8	68.1	26.5	13	253.6	62.9	24.8
	A	16	300.6	NA	NA	14	257.1	NA	NA	13	253.4	NA	NA
Thyroid, fixed (mg)	U	16	12.93	3.46	26.8	14	11.94	1.32	11.0	13	12.63	2.08	16.5
	A	16	12.93	NA	NA	14	11.92	NA	NA	13	12.65	NA	NA

a Data were obtained from Table 8 on page 29 of the study report. Percent differences from controls, calculated by the reviewers, are included in parentheses.
b Weight of one uterus excluded due to fluid leakage.
U Unadjusted for body weight on PND 21
A Adjusted for body weight on PND 21
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
R Organ-to-body weight ratio (relative to body weight)
* Significantly different from controls at $p<0.05$.

E. ESTROUS CYCLICITY: Estrous cycle data are provided in Table 4. There were no effects of treatment on mean age at first vaginal estrus, mean cycle length, percent cycling, or percent regularly cycling.

TABLE 4. Estrous Cyclicity[a]

Treatment Groups	N	Mean Age at First Vaginal Estrus (PND)	Mean Cycle Length (days)	Cycling (%)	Regularly Cycling (%)	Cycle Status at Necropsy (# Females)			
						Diestrus	Proestrus	Estrus	Not Cycling
Vehicle	16	34.6	4.8	100	81.3	3	6	7	0
Folpet (400 mg/kg/day)	14	34.9	4.7	100	100	5	6	3	0
Folpet (800 mg/kg/day)	13	35.2	4.4	100	84.6	7	2	4	0

a Data were obtained from Tables 8 and 9 on pages 29 and 30 of the study report.
N Number of animals

F. CLINICAL CHEMISTRY AND HORMONE LEVELS: Mean hormone and clinical chemistry levels are presented in Table 5. The study author noted that the performing

laboratory did not have a database of historical hormone and clinical chemistry values for female Sprague Dawley rats. However, reference ranges, obtained from published literature, were reported and are attached an Appendix to this document.

Serum T_4 levels were decreased ($p<0.01$) by 33-34% in rats dosed at 400 mg/kg/day and above; however, no concomitant change was observed in the TSH concentration. Sodium and chloride were increased ($p<0.01$) by 2% and 4%, respectively, in rats dosed at 800 mg/kg/day; chloride was also increased ($p<0.05$) in rats dosed at 400 mg/kg/day (↑2%). Alanine aminotransferase and alkaline phosphatase were decreased ($p<0.05$) in rats dosed at 400 and 800 mg/kg/day (↓30% and ↓52%, respectively, for alanine aminotransferase, and ↓20% and ↓18%, respectively, for alkaline phosphatase). There were no dose-related effects on levels of serum TSH, potassium, calcium, phosphorus, aspartate aminotransferase, gamma glutamyl transferase, blood urea nitrogen, creatinine, total bilirubin, sorbitol dehydrogenase, total protein, or albumin.

The results for serum T_4 in the control group were within the acceptable range of the performance criteria provided in the Guideline (890.1450).

TABLE 5. Hormone Levels and Clinical Chemistry[a]

Parameter Evaluated	Vehicle Control				Folpet (400 mg/kg/day)				Folpet (800 mg/kg/day)			
	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Hormones												
Serum T_4, Total (µg/dL)	16	3.22	0.77	24.1	14	2.15** (↓33)	0.60	28.0	13	2.14** (↓34)	0.40	18.7
Serum TSH (ng/mL)	16	2.62	1.26	48.1	14	2.37	0.75	31.6	13	3.48	1.46	41.9
Clinical Chemistry												
Sodium (mEq/L)	16	138	3	2	14	139	2	1	13	141** (↑2)	3	2
Potassium (mEq/L)	16	7.3	0.4	6.1	14	7.2	0.4	5.8	13	7.1	0.4	5.9
Chloride (mEq/L)	16	103	2	2	14	105* (↑2)	1	1	13	107** (↑4)	2	2
Calcium (mg/dL)	16	10.8	0.3	3.0	14	10.8	0.3	2.5	13	10.8	0.4	3.3
Phosphorus (mg/dL)	16	12.6	0.6	4.4	14	12.4	0.4	3.1	13	12.4	0.8	6.7
Aspartate aminotransferase (U/L)	16	258	50	19	14	245	26	10	13	221	28	13
Alanine aminotransferase (U/L)	16	56	9	17	14	39** (↓30)	11	28	13	27** (↓52)	8	30
Gamma glutamyl transferase (U/L)[b]	16	0	1.0	236	14	0	1	289	13	1	1.0	170
Alkaline phosphatase (U/L)	16	304	60	20	14	243** (↓20)	39	16	13	249* (↓18)	45	18
Blood urea nitrogen (mg/dL)	16	13	2	16	14	15	2	16	13	15	2	15
Creatinine (mg/dL)	16	0.4	0.1	15.4	14	0.4	0.03	6.6	13	0.4	0.04	9.8
Total bilirubin (mg/dL)[b]	16	0.0	0.0	NA	14	0.1	0.04	254.2	13	0.01	0.03	360.6
Sorbitol dehydrogenase (U/L)	16	16	4	26	14	17	2	14	13	16	3	18
Total protein (g/dL)	16	5.9	0.3	4.7	14	5.7	0.2	3.9	13	5.7	0.4	7.5
Albumin (g/dL)	16	4.8	0.3	5.4	14	4.7	0.2	4.9	13	4.6	0.3	7.2

a Data were obtained from Tables 10 and 11 on pages 30 and 31 of the study report. Percent differences from controls, calculated by the reviewers, are included in parentheses.
b Data as reported by study author. No limit of quantitation was reported.
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
NA Not applicable
* Significantly different from controls at $p<0.05$.
** Significantly different from controls at $p<0.01$.

G. GROSS PATHOLOGY: At necropsy, cecal dilatation was observed in 5/14 rats at 400 mg/kg/day and in 1/13 rats at 800 mg/kg/day. Enlarged kidneys were observed in one 400 mg/kg/day rat and colon dilatation was observed in one 800 mg/kg/day rat. There were no other gross observations at necropsy in rats surviving until scheduled termination.

H. HISTOPATHOLOGY: There were no dose-related histopathological changes in the uterus, thyroid glands, or kidneys.

Thyroid gland follicular cell height and colloid area data for rats in the study are summarized in Table 6. There were no apparent or statistical differences in follicular cell height and colloid area in the thyroid glands of folpet treated rats compared to the vehicle controls.

TABLE 6. Thyroid Gland Follicular Cell Height and Colloid Area[a]			
Findings	**Vehicle Control**	**Folpet (400 mg/kg/day)**	**Folpet (800 mg/kg/day)**
Number of animals examined	16	14	13
Follicular cell height[b]			
1	0	0	0
2	14 (87.5%)	11 (78.6%)	7 (53.8%)
3	2 (12.5%)	3 (21.4%)	6 (46.2%)
4	0	0	0
5	0	0	0
Follicular colloid area[b]			
1	0	0	0
2	0	0	0
3	2 (12.5%)	3 (21.4%)	6 (46.2%)
4	14 (87.5%)	11 (78.6%)	7 (53.8%)
5	0	0	0

a Data were obtained from Table 14 on page 34 of the study report.
b A five-point grading scale (1 = shortest / smallest; 5 = tallest / largest) was used.

The number of ovarian follicles, follicular cysts, and corpora lutea are presented in Table 7. At 800 mg/kg/day, the number of antral ovarian follicles was increased ($p<0.05$) by 58% compared to the controls, which the study author concluded was likely due to the stage of estrous cycle. The number of other types of ovarian follicles (small, medium, and atretic), follicular cysts, and corpora lutea were not significantly changed in rats from the 400 or 800 mg/kg/day folpet groups compared to vehicle control.

TABLE 7. Number of Ovary Follicles, Follicular Cysts, and Corpora Lutea[a]												
	Vehicle Control				**Folpet (400 mg/kg/day)**				**Folpet (800 mg/kg/day)**			
Parameter	**N**	**Mean**	**SD**	**CV (%)**	**N**	**Mean**	**SD**	**CV (%)**	**N**	**Mean**	**SD**	**CV (%)**
Small Follicles	16	29	14.2	49.0	14	24	13.2	55.0	13	26	8.4	32.3
Medium Follicles	16	11	5.1	46.4	14	10	4.6	46.0	13	10	3.6	36.0
Antral Follicles	16	4.5	2.4	53.3	14	4.6	1.8	39.1	13	7.1* (↑58)	2.6	36.6
Atretic Follicles	16	6.0	3.1	51.7	14	5.5	1.9	34.5	13	4.9	1.7	34.7
Follicular Cysts	16	0.0	0.1	0	14	0.0	0.0	NA	13	0.0	0.0	NA
Corpora Lutea	16	6.2	2.1	33.8	14	5.5	2.1	38.2	13	6.6	2.2	33.3

a Data were obtained from Table 13 on page 33 of the study report. Percent differences from controls, calculated by the reviewers, are included in parentheses.
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
NA Not applicable
* Significantly different from controls at $p<0.05$.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATORS' CONCLUSIONS: Administration of 400 or 800 mg/kg/day folpet did not show changes in endpoints that would suggest an effect on pubertal development. Although serum T_4 concentrations were decreased following administration of 400 or 800 mg/kg/day folpet, no other signs of thyroid gland modulation were observed.

B. AGENCY COMMENTS: There were no treatment related clinical signs of toxicity, and no dose-related effects were noted on final body weights, body weight gain, age and body weight at VO, mean age at first vaginal estrus, mean cycle length, percent cycling, or percent regularly cycling.

Two rats in the 400 mg/kg/day group and three rats in the 800 mg/kg/day group were euthanized prior to study termination due to body weight loss and abnormal breathing. No signs of dosing error were observed at necropsy; however, gastrointestinal dilatation was observed in all five animals. All other rats survived until scheduled sacrifice.

At 400 mg/kg/day, relative liver weights were decreased ($p<0.05$) by 5% compared to the vehicle controls; no changes in liver weights were observed in rats in the 800 mg/kg/day group. There were no dose-related effects on the weights of kidneys, pituitary, adrenals, ovaries, uterus (wet and blotted), or thyroid.

Serum T_4 levels were decreased ($p<0.01$) by 33-34% in rats dosed at 400 800 mg/kg/day. Sodium and chloride were increased ($p<0.01$) by 2% and 4%, respectively, in rats dosed at 800 mg/kg/day; Chloride was also increased ($p<0.05$) in rats dosed at 400 mg/kg/day (↑2%). Alanine aminotransferase and alkaline phosphatase were decreased ($p<0.05$) in rats dosed at 400 and 800 mg/kg/day (↓30% and ↓52%, respectively, for alanine aminotransferase, and ↓20% and ↓18%, respectively, for alkaline phosphatase). Sodium, chloride, and alanine aminotransferase were all within or slightly below the range of literature values provided by the analytical laboratory (see Appendix). There were no dose-related effects on levels of serum TSH, potassium, calcium, phosphorus, aspartate aminotransferase, gamma glutamyl transferase, blood urea nitrogen, creatinine, total bilirubin, sorbitol dehydrogenase, total protein, or albumin.

There were no dose-related histopathological changes in the uterus, thyroid glands, or kidneys. At 800 mg/kg/day, the number of antral ovarian follicles was increased ($p<0.05$) by 58% compared to the controls. The number of other types of ovarian follicles (small, medium, and atretic), follicular cysts, and corpora lutea were not significantly changed in rats from the 400 or 800 mg/kg/day folpet groups compared to vehicle control.

Initially, the high-dose of folpet selected for this study (800 mg/kg/day) was considered appropriate based on data from a dose range-finding study showing body weight loss at 1,000 mg/kg/day; however, in this assay both doses tested were determined to be overtly toxic based on 2/16 rats at the low dose and 3/16 rats at the high dose euthanized due to body weight loss, abnormal breathing, and gastrointestinal dilation.

C. STUDY DEFICIENCIES: The following deficiencies were noted that were not considered to have had an adverse effect on the results, interpretations or conclusions of this study:

- Control mean adrenal glands weight (35.6 mg) was below the Guideline performance criteria (38.34-48.84 mg).

DATA EVALUATION RECORD

FOLPET

Study Type: OCSPP 890.1500, Male Pubertal Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-68-2012 (MRID 48671202)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
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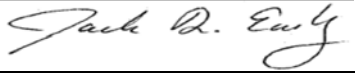
Sandra Hastings	Date:	6/25/2012
Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	6/29/2012
Quality Assurance:	Signature:	
Michael E. Viana, Ph.D., D.A.B.T.	Date:	6/29/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Ayaad Assaad, D.V.M., Ph.D. **Signature:**
Health Effects Division **Date:**
Secondary Reviewer: John Liccione, Ph.D. **Signature:**
Health Effects Division **Date:**

DATA EVALUATION RECORD

STUDY TYPE: Male Pubertal Assay; OCSPP 890.1500

PC CODE: 081601 **DP BARCODE:** D401689

TXR#: 0055725 **CAS No:** 133-07-3

TEST MATERIAL (PURITY): Folpet (97.6% a.i.)

SYNONYMS: Folpan; 2-[(trichloromethyl)thio]-1*H*-isoindole-1,3(2*H*)-dione

CITATION: Davis, J. P. (2012) Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Male Rats (OPPTS 890.1500); Folpet. Integrated Laboratory Systems, Inc., Durham, NC. Laboratory Project Study No.: C200-301, April 18, 2012. MRID 48671202. Unpublished.

SPONSOR: Makhteshim Chemical Works, Ltd., c/o Makhteshim Agan of North America, Inc., 4515 Falls of Neuse Road, Suite 300, Raleigh, NC

TEST ORDER #: EDSP-081601-175

EXECUTIVE SUMMARY: In a Male Pubertal Assay (MRID 48671202), 16 Sprague Dawley rats/dose group were treated daily via oral gavage with folpet (97.6% a.i., Batch/lot # 00138518) in aqueous 1% carboxymethylcellulose (CMC) at doses of 0 (vehicle), 200, 400, or 800 mg/kg/day from post-natal day (PND) 23 to 53 or 54. Animals were examined for preputial separation (PPS) daily beginning on PND 30 and age and weight at day of attainment was recorded. Following sacrifice on PND 53 or 54, blood was collected for clinical chemistry analyses; total serum testosterone, thyroxine (T_4), and thyroid stimulating hormone (TSH) levels were determined using radioimmunoassays. Adrenal, liver, pituitary, thyroid, and urogenital organs were weighed and microscopic examinations of the testes, epididymides, thyroid, and kidneys were performed.

One 200 mg/kg/day male was found dead on Day 25 as a result of a gavage error. At 400 mg/kg/day, 6 males were euthanized before scheduled termination due to moribundity, and one additional rat was found dead on Day 25. At 800 mg/kg/day, 5 males were euthanized prior to scheduled termination due to moribundity. All other rats survived until scheduled sacrifice.

