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

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

Date: 6/29/2015

SUBJECT: 2,4-D: Data Evaluation Records (DERs) for EDSP Tier 1 Assays

PC Code: 030001
Decision No.: NA
Petition No.: NA
Risk Assessment Type: NA
TXR No.: 0052104
MRID No.: See Table

DP Barcode: D398637, D398638, D398640
Registration No.: NA
Regulatory Action: NA
Case No.: NA
CAS No.: 94-75-7
40 CFR: NA

Ver.Apr. 2010

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

THROUGH: Jess Rowland
Deputy Director
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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 2,4-D: Test Order # CON-030001-1.

II. RESPONSE

Attached are the EDSP Tier 1 assay DERs for 2,4-D.

III. MRID Table

Chemical: 2,4-D		**PC Code: 030001**
Guideline	**Assay**	**MRID**
890.1100	Amphibian Metamorphosis Assay (Frog)	48317002
890.1150	Androgen Receptor Binding (Rat Prostate)	48614301
890.1200	Aromatase Assay (Human Recombinant)	48614302
890.1250	Estrogen Receptor Binding	48614303
890.1300	Estrogen Receptor Transcriptional Activation (Human Cell Line HeLa-9903)	48614304
890.1350	Fish Short-Term Reproduction	48317001
890.1400	Hershberger (Rat)	NA
890.1450	Female Pubertal (Rat)	NA
890.1500	Male Pubertal (Rat)	NA
890.1550	Steroidogenesis (Human Cell Line – H295R)	48614305
890.1600	Uterotrophic (Rat)	NA

NA= Not Applicable (Requirement satisfied by other scientifically relevant information (OSRI)).

Data Requirement:	EPA DP Barcode	388579
	OECD Data Point	231
	EPA MRID	48317002
	EPA Guideline	890.1100
		Amphibian Metamorphosis Assay (Frog)

Test Material:	**2,4-Dichlorophenoxyacetic acid**		**Purity (%):** 98.6
Common Name	2,4-D		
Chemical Name	IUPAC		
	CAS Name		
	CAS No.	94-75-7	
	Synonyms	2,4-D	
		2,4-D acid	
	EPA PC Code	030001	

Primary Reviewer: Catherine Aubee
Biologist, USEPA/OCSPP/OPP/EFED/ERB1

Signature: 2015.06.01 13:15:13 -04'00'
Date: 07/30/2012

Additional Reviewer: Alicia Korol
Biologist, USEPA/OCSPP/OPP/EFED/ERB1

Signature: No longer with EPA
Date: 06/24/2011

Additional Reviewer: Anita Ullagaddi
Biologist, USEPA/OCSPP/OPP/EFED/ERB1

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Date: 02/24/2012

Final Additional Reviewer: Robin Sternberg
Wildlife Biologist, USEPA/OCSPP/OPP/EFED/ERB1

Signature: Digitally signed by ROBIN STERNBERG DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=ROBIN STERNBERG, dnQualifier=0000039126 Date: 2015.06.01 13:09:51 -04'00'
Date: 05/27/2015

Date Evaluation Completed: 05/27/2015

CITATION: Coady, K.K., T.A. Marino, and J. Thomas. November 29, 2010. 2,4-Dichlorophenoxyacetic Acid: The Amphibian Metamorphosis Assay Using the South African Clawed Frog, Xenopus laevis. The Dow Chemical Company. Midland, Michigan. Laboratory Project Study ID 101025. Sponsor: Industry Task Force II on 2,4-D Research Data.

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 2,4-D (purity 98.6%) on amphibian metamorphosis of South 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 a negative control and test chemical nominal concentrations of 0.4, 4.0, 40.0, and 100.0 mg a.i./L. Mean measured concentrations were <0.120 (<LOQ, negative control), 0.273, 3.24, 38.0, and 113 mg a.i./L. The test system was maintained at 21.9 to 22.7 °C and a pH of 7.0 to 7.8.

Only one incidence of tadpole mortality occurred in the mid-low treatment group; the cause of death was unknown. No clinical signs of toxicity were noted.