One control and one 200 mg/kg/day rat were observed with a rough coat on Day 2; no abnormal findings were noted in the remaining animals in these groups. Abnormal breathing/rales, hunched posture, and/or thick red nasal discharge was observed in all six 400 mg/kg/day rats euthanized prior to study termination and two of the surviving rats. No abnormal findings were

noted in the remaining eight 400 mg/kg/day rats. In the 800 mg/kg/day group, clinical observations noted in animals 24 hours after dosing included abnormal breathing, distended abdomen, and piloerection in three of five rats euthanized prior to study termination and four of the surviving rats. No abnormal observations were noted in the remaining nine 800 mg/kg/day rats.

Final body weight was decreased ($p<0.05$) at 200, 400, and 800 mg/kg/day, by 8%, 14%, and 11%, respectively. Overall body weight gains were also decreased ($p<0.05$) at 200, 400, and 800 mg/kg/day, by 10%, 17%, and 14%, respectively. There were no treatment-related effects on age or weight at attainment of PPS.

There were no effects of treatment on the weights of adrenal glands, seminal vesicle plus coagulating gland, ventral prostate, dorso-lateral prostate, testes, or thyroid glands. At 800 mg/kg/day, absolute and adjusted (for body weight on PND 21) liver weights were decreased ($p<0.01$) by 15%, and absolute and adjusted levator ani-bulbocavernosus (LABC) and epididymis (right only) weights were decreased ($p<0.05$) by 12% (LABC) and 9% (epididymis). Absolute pituitary weights were decreased ($p<0.05$) by 15% and relative kidney weights were increased ($p<0.05$) by 6%.

At 400 mg/kg/day, absolute and adjusted weights for the following organs were decreased ($p<0.05$): liver (↓18%), kidney (↓10-11%), epididymides (↓13%), and LABC (↓11-13%). Absolute pituitary weights were decreased ($p<0.05$) by 16%.

At 200 mg/kg/day, absolute and adjusted liver weights were decreased ($p<0.05$) by 11%. The weights of kidneys, pituitary, LABC, and epididymides were comparable to the controls.

Serum T_4 was decreased ($p<0.01$) in rats dosed at 200 (↓23%) and 800 (↓33%) mg/kg/day. At 200 mg/kg/day, sodium, chloride, and sorbitol dehydrogenase were increased ($p<0.05$) by 3%, 5%, and 20%, respectively. At 800 mg/kg/day, chloride was increased ($p<0.01$; ↑3%), and the following parameters were decreased ($p<0.05$): alanine aminotransferase (↓42%), alkaline phosphatase (↓27%), total protein (↓5%), and albumin (↓5%). There were no dose-related effects on serum TSH, testosterone, potassium, calcium, phosphorus, aspartate aminotransferase, gamma glutamyl transferase, blood urea nitrogen, creatinine, or total bilirubin. Serum from rats in the 400 mg/kg/day group was not evaluated due to the low survival of rats in this group and the Guideline requirement of a minimum of two treatment levels.

There were no dose-related histopathological changes in the testes, epididymides, thyroid glands, or kidneys. No changes in follicular cell height or colloid area were observed in the thyroid glands of rats dosed at 200 or 800 mg/kg/day compared to the controls. Histopathological analyses were not conducted for rats in the 400 mg/kg/day group.

The most common clinical observations noted 24 hours post dosing in rats at 400 and 800 mg/kg/day were abnormal breathing/rales, distended abdomen, piloerection, hunched posture and/or thick red nasal discharge occurring coincident with decreased body weight (14% at 400 mg/kg/day and 11% at 800 mg/kg/day) when compared to controls. Necropsy of the dead and moribund animals did not show evidence for dosing error.

The agency does not concur with the study author's rationale for the mortality. The clinical signs such as abnormal breathing/rales, gasping, and hunched posture indicate that the cause of these signs is likely dosing errors. The mortalities cannot clearly be attributed to gavage-reflux, as rationalized by the study author, since in the range finding study, except for one rat at 800 mg/kg/day, all rats survived comparable doses, and there were no dosing errors. Additionally, it is also possible that the doses tested were excessive based on significant decreases (11-14%) in body weight in rats at the 400 and 800 mg/kg/day groups. Overt toxicity was not seen at the low dose (200 mg/kg/day).

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Male Pubertal Assay (OCSPP 890.1500).

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

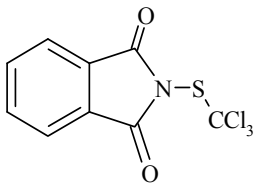
A. MATERIALS

1. Test Facility: Integrated Laboratory Systems, Inc. (ILS)

Location:	Durham, NC
Study Director:	J. P. Davis
Other Personnel:	S. Borghoff (Study Toxicologist); P. Sproul (Toxicology Study Manager); A. Glasscock (Animal Facility Operations Manager); J. Pope (Necropsy Manager); T. Hackett (Histology Manager); D. Giri (Study Pathologist); K. Cummings (QA Manager); K. Taylor (Facility Veterinarian); C. Cachafeiro (Health and Safety Manager)
Study Period:	September 2, 2011 to April 18, 2012

2. Test Substance: Folpet

Description:	Technical; fine white powder
Source:	Makhteshim Chemical Works, Ltd
Lot/Batch #:	00138518 (expiration date 5/26/2012)
Purity:	97.6% a.i.
Stability:	Dose formulations in 1% CMC stable for 8 days at 1-10 °C
CAS #:	133-07-3
Structure:	



3. Vehicle: 1% Carboxymethylcellulose (CMC) in deionized water

4. Test Animals:

Species:	Rat (males only)	
Strain:	Sprague Dawley [Crl:CD®(SD) IGS]	
Age/Weight at Study Initiation:	PND 23/ 50.6-74.2 g	
Source:	Charles River Laboratories (Raleigh, NC)	
Housing:	Animals were housed in polycarbonate cages with absorbent heat-treated hardwood bedding; dams were housed one per cage with litter and F_1 rats were housed two per cage.	
Diet:	Teklad Global 16% Protein Rodent Diet (Teklad Diets, Madison WI), *ad libitum* Total genistein equivalents (genistein plus daidzein) of 8.6 µg/g feed.	
Water:	Reverse-osmosis treated tap water, *ad libitum*	
Environmental Conditions:	**Temperature:**	17-25 °C
	Humidity:	29-66%
	Air changes:	Not reported
	Photoperiod:	14 hrs light/ 10 hrs dark

B. STUDY DESIGN

1. In-Life Dates: Start: October 1, 2011 End: November 1, 2011

2. Mating: Time-mated pregnant dams were received from the supplier on gestation day 8. Between PND 3 and 5, litters with the same date of birth were standardized to 8 pups with equal numbers of males and females.

3. Animal Assignment: Animals were assigned to the test groups noted in Table 1 using a procedure that stratified animals across groups by body weight such that mean body weight of each group was not statistically different from any other group. Littermates were not assigned to the same treatment group.

TABLE 1. Study Design[a]

Test group	Dose (mg/kg/day)	# of Males
Control	0	16
Low	200	16
Mid	400	16
High	800	16

a Data were obtained from Table 1 on page 16 of the study report.

4. Dose Selection Rationale: The dose levels were selected based on the results from a dose range finding study[1] and a previously reviewed Hershberger study (MRID 48616905). In the range finding study, four male rats (PND 36) per group were orally dosed with folpet in 1% CMC by oral gavage at 0, 200, 400, 600, 800, or 1000 mg/kg/day for 14 consecutive days. All rats survived until scheduled termination, with the exception of one 800 mg/kg/day rat that was euthanized due to body weight loss after 10 days on study. No signs of dosing error were observed during the necropsy of this animal. There were no effects of treatment observed on terminal body weights. There were no abnormal cage-side or clinical observations in animals dosed at 0, 200, or 400 mg/kg/day, except for two 400 mg/kg/day males (cage mates) that were thin (from Day 6) and exhibited rales (Day 12 to termination). Abnormal breathing/wheezing/rales were the most common cage-side or clinical observations in animals at 600 (2 rats), 800 (1 rat) and 1000 (2 rats) mg/kg/day. In four of these animals, this observation was coincident with body weight loss. There was no difference in weekly food consumption between the controls and folpet-dosed male rats, and no changes in relative liver or kidney weights. At necropsy, no gross lesions were observed in the stomach or the jejunum.

In the Hershberger study, adult castrated male rats were dosed at 250 or 800 mg/kg/day for 10 consecutive days. Respiratory distress and body weight loss led to the death or early euthanasia of 4/8 rats in the 800 mg/kg/day group; no significant decreases in body weight (compared to the controls) or clinical signs of toxicity were noted in the four remaining animals. The deaths were not anticipated based on the range finding study and were thought to be a result of gavage-related reflux and likely a reflection of the irritancy of the test substance when administered by oral gavage in a viscous vehicle, rather than systemic toxicity of the compound. Animals in the 250 mg/kg/day group survived to the scheduled termination.

In consideration of the fact that in the pubertal study, immature rats are dosed for an extended period (31/32 days), a high dose level of 800 mg/kg/day was selected as the high dose. In the event that animals in the 800 mg/kg/day group did not survive until scheduled termination, an additional dose group (200 mg/kg/day) was included in addition to the half-

[1] Davis, J. (2012). Range Finder Study for *In Vivo* Mammalian Assays for Folpet. Unpublished study report prepared by ILS Inc. Study No. C200-500.

dose level (400 mg/kg/day).

5. **Dose Preparation and Analysis:** Dose formulations at 40, 80, and 160 mg/mL were prepared seven times during the study by mixing appropriate amounts of test substance with 1% CMC in deionized water. Dose concentrations and homogeneity were tested by Smithers Viscient LLC (Wareham, MA) for each preparation of each formulation prepared by ILS. Three samples (top, middle, and bottom) were analyzed for each concentration level. Analyses to demonstrate stability of the test substance in 1% CMC were conducted previously.[2] It was stated that dose formulations in 1% CMC stored at 1-10 °C were stable for 8 days.

   Results of Dose Analysis

   Homogeneity (%CV): 0.00534-5.97%

   Stability: Not provided

   Concentration (% of nominal): 95.2-104%

   The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable.

6. **Dosage Administration:** All doses were administered once daily by gavage, from PND 23 through PND 53 (half the animals in each treatment group) or 54 (remaining animals in each group), in a volume of 5 mL/kg. Dose volume was based on individual animal daily body weight. According to the protocol, dosing was performed between 0700 and 0900 hours daily.

7. **Statistics:** Descriptive statistics (mean, standard deviation, coefficient of variance, and sample size) were calculated using Microsoft Excel 2003/2007. Data sets were statistically analyzed using SAS version 9.2. Studentized residual plots were used to detect possible outliers in the data and Levene's test was used to assess homogeneity of variance. Heterogeneous data were transformed (logarithm, multiplicative inverse, or square root) and if still heterogeneous, analyzed using the non-parametric Kruskal-Wallis and Dunn's test. Homogenous data sets [initial body weights, final body weights (using last day all body weights collected), final body weight gains (using last day all body weights collected), and age and body weight at PPS] were analyzed using a one-way analysis of variance (ANOVA) followed by pair-wise comparisons performed using Dunnett's two-tailed t tests. For tissue weights, relative tissue weights (liver, kidneys, pituitary, and adrenals), hormone levels, and clinical chemistry levels, data were analyzed using a two-way ANOVA with treatment and necropsy day (if >1 day) as main effects. Pair-wise comparisons were performed using Dunnett's two-tailed t tests. For initial body weights, final body weight, final body weight gain, age and body weight at PPS, and tissue weights, the data were analyzed using a two-way analysis of covariance (ANCOVA) with PND 21 body weight as the covariable. Pair-wise comparisons were performed using Dunnett's two-tailed t test. If data sets were not homogenous, ANCOVA analyses were not performed. Trend tests were performed on body

[2] Dix, M. (2011). Storage Stability of Folpet in 1% Carboxymethylcellulose Solutions. Unpublished study report prepared by Smithers Viscient Inc. Study No. 11742.6182.

weight and tissue weight data sets and reported when significant ($p<0.05$) for endpoints that did not show any significant pair-wise comparisons.

Dose-dependent changes were evaluated using a linear regression model for both adjusted and unadjusted values if the values were not significant. Statistical analyses of thyroid scoring (colloid area and follicular cell height) were performed by Fisher's Exact test, and when statistically significant, followed by Kruskal-Wallis and Dunn's test.

Statistically significant effects were reported when $p<0.05$. The statistical analyses were considered adequate.

C. METHODS

1. **Mortality and Clinical Examinations:** All animals were examined twice daily (once daily on weekends and holidays) for mortality and moribundity. Clinical examinations were conducted at study allocation, daily prior to dose administration, and at termination. In addition, cage-side observations were performed one hour (± 30 minutes) following dosing each day.

2. **Body Weight:** Animals were weighed at study allocation, daily prior to dosing, and prior to termination.

3. **Preputial Separation (PPS):** Beginning on PND 30, all animals were examined daily for onset of PPS. Age and weight at on the day of completion of PPS were recorded.

4. **Sacrifice and Pathology:** Beginning at the initiation of dosing, any rats found moribund or dead were necropsied and the cause of death determined, if possible. Moribund rats were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. On the day of termination, rats were removed to a holding room at least 2 hours before termination. All surviving animals were sacrificed by decapitation on PND 53 or 54 approximately 2 hours after dosing; according to the protocol, sacrifices were completed by 1300 hours. Blood from the trunk of the animals was collected immediately into serum separation tubes, processed by centrifugation, and the serum was stored at ≤−70°C for subsequent hormone and clinical chemistry evaluations.

a. **Hormone Analysis:** Total testosterone, total T_4, and TSH levels were determined using radioimmunoassays by a commercial laboratory (AniLytics Inc., Gaithersburg, MD).

b. **Clinical Chemistry:** The following CHECKED (X) parameters were examined by a commercial laboratory (AniLytics Inc., Gaithersburg, MD).

	ELECTROLYTES		OTHER
X	Calcium	X	Albumin
X	Chloride	X	Creatinine*
	Magnesium	X	Urea nitrogen*
X	Phosphorus		Total cholesterol
X	Potassium		Globulins
X	Sodium		Glucose
	ENZYMES	X	Total bilirubin
X	Alkaline phosphatase	X	Total protein
	Cholinesterase		Triglycerides
	Creatine phosphokinase		Serum protein electrophoresis
	Lactic acid dehydrogenase		
X	Alanine aminotransferase		
X	Aspartate aminotransferase		
X	Sorbitol dehydrogenase		
X	Gamma glutamyl transferase		
	Glutamate dehydrogenase		

* Recommended based on Guideline 890.1500.

c. <u>Organ Weights and Histopathology</u>: The following CHECKED (X) tissues were collected and weighed. The (XX) organs, in addition, were subjected to histological examination.

	UROGENITAL		OTHER
XX	Testes (left and right separately)*+	XX	Thyroid*+
XX	Epididymides (left and right separately)*+	X	Liver*
X	Seminal vesicle plus coagulating glands (with and without fluid)*	X	Adrenals (paired)*
X	Ventral prostate*	X	Pituitary*
X	Dorsolateral prostate*		
X	Levator ani-bulbocavernosus (LABC) muscle complex*		
XX	Kidneys (paired)*+		

* Weights required based on Guideline 890.1500
\+ Histopathological examination required based on Guideline 890.1500

The testis and epididymis (left) and kidneys were weighed prior to fixation. Following weighing, the testis and epididymis were fixed in Bouin's solution for 18 to 24 hours and washed in 70% histology grade alcohol. The thyroid (with parathyroid) was collected with the trachea and fixed in 10% neutral buffered formalin for at least 24 hrs. Following fixation, the thyroid was dissected free of the trachea and weighed. The kidney (left) was fixed in 10% neutral buffered formalin for at least 24 hrs. All collected tissues were transferred to 70% histology grade alcohol, routinely processed into paraffin blocks, sectioned, stained with hematoxylin and eosin, and examined microscopically.

Two serial sections from each of the two lobes of the thyroid were subjectively evaluated for follicular cell height and colloid area, using a five point grading scale (1 = shortest/smallest; 5 = tallest/largest).

II. RESULTS

A. MORTALITY: One 200 mg/kg/day male (#53) was found dead on Day 25 (PND 47) as a result of a gavage error (perforated esophagus). Six 400 mg/kg/day males (#20, #21, #23, #28, #29, and #30) were euthanized prior to scheduled termination due to moribundity, and one additional rat (#26) was found dead on Day 25 (PND 47). Five 800 mg/kg/day males (#35, #36, #40, #41, and #42) were euthanized prior to scheduled termination due to moribundity. All other rats survived until scheduled sacrifice.

B. CLINICAL SIGNS OF TOXICITY: One control male was observed with a rough coat 1 hour after dosing on Day 2 (PND 24); the same rat was observed with a rough coat 24 hours after dosing on Days 2 and 5 (PND 27). No other abnormal findings were observed in rats in the vehicle control group. One 200 mg/kg/day male was observed with a rough coat 1 and 24 hours after dosing on Day 2. No other abnormal findings were observed in rats in the 200 mg/kg/day group.

At 400 mg/kg/day, abnormal breathing (gasping) was noted in one male 1 hour after dosing on Day 24 (PND 46). The most common clinical observations noted 24 hours after dosing in animals in this group were abnormal breathing/rales, hunched posture, and/or thick red nasal discharge. These occurred in all six of the animals euthanized prior to study termination and two of the surviving rats (PND 47- 52). No abnormal findings were noted in the remaining eight males.

At 800 mg/kg/day, abnormal breathing (gasping) was noted in one male 1 hour after dosing on Day 18 (PND 40); a rough coat was noted in one rat on Day 2 and piloerection in one animal on Day 3 (PND 25) 1 hour post-dose. Clinical observations noted in animals 24 hours after dosing included abnormal breathing, distended abdomen, and piloerection in three of five rats euthanized prior to study termination and four of the surviving rats. No abnormal observations were noted in the remaining nine males.

C. GENERAL GROWTH AND PREPUTIAL SEPARATION: Body weights, body weight gains, age of attainment of PPS, and weight at day of PPS are presented in Table 2.

Final body weight was decreased ($p<0.05$) at 200, 400, and 800 mg/kg/day, by 8%, 14%, and 11%, respectively. Overall body weight gains were also decreased ($p<0.05$) at 200, 400, and 800 mg/kg/day, by 10%, 17%, and 14%, respectively. There were no treatment-related effects on age or weight at PPS. The number of animals that attained PPS prior to euthanasia/study termination was 16/16 controls, 15/16 at 200 mg/kg/day, 16/16 at 400 mg/kg/day, and 13/13 at 800 mg/kg/day.

The mean age at PPS, body weight at PPS, weaning weight, and final body weight in the control group were within the acceptable range of the performance criteria provided in the Guideline (890.1500); however, the CV for age at PPS, weight at PPS and final body weight were outside the acceptable range, as follows: 6.3% CV for age at PPS (maximum 5.67%), 10.2% CV for weight at PPS (maximum 7.57%), and 7.7% CV for final body weight (maximum 7.47%).

TABLE 2. General Growth and Preputial Separation (PPS)[a]

Parameter Evaluated		Vehicle Control				Folpet (200 mg/kg/day)				Folpet (400 mg/kg/day)				Folpet (800 mg/kg/day)			
		N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Initial body weight (PND 23; g)	U	16	67.2	4.7	6.9	16	67.3	6.0	8.9	16	67.0	4.5	6.8	16	66.9	4.7	7.0
	A	16	67.1	NA	NA	16	67.1	NA	NA	16	67.3	NA	NA	16	67.0	NA	NA
Body weight at PPS (g)	U	16	236.5	24.1	10.2	16	232.6	32.3	13.9	16	222.2	25.4	11.4	13	221.2	22.7	10.3
	A	16	236.2	NA	NA	16	232.1	NA	NA	16	222.4	NA	NA	13	222.0	NA	NA
Final body weight (g)	U	16	323.2	25.0	7.7	15	298.8* (↓8)	26.6	8.9	9	278.1** (↓14)	34.9	12.5	11	286.8** (↓11)	26.8	9.3
	A	16	322.8	NA	NA	15	298.0* (↓8)	NA	NA	9	281.9** (↓13)	NA	NA	11	285.4** (↓12)	NA	NA
Final body weight (% of control)	U	NA	NA	NA	NA	15	92.5	NA	NA	9	86.1	NA	NA	11	88.8	NA	NA
	A	NA	NA	NA	NA	15	92.3	NA	NA	9	87.3	NA	NA	11	88.4	NA	NA
Body weight gain (final – initial; g)	U	16	256.0	22.6	8.8	15	231.5* (↓10)	25.2	10.9	9	212.5** (↓17)	31.6	14.9	11	219.2** (↓14)	23.9	10.9
	A	16	255.7	NA	NA	15	231.0* (↓10)	NA	NA	9	214.7** (↓16)	NA	NA	11	218.4** (↓15)	NA	NA
Age at PPS (PND)	U	16	43.9	2.8	6.3	16	44.5	3.8	8.6	16	44.3	3.0	6.7	13	44.0	1.6	3.7
	A	16	43.9	NA	NA	16	44.6	NA	NA	16	44.2	NA	NA	13	44.0	NA	NA
Proportion unseparated (#/N)		0/16				1/16				0/16				0/13			

a Data were obtained from Table 10 on page 31 of the study report. Percent differences from controls, calculated by the reviewers, are included in parentheses.
U Unadjusted for body weight on PND 21
A Adjusted for body weight on PND 21
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
NA Not applicable
* Significantly different from controls at $p<0.05$.
** Significantly different from controls at $p<0.01$.

D. <u>ORGAN WEIGHTS</u>: Organ weights at necropsy are presented in Table 3. There were no effects of treatment on the weights of adrenal glands, seminal vesicle plus coagulating gland, ventral prostate, dorso-lateral prostate, testes, or thyroid.

At 800 mg/kg/day, absolute and adjusted (for body weight on PND 21) liver weights were decreased ($p<0.01$) by 15%, and absolute and adjusted LABC and epididymis (right only) weights were decreased ($p<0.05$) by 12% (LABC) and 9% (epididymis). Absolute pituitary weights were decreased ($p<0.05$) by 15% and relative kidney weights were increased ($p<0.05$) by 6%.

At 400 mg/kg/day, absolute and adjusted weights for the following organs were decreased ($p<0.05$): liver (↓18%), kidney (↓10-11%), epididymides (left and right; ↓13%), and LABC (↓11-13%). Absolute pituitary weights were decreased ($p<0.05$) by 16%.

At 200 mg/kg/day, absolute and adjusted liver weights were decreased ($p<0.05$) by 11%. The weights of kidneys, pituitary, LABC, and epididymides were comparable to the controls.

The unadjusted values for all organ weights in the control group were within the acceptable range of the performance criteria provided in the Guideline (890.1500), with the exception of mean kidneys weight (2.20 g; acceptable range 2.242-3.050 g).

TABLE 3. Organ Weights at Necropsy[a]

Organ		Vehicle Control				Folpet (200 mg/kg/day)				Folpet (400 mg/kg/day)				Folpet (800 mg/kg/day)			
		N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Liver (g)	U	16	14.52	1.60	11.0	15	12.94* (↓11)	1.85	14.3	9	11.84** (↓18)	1.60	13.5	11	12.35** (↓15)	1.56	12.6
	A	16	14.51	NA	NA	15	12.91* (↓11)	NA	NA	9	11.96** (↓18)	NA	NA	11	12.30** (↓15)	NA	NA
	R	16	4.43	0.22	4.9	15	4.25	0.30	7.1	9	4.22	0.32	7.6	11	4.25	0.25	5.8
Kidneys (g)	U	16	2.20	0.19	8.8	15	2.11	0.21	9.7	9	1.96* (↓11)	0.20	10.1	11	2.07	0.20	9.7
	A	16	2.20	NA	NA	15	2.11	NA	NA	9	1.98* (↓10)	NA	NA	11	2.06	NA	NA
	R	16	0.67	0.04	6.5	15	0.70	0.04	5.6	9	0.70	0.04	5.9	11	0.71* (↑6)	0.04	6.3
Pituitary (mg)	U	16	10.0	1.4	14.3	15	9.1	2.2	23.9	9	8.4* (↓16)	1.4	17.0	11	8.5** (↓15)	1.0	11.4
	A	16	9.9	NA	NA	15	9.1	NA	NA	9	8.5	NA	NA	11	8.4	NA	NA
	R	16	3.0	0.4	12.5	15	3.0	0.6	21.5	9	3.0	0.3	11.3	11	2.9	0.04	12.1
Adrenals (mg)	U	16	48.5	7.0	14.5	15	46.5	6.3	13.5	9	43.1	7.0	16.3	11	47.0	6.5	13.8
	A	16	48.5	NA	NA	15	46.4	NA	NA	9	43.6	NA	NA	11	46.8	NA	NA
	R	16	14.8	1.7	11.3	15	15.4	1.9	12.3	9	15.3	1.6	10.6	11	16.3	2.6	15.9
Seminal vesicle + coagulating gland, with fluid (mg)	U	16	489.1	82.1	16.8	15	462.6	104.8	22.6	9	464.7	120.2	25.9	11	479.4	89.2	18.6
	A	16	489.0	NA	NA	15	462.5	NA	NA	9	465.0	NA	NA	11	479.3	NA	NA
Seminal vesicle + coagulating gland, without fluid (mg)	U	16	291.8	43.9	15.0	15	282.3	57.8	20.5	9	277.2	78.9	28.5	11	278.7	38.0	13.6
	A	Not reported				Not reported				Not reported				Not reported			
Ventral prostate (mg)	U	16	282.6	57.8	20.4	15	270.5	55.0	20.3	9	234.2	54.6	23.3	11	244.8	42.4	17.3
	A	16	282.8	NA	NA	15	270.8	NA	NA	9	232.7	NA	NA	11	245.4	NA	NA
Dorsolateral prostate (mg)	U	16	148.5	37.1	25.0	15	155.9	23.7	15.2	9	139.1	32.6	23.4	11	128.9	12.5	9.7
	A	16	148.4	NA	NA	15	155.6	NA	NA	9	140.5	NA	NA	11	128.4	NA	NA
LABC (mg)	U	16	561.0	65.6	11.7	15	513.4	42.5	8.3	9	489.9* (↓13)	66.6	13.6	11	496.3* (↓12)	69.9	14.1
	A	16	560.4	NA	NA	15	512.1	NA	NA	9	496.0* (↓11)	NA	NA	11	493.9* (↓12)	NA	NA
Epididymis, left (mg)	U	16	221.0	19.4	8.8	15	211.6	21.2	10.0	9	192.0** (↓13)	24.3	12.6	11	210.1	25.2	12.0
	A	16	220.8	NA	NA	15	211.4	NA	NA	9	193.2* (↓13)	NA	NA	11	209.6	NA	NA
Epididymis, right (mg)	U	16	230.2	15.7	6.8	15	219.8	19.2	8.7	9	200.1** (↓13)	24.1	12.1	11	209.3* (↓9)	24.4	11.7
	A	16	230.1	NA	NA	15	219.5	NA	NA	9	201.2** (↓13)	NA	NA	11	208.9* (↓9)	NA	NA
Testis, left (mg)	U	16	1466.6	82.6	5.6	15	1606.0	411.4	25.6	9	1414.9	85.9	6.1	11	1398.2	127.1	9.1
	A	16	1465.5	NA	NA	15	1603.6	NA	NA	9	1426.5	NA	NA	11	1393.7	NA	NA

TABLE 3. Organ Weights at Necropsy[a]

Organ		Vehicle Control				Folpet (200 mg/kg/day)				Folpet (400 mg/kg/day)				Folpet (800 mg/kg/day)			
		N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Testis, right (mg)	U	16	1543.6	206.0	13.3	15	1503.2	187.1	12.4	9	1426.2	116.3	8.2	11	1404.0	133.5	9.5
	A	16	1542.6	NA	NA	15	1501.1	NA	NA	9	1436.4	NA	NA	11	1400.0	NA	NA
Thyroid, fixed (mg)	U	16	15.21	2.49	16.4	15	13.11	2.52	19.2	9	14.55	2.66	18.3	11	13.41	2.06	15.4
	A	16	15.21	NA	NA	15	13.11	NA	NA	9	14.55	NA	NA	11	13.41	NA	NA

a Data were obtained from Table 11 on pages 32-33 of the study report. Percent differences from controls, calculated by the reviewers, are included in parentheses.
U Unadjusted for body weight on PND 21
A Adjusted for body weight on PND 21
R Organ-to-body weight ratio (relative to body weight)
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
* Significantly different from controls at $p<0.05$.
** Significantly different from controls at $p<0.01$.

E. CLINICAL CHEMISTRY AND HORMONE LEVELS: Mean hormone and clinical chemistry levels are presented in Table 4. The study author noted that the performing laboratory did not have a database of historical hormone and clinical chemistry values for male Sprague Dawley rats. However, reference ranges, obtained from published literature, were reported and are attached as an Appendix to this document. No reference range was provided for testosterone.

Serum T_4 was decreased ($p<0.01$) in rats dosed at 200 (↓23%) and 800 (↓33%) mg/kg/day. At 200 mg/kg/day, sodium, chloride, and sorbitol dehydrogenase were increased ($p<0.05$) by 3%, 5%, and 20%, respectively. At 800 mg/kg/day, chloride was increased ($p<0.01$) by 3%, and the following parameters were decreased ($p<0.05$): alanine aminotransferase (↓42%), alkaline phosphatase (↓27%), total protein (↓5%), and albumin (↓5%). There were no dose-related effects on serum TSH, testosterone, potassium, calcium, phosphorus, aspartate aminotransferase, gamma glutamyl transferase, blood urea nitrogen, creatinine, or total bilirubin.

Only serum from animals in the control and the 200 and 800 mg/kg/day groups was evaluated for hormone and clinical chemistry levels, due to the low survival of rats in the 400 mg/kg/day group and the Guideline requirement of a minimum of two treatment levels.

The results for serum T_4, TSH, and testosterone in the control group were within the acceptable range of the performance criteria provided in the Guideline (890.1500).

TABLE 4. Hormone Levels and Clinical Chemistry[a]

Parameter Evaluated	Vehicle Control				Folpet (200 mg/kg/day)				Folpet (800 mg/kg/day)			
	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)	N	Mean	SD	CV (%)
Hormones												
Serum T_4, Total (µg/dL)	16	4.58	0.62	13.5	15	3.54** (↓23)	0.84	23.9	11	3.06** (↓33)	0.63	20.6
Serum TSH (ng/mL)	16	4.23	1.96	46.3	15	4.42	2.35	53.2	11	5.67	2.72	47.9
Serum testosterone (ng/mL)	16	2.40	1.33	55.2	15	2.14	1.57	73.1	11	1.53	0.94	61.3
Clinical Chemistry												
Sodium (mEq/L)	16	138	2	1.2	15	142** (↑3)	4	2.6	11	140	2	1.3
Potassium (mEq/L)	16	7.6	0.5	5.9	15	7.7	0.6	7.4	11	7.4	0.3	4.0
Chloride (mEq/L)	16	102	1	1.5	15	107** (↑5)	1	1.0	11	105** (↑3)	1	1.3
Calcium (mg/dL)	16	10.6	0.3	3.2	15	10.3	0.4	4.3	11	10.2	0.3	2.7
Phosphorus (mg/dL)	16	11.0	0.8	7.6	15	11.1	0.8	6.8	11	11.0	0.7	6.3
Aspartate aminotransferase (U/L)	16	232	75	32.3	15	245	55	22.5	11	234	32	13.6
Alanine aminotransferase (U/L)	16	62	36	57.6	15	47	19	40.6	11	36* (↓42)	21	57.9
Gamma glutamyl transferase (U/L)[b]	16	0.3	0.7	225.3	15	0	0	NA	11	0.6	1.1	176.0
Alkaline phosphatase (U/L)	16	327	67	20.4	15	284	69	24.3	11	239** (↓27)	39	16.4
Blood urea nitrogen (mg/dL)	16	14	4	31.7	15	14	3	21.6	11	16	3	21.7
Creatinine (mg/dL)	16	0.5	0.2	36.5	15	0.4	0.1	27.4	11	0.4	0.1	35.3
Total bilirubin (mg/dL)[b]	16	0.1	0.1	171.4	15	0.0	0.0	263.9	11	0.0	0.1	331.7
Sorbitol dehydrogenase (U/L)	16	25	6	22.5	15	30* (↑20)	6	21.5	11	28	6	22.4
Total protein (g/dL)	16	5.9	0.2	3.7	15	5.8	0.2	3.9	11	5.6** (↓5)	0.2	3.5
Albumin (g/dL)	16	4.4	0.1	2.7	15	4.5	0.1	3.2	11	4.2** (↓5)	0.2	5.7

a Data were obtained from Tables 12 and 13 on pages 34-35 of the study report. Percent differences from controls, calculated by the reviewers, are included in parentheses.
b Data as reported by study author. No limit of quantitation was reported.
N Number of animals examined
SD Standard Deviation
CV Coefficient of Variation
* Significantly different from controls at $p<0.05$.
** Significantly different from controls at $p<0.01$.

F. GROSS PATHOLOGY: At necropsy, enlarged testis was observed in one control and one 200 mg/kg/day rat; another 200 mg/kg/day rat had bilateral enlarged testes. One 200 mg/kg/day rat was observed with intestinal dilatation, and one 800 mg/kg/day rat was observed with one dilated kidney. There were no other gross observations at necropsy in rats surviving until scheduled termination.

G. HISTOPATHOLOGY: There were no dose-related histopathological changes in the testes, epididymides, thyroid glands, or kidneys. Thyroid follicular cell height and colloid area data for rats in the study are summarized in Table 5. No changes in follicular cell

height or colloid area were observed in the thyroid of rats dosed at 200 or 800 mg/kg/day compared to the controls.

Histopathological analyses were only conducted for animals in the control and the 200 and 800 mg/kg/day groups due to the low survival of rats in the 400 mg/kg/day group and the Guideline requirement of only two treatment levels.

TABLE 5. Thyroid Gland Follicular Cell Height and Colloid Area[a]

Findings	Vehicle Control	Folpet (200 mg/kg/day)	Folpet (800 mg/kg/day)
Number of animals examined	16	15	11
Follicular cell height[b]			
1	0	0	0
2	5 (31%)	1 (7%)	0
3	11 (69%)	14 (93%)	11 (100%)
4	0	0	0
5	0	0	0
Follicular colloid area[b]			
1	0	0	0
2	0	0	0
3	11 (69%)	14 (93%)	11 (100%)
4	5 (31%)	1 (7%)	0
5	0	0	0

a Data were obtained from Table 15 on page 37 of the study report.
b A five-point grading scale (1 = shortest / smallest; 5 = tallest / largest) was used.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Administration of 400 or 800 mg/kg/day folpet did not show changes in endpoints that would suggest an effect on pubertal development. Although serum T4 concentrations were decreased following administration of 400 or 800 mg/kg/day folpet, no other signs of thyroid gland modulation were observed.

The study author attributed the mortality to oral gavage dosing that can lead to reflux and serious respiratory effects and mortality (Damsch et al. 2011). Therefore, it is reasonable to attribute the adverse clinical signs to gavage-related reflux of folpet since it is an irritant. This is further supported by the findings in the Part 158 studies with folpet. In the 90-day study, dietary administration of folpet results in remarkable histopathological lesions in the non-glandular portions of the stomach characterized as acanthosis, hyperkeratosis, submucosal edema, and oleo cellular infilterate (inflammatory reaction) focal erosion and ulcerations in both sexes. In the two-generation reproduction study, folpet caused lesions in the esophagus (hyperkeratosis) and the stomach (keratosis, squamous epithelial hyperplasia) of F0 males and F0 females, and in the F1 females. In the developmental toxicity study with rats, following gavage dosing dams exhibited salivation, rales, soft/liquid feces and decreased motor activity.

B. AGENCY COMMENTS: One 200 mg/kg/day male was found dead on Day 25 as a result of a gavage error. Six 400 mg/kg/day males were euthanized before scheduled termination due to moribundity, and one additional rat was found dead on Day 25. Five 800 mg/kg/day males were euthanized prior to scheduled termination due to moribundity. All other rats survived until scheduled sacrifice.

One control and one 200 mg/kg/day rat were observed with a rough coat on Day 2; no abnormal findings were noted in the remaining animals in these groups. Abnormal breathing/rales, hunched posture, and/or thick red nasal discharge was observed in all six of the 400 mg/kg/day rats euthanized prior to study termination and two of the surviving rats. No abnormal findings were noted in the remaining eight 400 mg/kg/day rats. In the 800 mg/kg/day group, clinical observations noted in animals 24 hours after dosing included abnormal breathing, distended abdomen, and piloerection in three of five rats euthanized prior to study termination and four of the surviving rats. No abnormal observations were noted in the remaining nine 800 mg/kg/day rats. The study author attributed the adverse clinical signs noted in this study to gavage-related reflux[3] of folpet, since it is an irritant, rather than direct systemic toxicity; the tolerability of a systemic dose of 12,000 ppm in the diet (approximately 1,000 mg/kg) for three weeks has been demonstrated in a repeat dose study.[4]

Final body weight was decreased ($p<0.05$) at 200, 400, and 800 mg/kg/day, by 8%, 14%, and 11%, respectively. Overall body weight gains were also decreased ($p<0.05$) at 200, 400, and 800 mg/kg/day, by 10%, 17%, and 14%, respectively. There were no treatment-related effects on age or weight at attainment of PPS.

There were no effects of treatment on the weights of adrenal glands, seminal vesicle plus coagulating gland, ventral prostate, dorso-lateral prostate, testes, or thyroid glands. At 800 mg/kg/day, absolute and adjusted (for body weight on PND 21) liver weights were decreased ($p<0.01$) by 15%, and absolute and adjusted LABC and epididymis (right only) weights were decreased ($p<0.05$) by 12% (LABC) and 9% (epididymis). Absolute pituitary weights were decreased ($p<0.05$) by 15% and relative kidney weights were increased ($p<0.05$) by 6%.

At 400 mg/kg/day, absolute and adjusted weights for the following organs were decreased ($p<0.05$): liver (↓18%), kidney (↓10-11%), epididymides (↓13%), and LABC (↓11-13%). Absolute pituitary weights were decreased ($p<0.05$) by 16%.

At 200 mg/kg/day, absolute and adjusted liver weights were decreased ($p<0.05$) by 11%. The weights of kidneys, pituitary, LABC, and epididymides were comparable to the controls.

Serum T_4 was decreased ($p<0.01$) in rats dosed at 200 mg/kg/day (↓23%) and 800 mg/kg/day (↓33%). At 200 mg/kg/day, sodium, chloride, and sorbitol dehydrogenase were increased ($p<0.05$) by 3%, 5%, and 20%, respectively. At 800 mg/kg/day, chloride was

[3] Damsch, S., et al. (2011). Gavage-Related Reflux in Rats: Identification, Pathogenesis, and Toxicological Implications (Review). *Toxicol Pathol*, 39: 348-360.

[4] Bullock, C.H. (1979) A 21-Day Feeding Study of Technical Phaltan in Rats. Unpublished study report prepared by Chevron Environmental Health Center Study No. S-1407.

increased ($p<0.01$; ↑3%), and the following parameters were decreased ($p<0.05$): alanine aminotransferase (↓42%), alkaline phosphatase (↓27%), total protein (↓5%), and albumin (↓5%). Sodium, chloride, alanine aminotransferase, sorbitol dehydrogenase, total protein, and albumin were all within or slightly below the range of values provided by the analytical laboratory (see Appendix). There were no dose-related effects on serum TSH, testosterone, potassium, calcium, phosphorus, aspartate aminotransferase, gamma glutamyl transferase, blood urea nitrogen, creatinine, or total bilirubin. Serum from rats in the 400 mg/kg/day group was not evaluated due to the low survival of rats in this group and the Guideline requirement of a minimum of two treatment levels.

There were no dose-related histopathological changes in the testes, epididymides, thyroid glands, or kidneys. No changes in follicular cell height or colloid area were observed in the thyroid glands of rats dosed at 200 or 800 mg/kg/day compared to the controls. Histopathological analyses were not conducted for rats in the 400 mg/kg/day group.

The decreases in final body weights of 14% and 11% in the 400 and 800 mg/kg/day groups, respectively, along with the low survival rates in these groups, indicated that these dose levels were excessive.

The study author concluded that the decrease in absolute LABC and epididymis (left and right) weights in animals administered 400 or 800 mg/kg/day, compared to control animals, as well as the decrease in circulating T_4 concentrations in males administered 800 mg/kg/day were most likely a reflection of the decreased body weight of the surviving animals, based on a study on the effects of body weight loss on pubertal development in Wistar rats.[5] In the study, male rat body weight decreases of 9-19% (of control animals) resulted in significant decreases in serum T_4 levels and androgen dependent tissue weights (ventral prostate, epididymis, and seminal vesicle).

The most common clinical observations noted 24 hours post dosing in rats at 400 and 800 mg/kg/day were abnormal breathing/rales, distended abdomen, piloerection, hunched posture and/or thick red nasal discharge occurring coincident with decreased body weight (14% at 400 mg/kg/day and 11% at 800 mg/kg/day) when compared to controls. Necropsy of the dead and moribund animals did not show evidence for dosing error.

The agency does not concur with the study author's rationale for the mortality. Clinical signs such as abnormal breathing/rales, gasping, and hunched posture clearly indicate that the cause of these signs is likely dosing errors. The mortalities cannot be attributed to gavage-reflux, as rationalized by the study author, since in the range finding study, except for one rat at 800 mg/kg/day, all rats survived comparable doses, and there were no dosing errors. Additionally, it is also possible that the doses tested were excessive based on significant decreases (11-14%) in body weight in rats at the 400 and 800 mg/kg/day groups. Overt toxicity was not seen at the low dose (200 mg/kg/day).

C. STUDY DEFICIENCIES: Major deficiencies noted in this study include 44% mortality/moribundity at 400 mg/kg/day and 33% mortality/moribundity at 800 mg/kg/day.

[5] Laws, S.C., Stoker, T.E., Ferrell, J.M., Hotchkiss, M.G., and Cooper, R.G. (2007). Effects of Altered Food Intake during Pubertal Development in Male and Female Wister Rats. *Toxicol. Sci.* 100(1): 194-202.

The following deficiencies were noted that were not considered to have had an adverse effect on the results, interpretations or conclusions of this study:

- Control CV for age at PPS (6.3%) exceeded the Guideline performance criteria maximum (5.67%).
- Control CV for weight at PPS (10.2%) exceeded the Guideline performance criteria maximum (7.57%).
- Control CV for final body weight (7.7%) exceeded the Guideline performance criteria (maximum 7.47%).
- Control mean kidney weight (2.20 g) was below the Guideline performance criteria (2.242-3.050 g).

DATA EVALUATION RECORD

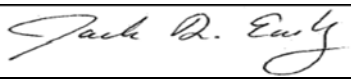
FOLPET

Study Type: OCSPP 890.1550, Steroidogenesis Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-41-2012 (MRID 48616906)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer: Scott D. Studenberg, Ph.D., D.A.B.T.	Signature: Date:	 4/23/2012
Secondary Reviewer: Michelle Sharpe-Kass, M.S	Signature: Date:	 5/10/2012
Program Manager: Jack D. Early, M.S.	Signature: Date:	 5/22/2012
Quality Assurance: Steven Brecher, Ph.D., D.A.B.T.	Signature: Date:	 5/22/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Ayaad Assaad, D.V.M., Ph.D. **Signature:**
Health Effects Division **Date:** 6/12/2015

Secondary Reviewer: John Liccione, Ph.D. **Signature:**
Health Effects Division **Date:** 6/16/15

DATA EVALUATION RECORD

STUDY TYPE: Steroidogenesis Assay (H295R Cells); OCSPP 890.1550

PC CODE: 081601 **DP BARCODE:** D398813

TXR#: 0055725 **CAS No.:** 133-07-3

TEST MATERIAL (PURITY): Folpet (94.5% a.i.)

SYNONYMS: 2-[(trichloromethyl)thio]-1H-isoindole-1,3(2H)-dione

CITATION: Wagner, H. (2012). Folpet: Steroidogenesis (Human Cell Line – H295R). CeeTox, Inc., Kalamazoo, MI. Laboratory Study No.: 9141V-100357STER, January 4, 2012. MRID 48616906. Unpublished.

SPONSOR: Makhteshim Chemical Works Ltd. c/o Makhteshim Agan of North America, Inc., 4515 Falls of Neuse Road, Suite 300, Raleigh, NC.

TEST ORDER #: EDSP-081601-175

EXECUTIVE SUMMARY: In a steroidogenesis assay (MRID 48616906), H295R cells cultured *in vitro* in 24-well plates were incubated with folpet, (94.5% purity, Batch # 00138518) at log concentrations from 0.0001 to 100 µM in triplicate for 48 hours. Dimethyl sulfoxide (DMSO) was used as the vehicle, at a final concentration of 0.05%.

Testosterone and estradiol concentrations were measured by HPLC/MS-MS with positive ion multiple reaction monitoring. Four independent experiments were performed. A Quality Control (QC) plate was run concurrently with each independent run of a test chemical plate to demonstrate that the assay responded properly to positive control agents at two concentration levels; positive controls included the known inhibitor (prochloraz) and inducer (forskolin) of estradiol and testosterone production.

Laboratory proficiency testing was not conducted in the current study, and details of a previous proficiency determination were not included in the study report. The data from Run #1 were not analyzed due to an error in one of the reference chemical concentrations on the QC plate. Because of precipitation, the highest suitable concentrations of folpet in Runs #2 and #4 were 0.1 µM and 1 µM. Cytotoxicity at the 100 µM concentration, and decreased cell viability (81.9%) at the 10 µM concentration, was noted in Run #3.

Guideline acceptability recommendations and requirements were met, including adequate production of testosterone and estradiol, acceptable reproducibility (low %CV), and appropriate induction and inhibition with positive controls.

The results of the steroidogenesis assay with folpet indicate that folpet is neither an inducer nor an inhibitor of testosterone synthesis in this assay. The results of this assay also indicate that, although folpet is not an inducer of estradiol synthesis, it may be an inhibitor of estradiol production at high concentrations (≥10 µM). This effect could not be confirmed due to precipitation in the test wells after incubation in two of the three runs at concentrations of 10 and 100 µM.

Based on the hormone responses in the three independent runs, folpet treatment did not result in statistically significant and reproducible alterations in testosterone or estradiol production.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Steroidogenesis assay (OCSPP 890.1550).

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, and Quality Assurance statements were provided.

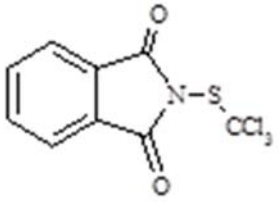
I. MATERIALS AND METHODS

A. MATERIALS

1. Test Facility: CeeTox, Inc.

Location:	Kalamazoo, MI
Study Director:	H. Wagner
Other Personnel:	C. Toole, Director of Project Management K. Rutherford, Director of Laboratory Operations D. Blakeman, Senior Scientist B. Wallace, Lead Cell Culture Scientist S. McColley, Scientist C. Haines, Scientist F. Wong, Scientist J. Burgam, Associate Scientist L. Blakeman, Associate Scientist
Study Period:	June 6, 2011 to January 4, 2012

2. Test Substance: Folpet

Description:	Technical Grade, white powder
Batch #: (Expiration Date)	00138518 (May 26, 2012)
Purity:	94.5%
Solubility (in Solvent):	Soluble in DMSO up to 200 mM
Volatility:	Not reported
Stability:	Stability in test preparations was not conducted.
Storage conditions:	Room temperature
CAS #:	133-07-3
Molecular weight:	296.6
Structure:	

3. Positive Control (Inducer): Forskolin

Description:	White powder, 410.50
Source:	Sigma Aldrich, St. Louis, MO (catalog # not reported)
Lot #: (Expiration Date):	109K50571V (July, 2016)
Purity:	98%
Solubility (in Solvent):	Soluble in DMSO up to 100 mM
Storage conditions:	Room temperature
CAS #:	66575-29-9

4. Positive Control (Inhibitor): Prochloraz

Description:	White powder, 376.67
Source:	Sigma Aldrich, St. Louis, MO (catalog # not reported)
Lot #: (Expiration Date)	SZE6220X (August 8, 2012)
Purity:	99.1%
Solubility (in Solvent):	Soluble in DMSO up to 100 mM
Storage conditions:	Room temperature
CAS #:	67747-09-5

5. Solvent/Vehicle Control: Dimethyl Sulfoxide (DMSO)

Description:	Not reported
Source:	Sigma Aldrich, St. Louis, MO (catalog # not reported)
Lot #: (Expiration Date)	RNBB7617 (expiration date not reported)
Purity:	Not reported
Storage conditions:	Not reported
CAS #:	Not reported
Justification for choice of solvent:	Not reported (Folpet, forskolin, and prochloraz were soluble at test concentrations)
Final concentration: (% volume in assay)	0.05% (v/v)

6. Other Materials: 22R-Hydroxycholesterol

Description (molecular weight):	White powder (402.65)
Source:	Sigma-Aldrich (St. Louis, MO)
Lot # (expiration date):	089K4132, 060M4098 (not provided)
Purity:	99.0%
Storage conditions:	Room temperature
Solvent used:	Ethanol, final concentration in assay, 0.025% v/v
CAS #:	17954-98-2

7. Stock Medium: Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (DMEM/Ham-F12)

Description:	Included 15 mM HEPES
Source:	Not reported
Lot / Batch #: (Expiration Date)	Not reported
Sodium bicarbonate:	Not reported
Nu-Serum:	Becton Dickinson, Catalog #355500, Lot #81515; tested for background hormone concentrations.
ITS+ premix:	Becton Dickinson, Catalog #354352, Lot #05245 and #09233.

8. Test Cells: H295R human adrenocortical carcinoma cells (ATCC, Manassas, VA, CLR-2128; Lot #7635054). Cells were maintained in DMEM/Ham-F12 with 15 mM HEPES, sodium bicarbonate, ITS+Premix, and 2.5% Nu-Serum in a 5% CO_2 incubator at 37±2ºC. The background hormone concentrations present in the Nu-Serum were 3754 pg/mL for testosterone and 3846 pg/mL for estradiol. Cells were initially grown for five passages, and then frozen in liquid nitrogen. After thawing, cells were cultured additionally for seven or eight passages prior to use in the steroidogenesis assay. 22R-Hydroxycholesterol (Sigma-Aldrich, Lot #089K4132 and #060M4098, 99.0% pure, 10 µM) was added to the culture medium at plating, dosing, and harvesting. Cells were plated into wells of 24-well culture plates at a density of approximately 3×10^5 cells/mL. Plates were incubated in a 5% CO_2 atmosphere at 37±2ºC for approximately 24 hours prior to exposure to the test chemical or the positive controls.

The following performance criteria were met (indicated by an "x"):

x	Cell passage identifier. Cell Passage #: ≥7.5
x	Cells frozen down at passage 5
x	Frozen cells cultured for 4 additional passages
	Total number of passages does not exceed 10 (not reported)

B. METHODS

1. Pre-Test Information

a. Hormone Assay Interference Test: Hormone cross-reactivity or interference tests were not conducted because testosterone and estradiol concentrations were determined by HPLC/MS-MS analysis; no interference was expected.

b. Hormone Extraction: Testosterone and estradiol were extracted from H295R-supplemented medium by liquid/liquid extraction with methyl tert-butyl ether spiked with [$^2H_5$]-testosterone and [$^2H_5$]-estradiol as internal standards. The extracts were analyzed by using HPLC/MS-MS with positive ion multiple reaction monitoring. The method MDL for testosterone and estradiol were 100 and 10 pg/mL, respectively, for a 300-µL portion of medium.

c. Laboratory Proficiency Test: Laboratory proficiency testing was not conducted for the current study. A protocol amendment stated that laboratory proficiency data was previously determined for this batch of cells. The details of this previous proficiency determination, including estimates of EC_{50}s for prochloraz and forskolin, were not reported in the study report.

2. Test Solutions: A 200-mM stock of folpet was prepared in DMSO, followed by serial dilutions (1:10) in DMSO. A 40-mM stock of 22R-hydroxycholesterol was dissolved in ethanol and further diluted in supplemented medium to achieve a final concentration of 10 µM. Mastermix solutions with folpet were prepared by 1:2000 dilutions in supplemented medium containing 10 µM 22R-hydroxycholesterol. Forskolin and prochloraz solutions were prepared in a similar manner by preparing 100 mM solutions in DMSO, followed by serial dilutions in DMSO and 1:2000 dilutions in supplemented medium containing 10 µM 22R-hydroxycholesterol. Final concentrations after addition to the 24-well culture plates were 1 and 10 µM for forskolin, 0.1 and 1 µM for prochloraz, and 0.0001 to 100 µM for folpet. Final concentrations of DMSO and ethanol in the medium were 0.05% and 0.025% (v/v), respectively.

3. Cell Plating and Preincubation: H295R cells (ATCC CLR-2128) were initially grown for five passages, and then frozen in liquid nitrogen. After thawing, cells were cultured additionally for seven or eight passages prior to use. 22R-Hydroxycholesterol (10 µM) was added to the culture medium at plating, dosing, and harvesting. Cells (1 mL) were seeded into wells of 24-well culture plates at a density of approximately 3×10^5 cells/mL. Plates were incubated in a 5% CO_2 atmosphere at 37±2ºC for approximately 24 hours prior to exposure to the test chemical or the positive controls. The percent confluency after 24-hour incubation was not reported, but the protocol stated that 50-60% confluency was expected after plating 2.5×10^5 cells/mL followed by a 24-hour incubation period.

4. Exposure: The cells were checked microscopically for good attachment and proper morphology, and the medium was removed and replaced with 1 mL of supplemented medium containing 10 µM 22R-hydroxycholesterol. The cells were then exposed to identical volumes of either each serial dilution of the test compound or DMSO (SC) in triplicate according to the schematic presented in Table 1.

TABLE 1. Dosing Schematic for the Exposure of H295R Cells to Folpet (Final Concentrations in µM)[a]						
	1	**2**	**3**	**4**	**5**	**6**
A	DMSO	DMSO	DMSO	0.1	0.1	0.1
B	100	100	100	0.01	0.01	0.01
C	10	10	10	0.001	0.001	0.001
D	1	1	1	0.0001	0.0001	0.0001

a Data were obtained from page 14 of the study report. Dosing was calculated based on a total volume of 1 mL per well.

A concurrent quality control (QC) plate was included with each of the four independent runs of the test chemical plates to demonstrate the assay's response to forskolin (an inducer of testosterone and estradiol production) and prochloraz (an inhibitor of testosterone and estradiol production). The QC plate was prepared and dosed in the same manner with either forskolin or prochloraz according to the schematic presented in Table 2.

TABLE 2. Dosing Schematic for the QC Plate for Positive Controls (Final Concentrations in µM)[a]						
	1	**2**	**3**	**4**	**5**	**6**
A	Blank[b]	Blank	Blank	Background[c]	Background	Background
B	DMSO	DMSO	DMSO	DMSO + MeOH[d]	DMSO + MeOH[d]	DMSO + MeOH[d]
C	Forskolin 1µM[e]	Forskolin 1µM[e]	Forskolin 1µM[e]	Prochloraz 0.1µM	Prochloraz 0.1µM	Prochloraz 0.1µM
D	Forskolin 10µM	Forskolin 10µM	Forskolin 10µM	Prochloraz 1µM	Prochloraz 1µM	Prochloraz 1µM

a Data were obtained from page 14 of the study report. Dosing was calculated based on a total volume of 1 mL per well.
b Blank wells received medium containing 10 µM 22R-hydroxycholesterol.
c Background wells received medium only.
d MeOH = 70% methanol was added to these wells for 30 minutes at room temperature following medium removal.
e In Run 1, the wells for 1µM forskolin were dosed with 3.33 µM forskolin; this run was not analyzed. Runs 2-4 contained 1µM forskolin.

Following dosing, the plates were incubated for 48±2 hours under the conditions described previously. After the incubation period, each well was examined microscopically, and images were taken of the solvent control wells and the two highest non-cytotoxic concentrations. Precipitation, if present, was noted. The medium from each well was removed, split into two equal volumes, and frozen at −80±10°C until hormone measurements were conducted by using HPLC/MS-MS. Cell viability was determined after media removal was completed.

5. <u>Cell Viability/Cytotoxicity Assay</u>: Cell viability was determined with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] test described by Mosman (1983)[1] immediately after removal of the culture medium after the 48-hour incubation. The QC plate wells designated to receive methanol (MeOH) were rinsed twice with phosphate buffered saline (PBS), followed by an incubation with MeOH for 30 minutes at room temperature, and three additional rinses with PBS. Next, 0.5-mL portions of a 0.5 mg/mL MTT solution in supplemented medium containing 10 µM 22R-hydroxycholesterol were added to each well on the test chemical and QC plates. MTT-treated plates were incubated in a 5% CO_2 atmosphere at 37±2ºC for 3 hours, followed by removal of the MTT solutions

1 Mosman T. (1983). Rapid colorimetric assay for growth and survival: application to proliferation and cytotoxicity. *J. Immunol. Methods*. 100:45-50.

and addition of 0.5 mL of isopropanol to each well. Plates were incubated for a further 20 minutes at room temperature with shaking. After the 20-minute incubation, the absorbance values at 570 and 650 nm were determined with a Packard Fusion plate reader, and the absorbance at 650 nm was subtracted from the absorbance at 570 nm to calculate the MTT value. A reduction of ≥20% in cell viability was considered evidence of cytotoxicity.

6. Hormone Measurement System: Concentrations of testosterone and estradiol in the supplemented medium were determined by HPLC-MS/MS at a separate analytical facility (OpAns, LLC, Durham, NC). The extraction method (with spiked, labeled internal standards) was described previously (B.1.b.). All back-calculated calibration standard concentrations, except for one 10 pg/mL standard measurement, and QC concentrations were acceptable. The percent recovery across the test runs for the QC samples ranged from 91.4-105.4% for testosterone and 85.2-106.1% for estradiol.

The following performance criteria were met (indicated by an "x"):

X	Method detection limit (100 pg/mL testosterone; 10 pg/mL estradiol)
X	Spiked sample recovery acceptable for two concentrations of testosterone and estradiol (mean measured amount from triplicate samples within 30% of nominal concentration)
NA	Hormone cross-reactivity (antibody-based assays only; ≤30% of basal production of the respective hormone)
X	Solvent control within 75% range below maximum response on standard curve
NA	Test compound tested for interference with measurement system

C. DATA ANALYSIS: Mean values (pg/mL) and standard deviations (SD) for testosterone and estradiol concentrations were calculated for each concentration of the reference chemicals and SC, as well as the blank and background wells, on the QC plates and for each concentration of folpet and SC on the test chemical plate. Relative changes in hormone production were calculated with the following equation:

Relative change = (hormone concentration in each well) ÷ (mean SC hormone concentration)

For forskolin induction of testosterone, the background hormone production was subtracted from the forskolin-treated wells, and blank and SC wells, prior to calculation of the relative change. Background hormone production was determined from three wells on the QC plate that received H295R cells with no 22R-hydroxycholesterol.

Concentrations that exhibited ≥20% cytotoxicity, or where precipitation was observed, were omitted from further analysis.

Normality of the data was evaluated with the Shapiro-Wilk's test. Homogeneity of the variances between the treatment groups was evaluated by using Levene's test. Statistical significance between each treatment group and control was evaluated with Dunnett's test if $p>0.05$ in both tests. If $p\leq0.05$ in the normality or homogeneity tests, a log transformation was performed on the data to attempt to approximate a normal distribution. Dunnett's test was conducted to evaluate statistical significance between the treatment and control groups if $p>0.05$ in both the normality and homogeneity tests after transformation. If $p\leq0.05$ in either the normality or homogeneity tests after transformation, the non-transformed data were analyzed with the nonparametric Kruskal-Wallis test, followed by Dunn's test, to evaluate statistical significance between the treatment and control groups.

Data analysis was conducted with Microsoft Excel, and statistics were calculated with the Unistat 6.0 Light program for Excel. The statistical analyses are considered appropriate.

II. RESULTS

A. TEST COMPOUND: The hormone concentrations after exposure to folpet, and the fold-difference change relative to SC and mean ± SD for the three suitable assay runs (2 through 4), are presented in Table 3. Samples from Run 1 were not analyzed due to an error with the forskolin concentration on the QC plate. The fold-changes in hormone concentration for folpet in the three runs are shown graphically in Figures 1 and 2 for testosterone and estradiol, respectively. The highest suitable concentration of folpet in Run 2 was 0.1 µM due to precipitation at concentrations of ≥1 µM. In Run 3 the highest suitable concentration of folpet was 10 µM due to cytotoxicity at the 100 µM concentration. Finally, 1 µM was the highest suitable concentration of folpet in Run 4 because precipitation was present at ≥10 µM.

The % CVs for absolute testosterone concentrations for the SC replicates within the test plates ranged from 0.77 to 7.51%, and met the ≤30% performance criteria recommended in the test Guideline. Similarly, the % CVs for absolute estradiol concentrations for the SC replicates within the test plates ranged from 1.50-3.34%. The between-plate % CVs for the absolute hormone concentrations of the SC were 17.3% for testosterone and 16.7% for estradiol (calculated by the reviewers). These data meet the recommended performance criteria of ≤30%.

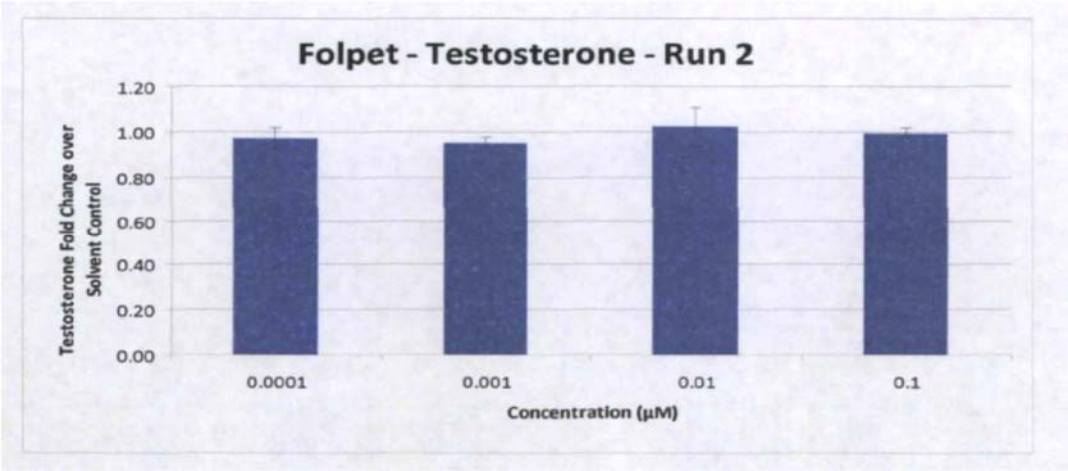
No statistically significant effects on testosterone production were observed in Runs 2 through 4 after incubation with folpet. A statistically significant decrease in estradiol production was observed in Run 3 at the highest evaluable concentration, 10 µM. The significance of this decrease in estradiol production is unknown due to the reduced cell viability (81.9%) in this well, the loss of the next higher concentration due to cytotoxicity (100 µM), and the lack of data at this concentration in the other two runs due to precipitation.

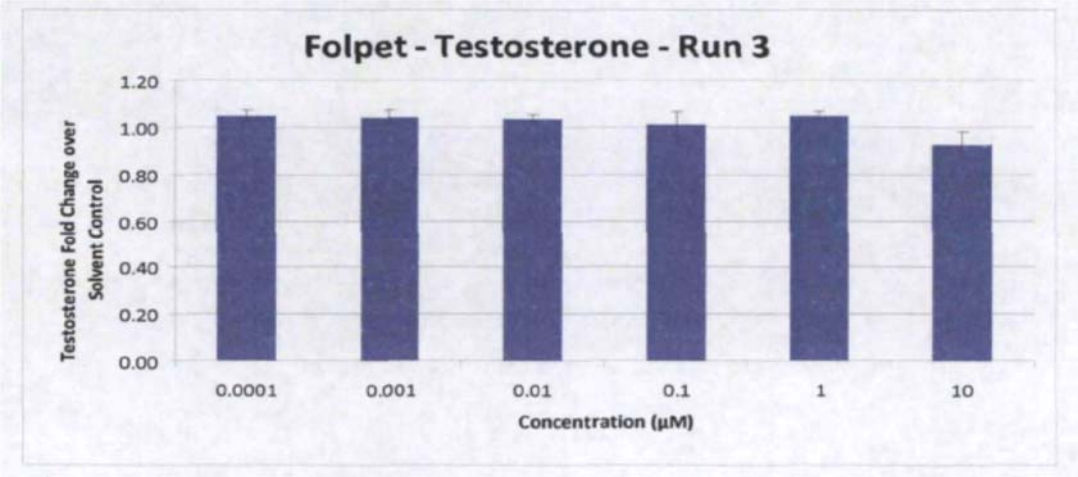
TABLE 3. Mean (±SD) Hormone Concentrations Following Treatment with Folpet for 48 Hours.[a]

Nominal Concentration (µM)	Run 2	Run 3	Run 4	Run 2	Run 3	Run 4	Mean[b]	± SD[b]	Statistical Significance
	Testosterone (pg/mL)			Fold Difference[c]					
DMSO (SC)	2832	2012	2332	—	—	—	—	—	NA
0.0001	2750	2109	2332	0.97	1.05	1.00	1.01	0.04	NA
0.001	2699	2093	2249	0.95	1.04	0.96	0.98	0.05	NA
0.01	2890	2083	2230	1.02	1.04	0.96	1.01	0.04	NA
0.1	2820	2034	2275	1.00	1.01	0.98	1.00	0.02	NA
1	2736	2108	2379	NA	1.05	1.02	1.04	NC[d]	NA
10	1489	1861	1817	NA	0.92	NA	0.92	NC[e]	NA
100	109	99.0	89.0	NA	NA	NA	NA	NA	NA
	Estradiol (pg/mL)			Fold Difference					
DMSO (SC)	254	185	203	—	—	—	—	—	NA
0.0001	252	177	192	0.99	0.96	0.95	0.97	0.02	NA
0.001	244	181	196	0.96	0.98	0.96	0.97	0.01	NA
0.01	255	184	201	1.00	1.00	0.99	1.00	0.01	NA
0.1	259	187	204	1.02	1.01	1.01	1.01	0.01	NA
1	246	176	206	NA	0.95	1.02	0.99	NC[d]	NA
10	155	168	173	NA	0.91*	NA	0.91	NC[e]	Run 3
100	49.0	73.0	56.0	NA	NA	NA	NA	NA	NA

a Data were obtained from page 26-27 and 33-35 of the study report.
b SEM were calculated by the reviewers.
c Fold difference relative to SC (DMSO = 1)
d n=2
e n=1
SC Solvent control
NA Not applicable
NC Not calculated
* Statistically significant ($p \leq 0.05$)

FIGURE 1. Change in Testosterone Production Relative to Folpet Concentration in Test Runs 2 to 4.





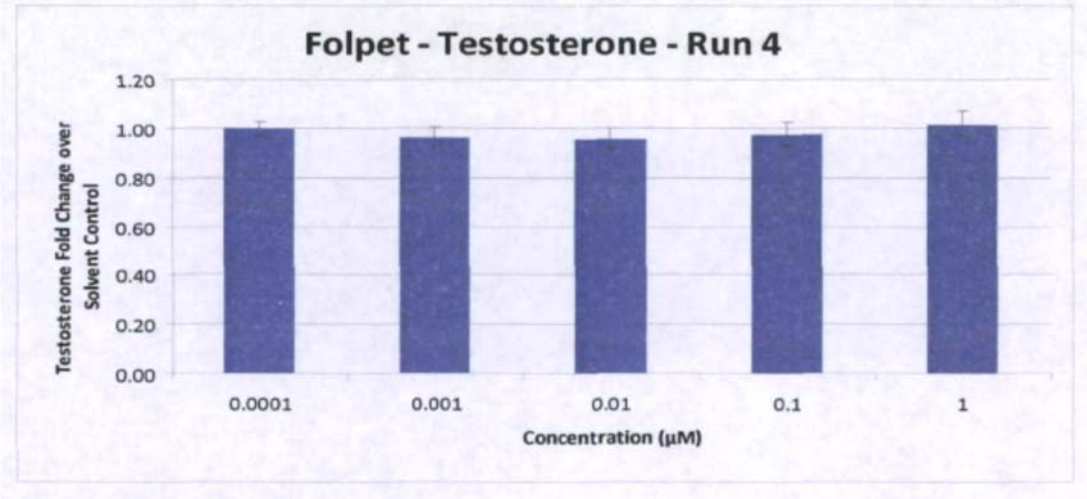
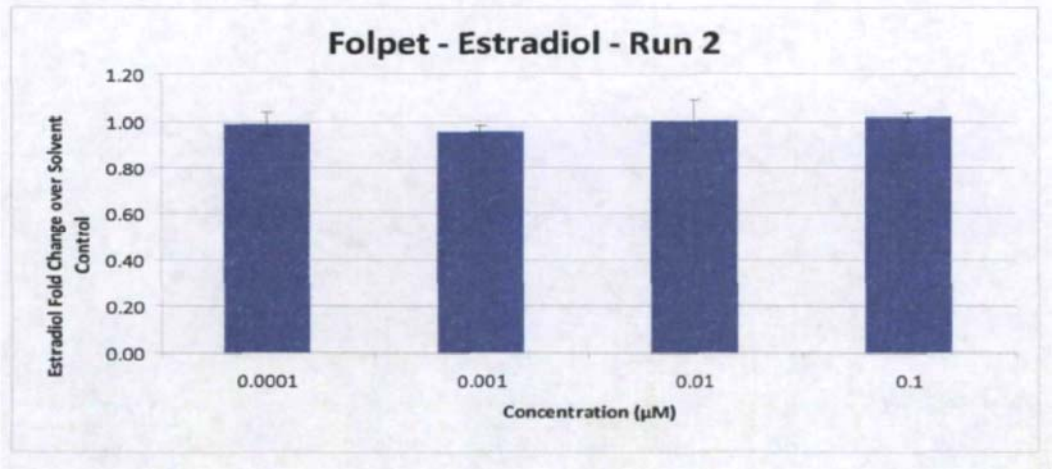
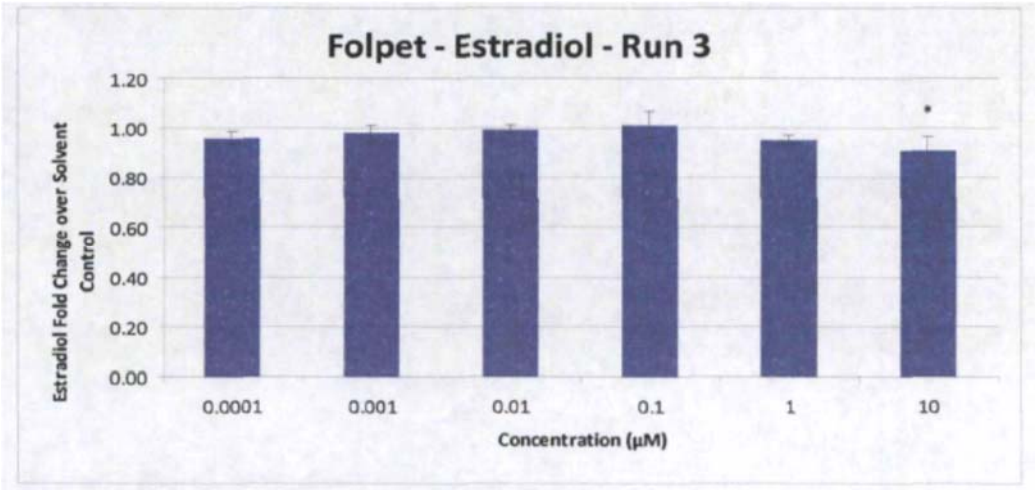
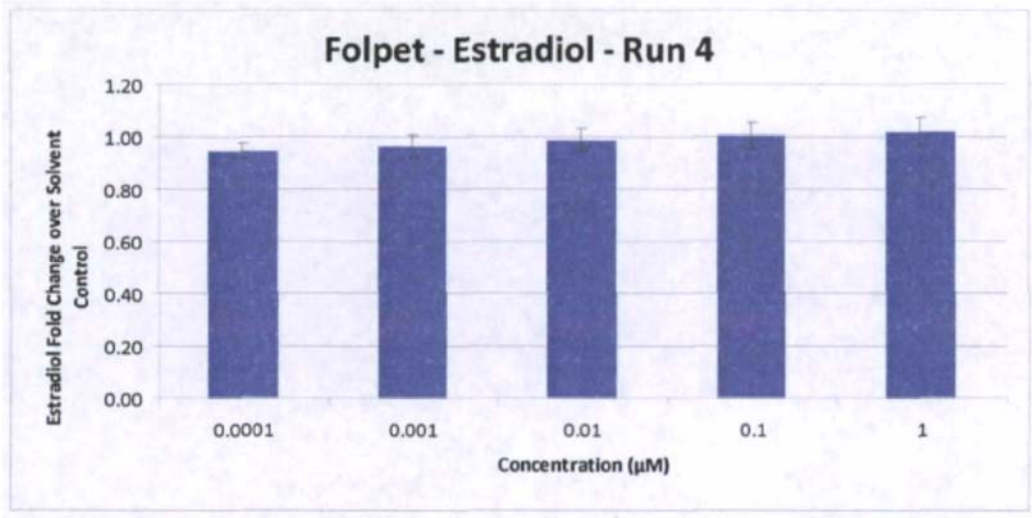


FIGURE 2. Change in Estradiol Production Relative to Folpet Concentration in Test Runs 2 to 4.





* Significantly different from the solvent control at $p \leq 0.05$.



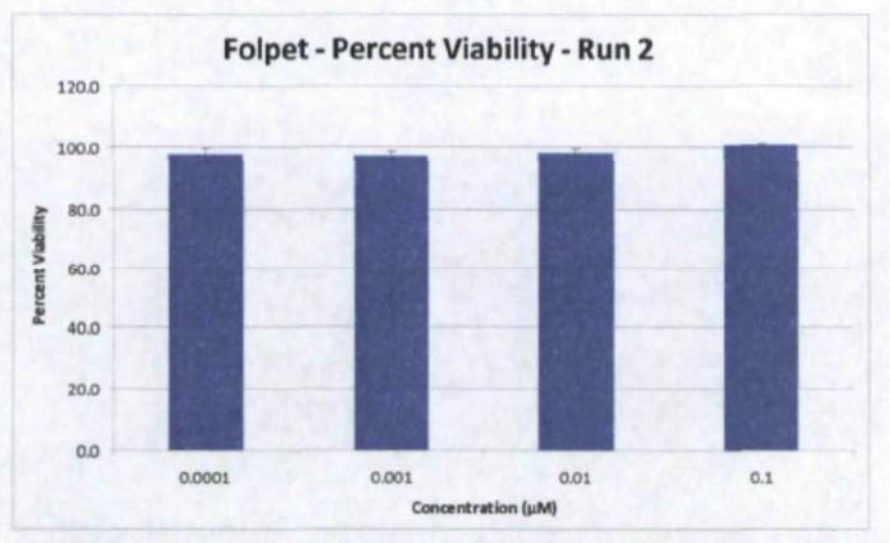
B. CYTOTOXICITY: No evidence of cytotoxicity was present from the MTT cell viability assay conducted on the QC and folpet test plates (Table 4 and Figure 3), except for cytotoxicity at the 100 µM concentration and decreased cell viability (81.9%) at the 10 µM concentration in Run 3. Otherwise, fold-change values ranged from 95.4-109.3% on the QC plates and 89.9-101.9% on the test chemical plates; methanol exposure reduced cell viability to 5.1-11.4%.

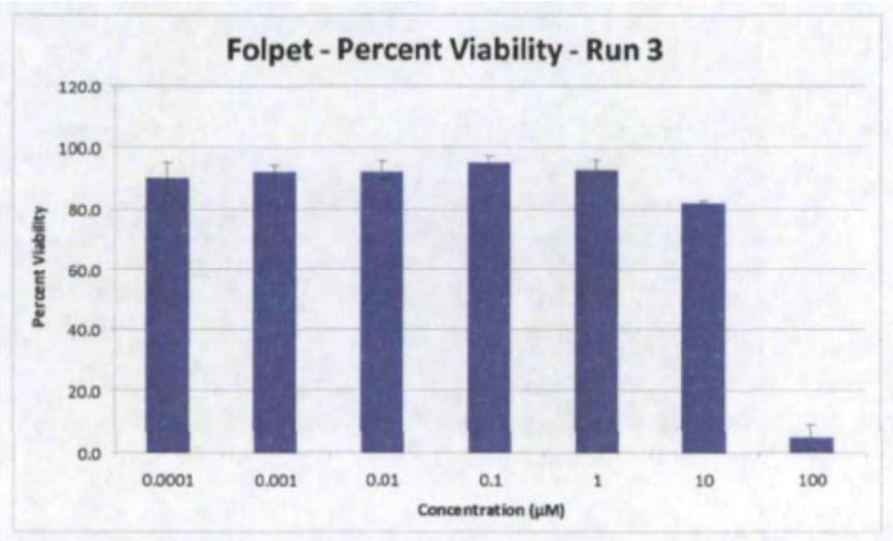
TABLE 4. Mean (±SD) MTT Cell Viability Results after Treatment with Forskolin, Prochloraz, or Folpet for 48 Hours.[a]

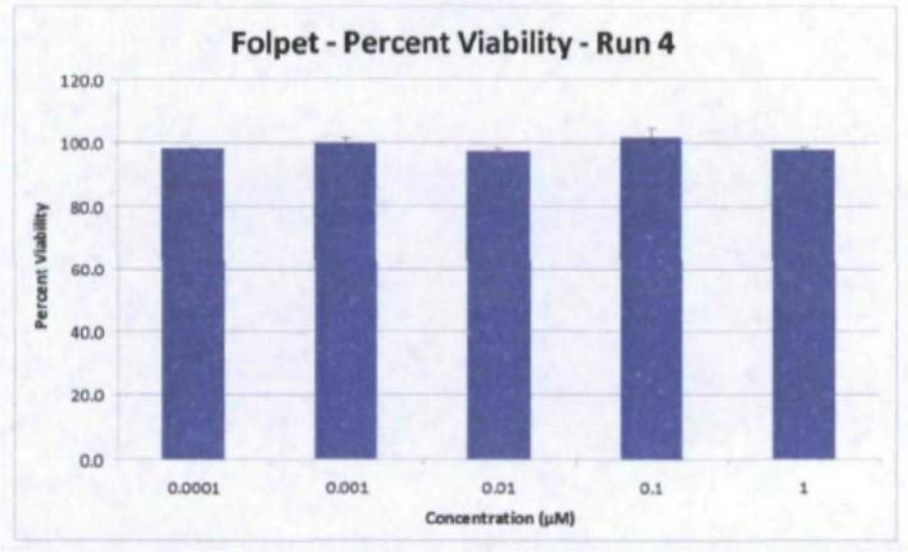
Compound	Concen. (µM)	Cell Viability – Run 2		Cell Viability – Run 3		Cell Viability – Run 4	
		Mean	SD	Mean	SD	Mean	SD
Blank	NA	104.3	2.69	104.3	2.81	100.7	3.98
Background	NA	95.4	4.14	102.5	1.79	98.9	0.94
SC + Methanol	NA	5.6	2.51	11.4	1.99	5.1	0.14
Forskolin	1	107.8	1.52	109.3	1.17	105	1.55
Forskolin	10	107	2.43	108.5	4.17	102.4	2.58
Prochloraz	0.1	98.3	2.20	101.2	0.47	100.7	0.86
Prochloraz	1	99.4	1.19	101.5	0.78	99.5	2.37
Folpet	0.0001	97.6	2.07	89.9	5.06	98.2	0.14
Folpet	0.001	97.4	1.29	92.5	1.83	99.8	1.35
Folpet	0.01	98.3	1.19	92.2	3.08	97.3	0.63
Folpet	0.1	100.6	0.56	94.8	2.38	101.9	2.56
Folpet	1	NC	NC	92.8	3.12	97.9	0.6
Folpet	10	NC	NC	81.9	0.79	NC	NC
Folpet	100	NC	NC	4.9	4.14	NC	NC

a Data were obtained from page 20-21 of the study report.
NC = Not calculated due to precipitation.
SC = Solvent control

FIGURE 3. MTT cell cytotoxicity results from Test Runs 2 to 4.







C. **QC PLATE:** The hormone concentrations after exposure to the reference chemical, SC, blank, and background samples, as well as the fold-difference change relative to SC (individual and mean ± SD) for the three suitable assay runs, are presented in Table 5. Minimum basal hormone production was met in all blank and SC wells (500 pg/mL for testosterone, 40 pg/mL for estradiol), and it met the basal hormone production increase criteria in SC of ≥5-fold for testosterone and ≥2.5-fold for estradiol above the MDL of the assay. The medium was supplemented with 10 µM 22R-hydroxycholesterol to ensure that estradiol production met the minimum Guideline levels. Exposure to 10 µM forskolin induced testosterone production an average of 1.71-fold which is below the Guideline recommendation of ≥2-fold; however, when the background is subtracted the average induction was 2.5-fold. Estradiol production was induced by 10 µM forskolin ≥7.5-fold (actual 9.3- to 11.1-fold) over SC. Exposure to 1 µM prochloraz inhibited synthesis of testosterone and estradiol by ≥50% (actual 51-73% and 63-71%, respectively) compared to SC. These data met the performance criteria recommended by the Guideline.

The % CVs for absolute testosterone concentration in SC well replicates within the QC plates ranged from 0.83 to 5.44%, and the % CVs for absolute estradiol concentration in SC well replicates within the QC plates ranged from 2.90 to 6.16%. The between-plate variability for the QC plates based on the absolute hormone concentrations in the SC wells yielded % CVs of 14.7% for testosterone and 11.0% for estradiol. These data, calculated by the reviewers, met the performance criteria recommended by the Guideline.

TABLE 6. Mean (±SD) Hormone Concentrations Following Treatment with Forskolin or Prochloraz for 48 Hours.[a]

Concentration (µM)	Run 2	Run 3	Run 4	Run 2	Run 3	Run 4	Mean[b]	± SD[b]
	Testosterone (pg/mL)			Fold Difference (Relative to DMSO)				
Background	1135	1073	1262	0.45	0.57	0.54	—	—
Blank	2581	1873	2304	—	—	—	—	—
DMSO	2511	1874	2339	—	—	—	—	—
1 µM Forskolin	3764	2595	3120	1.50[c]	1.38[c]	1.33[c]	1.40[c]	0.09
10 µM Forskolin	4751	3019	3780	1.89[d]	1.61[d]	1.62[d]	1.71[d]	0.16
0.1 µM Prochloraz	1816	1149	1818	0.72	0.61	0.78	0.70	0.09
1 µM Prochloraz	1188	501	1153	0.47	0.27	0.49	0.41	0.12
	Estradiol (pg/mL)			Fold Difference (Relative to DMSO)				
Blank	208	182	228	—	—	—	—	—
DMSO	200	179	223	—	—	—	—	—
1 µM Forskolin	1343	1254	1538	6.72	7.01	6.91	6.88	0.15
10 µM Forskolin	2116	1661	2477	10.60	9.28	11.13	10.34	0.95
0.1 µM Prochloraz	133	104	171	0.67	0.58	0.77	0.67	0.10
1 µM Prochloraz	57	52	83	0.29	0.29	0.37	0.32	0.05

a Data were obtained from page 22 and 23 of the study report.
b Calculated by the reviewers.
c When the background is subtracted, the values are 1.91-, 1.90-, and 1.73-fold difference for Runs 2, 3, and 4, respectively, with a mean of 1.85-fold change.
d When the background is subtracted, the values are 2.63-, 2.43-, and 2.34-fold difference for Runs 2, 3, and 4, respectively, with a mean of 2.47-fold change.

III. DISCUSSION AND CONCLUSIONS

A. <u>INVESTIGATOR'S CONCLUSIONS</u>: Folpet exposure was associated with a statistically significant decrease in estradiol concentration at the highest folpet concentration analyzed in one of the three independent runs of the assay. This change in estradiol was associated with a reduction in cell viability to 81.9%. No other statistically significant changes in testosterone or estradiol were observed in any of the independent runs of the assay at any of the folpet concentrations that could be analyzed.

B. AGENCY COMMENTS: Laboratory proficiency testing results were not conducted as detailed in a protocol amendment for the current study because laboratory proficiency data had previously been determined for this batch of cells. The details of this previous proficiency determination, including estimates of EC_{50}s for prochloraz and forskolin were not reported in the current study report.

Although four independent test runs of the steroidogenesis assay with folpet were conducted, samples from only three of the four runs were analyzed due to an error in one of the reference chemical concentrations on the QC plate in Run 1. Because of precipitation, the highest suitable concentrations of folpet in Runs 2 and 4 were 0.1 µM and 1 µM. Cytotoxicity at the 100 µM concentration, and decreased cell viability (81.9%) at the 10 µM concentration, was noted in Run 3.

The QC plate results met the minimum basal hormone production in all blank and SC wells (500 pg/mL for testosterone, 40 pg/mL for estradiol), and met the basal hormone production criteria in SC of ≥5-fold increases for testosterone and ≥2.5-fold increases for estradiol above the MDL of the assay. Exposure to 10 µM forskolin induced testosterone production an average of 1.71-fold which is below the Guideline recommendation of ≥2-fold; however, when the background is subtracted the average induction was 2.5-fold. Estradiol production was induced by 10 µM forskolin. Exposure to 1 µM prochloraz inhibited synthesis of testosterone and estradiol by ≥50% (actual 51-73% and 63-71%, respectively) compared to SC. These data meet the performance criteria recommended by the Guideline.

Within-plate and between-plate % CVs were not reported for the QC plate data, but were calculated by the reviewers. The within-plate % CVs for absolute testosterone concentration in SC well replicates ranged from 0.83 to 5.44%, and for absolute estradiol concentration in SC well replicates ranged from 2.90 to 6.16%. The between-plate variability data for the QC plates based on the absolute hormone concentrations in the SC wells yielded % CVs of 14.7% for testosterone and 11.0% for estradiol. These data met the performance criteria recommended by the Guideline.

The % CVs for absolute testosterone and estradiol concentrations for the SC replicates within the test plates ranged from 0.77 to 7.51% and 1.50 to 3.34%, respectively, and met the ≤30% performance criteria recommended in the test Guideline. The between-plate % CVs for the absolute hormone concentrations of the SC were 17.3% for testosterone and 16.7% for estradiol. These data also meet the recommended performance criteria.

No statistically significant effects on testosterone production were observed in Runs 2 through 4 after incubation with folpet. A statistically significant decrease in estradiol production was observed in Run 3 at the highest evaluable concentration, 10 µM. The significance of this decrease in estradiol production is unknown due to the reduced cell viability (81.9%) in this well, the loss of the next higher concentration (100 µM) due to cytotoxicity, and the lack of data at this concentration in the other two runs due to precipitation.

C. STUDY DEFICIENCIES: The following deficiency was noted that is not considered to have had an adverse impact on the results, interpretation or conclusions of this study:

- Laboratory proficiency testing prior to the initiation of the current study was not conducted, and the details of a previous proficiency determination with the current batch of cells, including estimates of EC_{50}s for prochloraz and forskolin, were not reported in the study report.
- Although it was stated that cells were thawed after being frozen down at passage 5, and cultured for an additional 7 or 8 passages, the specific cell passage identification for each test run was not provided in the study report.
- The number of suitable concentrations for evaluation from Run 2 (the first run deemed acceptable by the investigators) was 4 instead of 7 due to precipitation at concentrations ≥1 µM. The recommended Guideline strategy is revision of the test chemical concentration range to better define the dose response range that contains the lowest observable effect concentration (LOEC). Instead of this recommended approach, the investigators used the original concentration range without revision. Data from the two highest concentrations (10 and 100 µM) were either lost or questionable in Runs 3 and 4 due to cytotoxicity or precipitation at these concentrations.

DATA EVALUATION RECORD

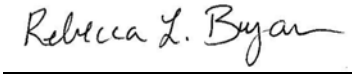
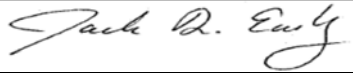
FOLPET

Study Type: OCSPP 890.1600, *In vivo* Uterotrophic Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-41-2012 (MRID 48616907)

Prepared for
Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
CSS-Dynamac Corporation
1910 Sedwick Road,
Building 100, Suite B
Durham, NC 27713

Primary Reviewer:	Signature:	
Kelly Luck, M.S.	Date:	04/25/2012
Secondary Reviewer:	Signature:	
Rebecca L. Bryan, B.S.	Date:	05/11/2012
Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	5/22/2012
Quality Assurance:	Signature:	
Steven Brecher, Ph.D., D.A.B.T.	Date:	5/22/2012

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CSS-Dynamac Corporation personnel**.**

The US EPA Endocrine Disruptor Screening Program (EDSP) Tier 1 screening battery is comprised of eleven screening assays intended to identify a chemical's likely endocrine bioactivity, i.e., its potential to interact with the estrogen, androgen, or thyroid (E, A, or T) pathways. The robustness of the Tier 1 battery is based on the strengths of each individual assay to identify potential endocrine bioactivity with complementary endpoints within the assay, where available, and redundancy across the battery. Thus, the results of each individual assay should not be considered in isolation but rather should be considered in the context of other assays in the battery as well as Other Scientifically Relevant Information (OSRI). In order to determine if a chemical has the potential to interact with the E, A or T pathways, a Weight of Evidence (WoE) evaluation of Tier 1 assay results, in combination with the findings in the OSRI, should be undertaken (refer to the WoE Document).

Primary Reviewer: Ayaad Assaad, D.V.M., Ph.D **Signature:**
Health Effects Division **Date:**
Secondary Reviewer: Jess Rowland **Signature:**
Health Effects Division **Date:** 6/4/15

Template version 09/2011

DATA EVALUATION RECORD

STUDY TYPE: Uterotrophic Assay (Rat); OCSPP 890.1600; OECD 440

PC CODE: 081601 **DP BARCODE:** D398813

TXR#: 0055725 **CAS#:** 133-07-3

TEST MATERIAL (PURITY): Folpet (97.6% a.i.)

SYNONYMS: Folpan; 2-[(trichloromethyl)thio]-1H-isoindole-1,3(2H)-dione

CITATION: Davis, J. P. (2012). The Uterotrophic Assay (OPPTS 890.1600); Folpet. Integrated Laboratory Systems, Inc., Durham, NC. Laboratory Project Study No.: C200-100, January 3, 2012. MRID 48616907. Unpublished.

SPONSOR: Makhteshim Chemical Works, Ltd., c/o Makhteshim Agan of North America, Inc., 4515 Falls of Neuse Road, Suite 300, Raleigh, NC

TEST ORDER #: EDSP-081601-175

EXECUTIVE SUMMARY: In a Uterotrophic Assay (MRID 48616907) conducted to screen for potential estrogenic activity, folpet (97.6% a.i., batch/lot # 00138518) in 1% carboxymethylcellulose was administered daily via oral gavage (5 mL/kg) to groups of eight ovariectomized female Sprague Dawley rats at dose levels of 0 (vehicle), 313, or 1,000 (limit dose) mg/kg/day on post-natal days (PND) 56-58. A positive control group was treated with a daily dose of 17α-ethynyl estradiol (EE) in corn oil at 0.05 mg/kg/day by oral gavage. Body weights were determined daily. All animals were terminated and necropsied on PND 59 approximately 24 hours after the final dose administration to determine wet and blotted uterine weights.

All animals survived until scheduled termination and no treatment-related clinical findings were observed in folpet treated animals. Final body weights, overall body weight gains, and uterine weights in the folpet treated groups were comparable to the vehicle controls.

Comparative statistical analyses were not conducted for the final body weights and body weight gain of animals in the positive control (EE) group. However, final body weights were slightly decreased (↓6%) in the EE group compared to the vehicle controls, with a larger decrease in overall body weight gain (↓94%). Absolute wet and blotted uterus weights for the EE group were increased ($p<0.05$) by 77% and 66%, respectively, as expected.

No statistically significant changes were seen in uterine weight in this assay. Folpet is negative in the uterotrophic assay.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for an uterotrophic assay (OCSPP 890.1600).

COMPLIANCE: Signed and dated GLP Compliance and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

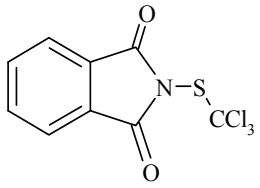
A. MATERIALS

1. Test Facility: Integrated Laboratory Systems, Inc. (ILS)

Location: Durham, NC
Study Director: J. P. Davis
Other Personnel: S. Borghoff (Study Toxicologist); P. Sproul (Toxicology Study Manager); A. Glasscock (Animal Facility Operations Manager); J. Pope (Necropsy Manager); K. Taylor (Facility Veterinarian); C. Cachafeiro (Health and Safety Manager)
Study Period: August 29, 2011 to January 3, 2012

2. Test Substance: Folpet

Description: Fine white powder
Source: Makteshim Chemical Works, Ltd
Lot/Batch #: 00138518 (expiration date 5/26/2012)
Purity: 97.6%
Stability: Dose formulations in carboxymethylcellulose stable for 8 days at 1-10 °C
CAS #: 133-07-3
Structure:



3. Reference Estrogen: 17α-ethynyl estradiol (EE)

Supplier: Sigma Aldrich (St. Louis, MO)
Lot/Batch #: 090M1241V (expiration date 2/2012)
Purity: ≥98%
CAS #: 57-63-6

4. Solvent/Vehicle Control (test substance): Carboxymethylcellulose (CMC)

Supplier: Sigma Aldrich (St. Louis, MO)
Lot/Batch #: 100M0113V
Rationale (if other than water): Not provided
Final concentration: 1%

Solvent/Vehicle Control (EE): Corn oil

Supplier: MP Biomedicals, LLC (Solon, OH)
Lot/Batch #: 7862K
Rationale (if other than water): Not applicable
Final concentration: Not applicable

5. Test Animals:

Species:	Rat (ovariectomized females only)	
Strain:	Sprague Dawley [Crl:CD®(SD) IGS]	
Age/weight at dose initiation:	PND 56/ 191.3-248.5 g	
Source:	Charles River Laboratories (Raleigh, NC)	
Housing:	Animals were housed in polycarbonate cages with micro-isolator tops and absorbent heat-treated hardwood bedding; rats were housed one per cage from receipt until study allocation and two per cage after allocation.	
Diet:	Teklad Global 16% Protein Rodent Diet (Teklad Diets, Madison WI) *ad libitum* Total genistein equivalents (genistein plus daidzein) of 8.6 µg/g of feed.	
Water:	Reverse-osmosis treated tap water, *ad libitum*	
Environmental conditions:	**Temperature:**	15-23 ºC
	Humidity:	38-64%
	Air changes:	Not stated
	Photoperiod:	12 hrs light/ 12 hrs dark
Acclimation period:	Seven days	

B. STUDY DESIGN

1. In Life Dates: Start: September 6, 2011 End: September 9, 2011

2. Study Design: Ovariectomized rats (date of ovariectomy not provided) were received from Charles River Laboratories (PND 49). Animals were acclimated for 7 days prior to initiation of dosing. Vaginal smears were taken daily for five days prior to assignment of animals to study, to verify that females were in persistent diestrus. The dose administration period was from PND 56 through 58. Rats were euthanized approximately 24 hours later on PND 59 and necropsied for uterine weight measurements.

3. Animal Assignment: Animals were assigned to the test groups noted in Table 1 using a procedure that stratified animals across groups by body weight such that mean body weight of each group was not statistically different from any other group using analysis of variance [ANOVA, Statistical Analysis System (SAS) version 9.2].

TABLE 1. Study Design[a]

Test Group	Dose (mg/kg/day)	# of Females
Estrogen Agonist Assay		
Vehicle Control	0	8
Low (Folpet)	313	8
High (Folpet)	1000	8
17α-ethynyl estradiol (EE), Reference Estrogen	0.05	8

a Data were obtained from Table 1 on page 16 of the study report.

4. **Dose Selection Rationale:** The dose levels were selected based on the results from a range-finding study conducted at ILS[1] in which rats (number and gender were not identified) received the test substance at 200, 400, 600, 800, or 1,000 for three consecutive days. All rats survived to study termination and did not show abnormal clinical signs 3 or 24 hours post-dose. The body weights of rats dosed at 1,000 mg/kg/day (limit dose) were not significantly affected. The dose level of 1,000 mg/kg/day was selected to meet the requirements of achieving a maximum tolerated dose.

5. **Dose Preparation and Analysis:** Dose formulations in 1% carboxymethylcellulose (CMC) were prepared once prior to initiation of treatment**.** Dose concentrations and homogeneity were tested by Smithers Viscient LLC (Wareham, MA) for both formulations (62.6 and 200 mg/mL) prepared by ILS. Three samples (top, middle, bottom) were analyzed for each concentration level. Analyses to demonstrate stability of the test substance in 1% CMC were conducted previously,[2] indicating that dose formulations were stable for up to 8 days of storage at 1-10 °C.

   Results of Dose Analysis

   Homogeneity (%CV): 0.429 and 3.15%

   Stability: It was stated that dose formulations in 1% CMC stored at 1-10 °C were stable for 8 days.

   Concentration (% of nominal): 86.0-94.6%

   The analytical data indicated that the mixing procedure was adequate and that the variation between nominal and actual dosage to the animals was acceptable. \

6. **Dosage Administration:** Animals were administered the test formulations and/or EE or vehicle daily via oral gavage for three consecutive days in a dose volume of 5 mL/kg body weight. Dose volumes were adjusted daily based on the concurrent body weight measurements.

7. **Statistics:** Descriptive statistics (mean, standard deviation and count) were calculated using MS Excel. Final body weight, body weight gain, and tissue weights were analyzed using SAS version 9.2. Studentized residual plots were used to detect possible outliers and Levene's test was used to assess homogeneity of variance.

[1] Davis, J. (2011). Range Finder Study for In Vivo Mammalian Assays for Folpet. Unpublished draft study report prepared by ILS Inc. Study No. C200-500.
[2] Dix, M. (2011). Storage Stability of Folpet in 1% Carboxymethylcellulose Solutions. Unpublished study report prepared by Smithers Viscient Inc. Study No. 11742.6182.

Final body weight, body weight gain, and uterine weights were analyzed by one-way ANOVA followed by pair-wise comparisons using a Dunnett's one tailed t-test (uterine weights) or Dunnett's two tailed t-test (final body weight and body weight gain). Positive controls (EE) were compared to vehicle controls by appropriate t-tests. Statistically significant effects were reported when $p<0.05$. The statistical analyses were considered adequate.

C. METHODS

1. **Clinical Examinations:** Cage-side checks for mortality and morbidity were conducted twice daily (once daily on weekends). Cage-side observations for clinical signs of toxicity were also conducted one hour following dose administration. Clinical observations were conducted within two days of arrival, at allocation to dose groups, daily prior to dose administration, and at termination.

2. **Body Weight:** Animals were weighed at randomization, study initiation, daily throughout the dosing period, and at termination.

3. **Food Consumption (Optional):** Food consumption was not measured.

4. **Necropsy and Measurement of Uterine Weight:** On PND 59 (approximately 24 hours after final administration of the test substance), all animals were euthanized by carbon dioxide asphyxiation and cervical dislocation, and subjected to a gross necropsy. Animals were checked for ovarian tissue at necropsy. The "wet" uterus (i.e., containing the luminal fluid) was weighed. Subsequently, the uterus was pierced and blotted to remove the luminal fluid, and the blotted uterus was weighed.

5. **Microscopic Examination (Optional):** Microscopic examinations were not conducted.

II. RESULTS

A. OBSERVATIONS

1. **Mortality:** All animals survived until scheduled termination.

2. **Clinical Signs of Toxicity:** No clinical signs of toxicity were observed in animals for any dose groups, with the exception of one animal from the 1,000 mg/kg/day dose group for which rales were noted on Days 3 and 4.

B. BODY WEIGHT AND WEIGHT GAIN: Body weight and body weight gain data are presented in Table 2. Final body weights and body weight gain in the folpet treatment groups were comparable to controls. Comparative statistical analyses were not conducted for the final body weights and body weight gain of animals in the positive control (EE) group. However, final body weights were slightly decreased (↓6%) in the EE group compared to the vehicle controls, with a larger decrease in overall body weight gain (↓94%).

TABLE 2. Group Body Weights and Cumulative Body Weight Gains (g) In the Estrogen Agonist Assay [a]												
Study Day #	Dose (mg/kg/day)											
	Vehicle Control			Folpet (313)			Folpet (1000)			Reference Estrogen EE (0.05)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
1	8	222.9	16.1	8	223.3	16.1	8	223.0	15.6	8	222.2	13.0
2	8	226.7	16.5	8	221.5	19.3	8	221.1	13.0	8	223.3	12.3
3	8	230.6	17.1	8	226.1	17.6	8	225.4	14.2	8	222.3	11.7
4	8	238.1	17.9	8	229.7	19.7	8	230.5	12.5	8	223.1 (↓6)	11.1
Body Weight Gain (1 - 4)	8	15.2	3.4	8	6.4	11.6	8	7.5	8.4	8	0.9 (↓94)	3.4

a Data were obtained from Table 3 on page 20 and Appendix 6 on page 59 of the study report. Percent differences from controls, calculated by the reviewers, are included in parentheses. Statistical analyses were only conducted for body weights on Day 4 and overall body weight gain in folpet treatment groups.
N Number of animals in the group
SD Standard Deviation

C. FOOD CONSUMPTION (Optional): Food consumption was not measured.

D. PATHOLOGY

1. Uterine Weights: Uterine weight data are presented in Table 3. Uterine weights in the folpet treatment groups were comparable to the vehicle controls. Absolute wet and blotted uterus weights for the positive control (EE) group were increased ($p<0.05$) by 77% and 66%, respectively. The increased uterine weights were within the expected range.

No macroscopic findings in the uterus were observed in the folpet treatment groups or the positive control group, with the exception of one animal in the 313 mg/kg/day group, which was found to have adhesion of the left uterine horn to the abdominal wall.

The mean blotted uterine weight for the vehicle control group was 0.034% of the mean vehicle control terminal body weight, which meets the Guideline requirement (<0.04%).

TABLE 3. Uterine Weights from Estrogen Agonist Assay in Sprague Dawley Rats[a]

Parameter	Dose (mg/kg/day)											
	Vehicle Control			Folpet (313)			Folpet (1000)			Reference Estrogen EE (0.05)		
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Terminal BW (g)	8	238.1	17.9	8	229.7	19.7	8	230.5	12.5	8	223.1	11.1
Wet, absolute (mg)	8	86.2	6.5	8	90.8	11.1	8	82.4	4.9	8	152.9* (↑77)	60.8
Wet, relative (%)[b]	8	0.0364	0.0039	8	0.0398	0.0054	8	0.0359	0.0029	8	0.0691	0.0300
Blotted, absolute (mg)	8	80.6	6.5	8	86.3	9.3	8	75.3	4.7	8	133.4* (↑66)	28.7
Blotted, relative (%)[b]	8	0.0340	0.0036	8	0.0378	0.0049	8	0.0328	0.0027	8	0.0601	0.0147

a Data were obtained from Table 4 on page 21 and Appendix 7 on page 61 of the study report. Percent differences from controls, calculated by the reviewers, are included in parentheses.
b With the exception of relative blotted uterine weight for the vehicle control group, relative weights (including mean and SD) were calculated by the reviewers using the individual terminal BW and uterine weights from Appendix 7 on page 61 of the study report. Comparative statistical analyses were not conducted for relative weights.
BW Body weight
N Number of animals in the group
SD Standard Deviation
* Significantly different from controls at $p<0.05$

2. Microscopic Examination (Optional): Microscopic examinations were not conducted.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Administration of folpet at dose levels of 313 or 1,000 mg/kg did not affect final body weights, body weight gain or uterine weights (wet and blotted). Based on these findings, oral administration of folpet up to the limit dose of 1,000 mg/kg showed no evidence of estrogen activity in the Uterotrophic Assay (OPPTS 890.1600).

B. AGENCY COMMENTS: All animals survived until scheduled termination and no treatment-related clinical findings were observed in folpet treated animals. Final body weights, overall body weight gains, and uterine weights in the folpet treated groups were comparable to the vehicle controls.

Comparative statistical analyses were not conducted for the final body weights and body weight gain of animals in the positive control (EE) group. However, final body weights were slightly decreased (↓6%) in the EE group compared to the vehicle controls, with a larger decrease in overall body weight gain (↓94%). Absolute wet and blotted uterus weights for the EE group were increased ($p<0.05$) by 77% and 66%, respectively, as expected. No statistically significant changes were seen in uterine weight in this assay. Folpet is negative in the uterotrophic assay.

C. STUDY DEFICIENCIES: None