2,4-D did not affect Day 7 normalized (for snout-vent length) hind-limb length (HLL). However, there was a statistically significant ($p<0.05$) decrease of 15% in Day 21 normalized HLL at the highest treatment level compared with the negative control. There was no significant effect on median NF developmental stage, snout-vent length (SVL), or body weight at Day 7 or Day 21. Asynchronous development was not observed. There were no effects on thyroid gland histopathology. Late stage (>NF stage 60) tadpoles were observed in the negative control and in all treatment levels; consistent with the guideline recommendations, these tadpoles were excluded from analyses of growth and normalized HLL.

The study met all performance and validity criteria with the exception that the coefficients of variation (CVs) for measured concentrations of the low and low-mid treatment groups were 56 and 22%, respectively, exceeding the guideline performance criterion of ≤20%. This was likely due to biodegradation of the test material in the test vessels.

The 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 2,4-D.

Treatment (mg a.i./L) [mean-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
0.273	No	No	No	No	No	No	No
3.24	No	No	No	No	No	No	No
38.0	No	No	No	No	No	No	No
113	No	No	No	**Yes**	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).

I. MATERIALS AND METHODS

Guideline Followed: This study was conducted following guidelines outlined in United States Environmental Protection Agency (USEPA) 2009, Endocrine Disrupter Screening Program Test Guidelines OPPTS 890.1100: Amphibian Metamorphosis (Frog). EPA 740-C-09-002, October 2009 and Organization for Economic Cooperation and Development (OECD). 2009. OECD Guideline for the Testing of Chemicals: The Amphibian Metamorphosis Assay. OECD231 Adopted 7 September 2009. The following deviations was noted:

1. The CVs for measured concentrations of the low and low-mid treatment groups were 56 and 22%, respectively, exceeding the guideline performance criterion of ≤20%. This was likely due to biodegradation of the test material in the test vessels.

This deviation did not impact the interpretation of the study.

Compliance: Signed and dated GLP, Quality Assurance and Data Confidentiality statements were provided. This study was conducted in compliance with the following: European Community (EC) — European Parliament and Council Directive 2004/1 0/EC (O.J. No. L 50/44, 20/02/2004); Organisation for Economic Co-Operation and Development (OECD) – OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1. OECD Principles on Good Laboratory Practice (as revised in 1 997) ENV/MC/CHEM(98)17; and US Environmental Protection Agency -- FIFRA GLPs Title 40 CFR, Part 160 - Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards, Final Rule.

A. Test Material 2,4-Dichlorophenoxyacetic acid

CAS No 94-75-7

Description:

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.: 2006 2433 8006-USA

Purity: 98.6%

Impurities: None reported

Stability of Compound: The CVs for mean-measured concentrations were 55, 22, 16, and 17% for the low, low-mid, high-mid, and high concentrations, respectively. This was likely due to biodegradation of the test material in the test vessels. Overall recoveries were 68, 81, 95, and 113% of nominal for the low, low-mid, high-mid, and high concentrations, respectively.

Storage Conditions of Test Chemicals: Not reported. Aqueous stock solutions held in amber glassware or covered during storage to prevent photodegradation.

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:	South 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):			

[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	Breeding pairs were ordered from Xenopus Express (Brooksville, Florida). Adult male and female frogs were injected with human chorionic gonadotropin at least twelve hours before the desired breeding events.	*EPA recommends that larvae used in the assay be derived from in-house adults.*
Were parental acclimation conditions same as definitive test?	No	Not reported	
Acclimation period for parental frogs (if applicable):	days		
Details on parental feeding:	Not reported		
Details on parental health:	Not reported		

Table 3: Larval Selection and Care.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Best single spawn?	No	Not reported	*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):		Not reported	
Number of eggs sampled per spawn:		Not reported	
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:	18 days post-fertilization (dpf)	Test initiation began when tadpoles were 16 days old (discrepancy)	
Mean total length at test initiation (if reported):	mm	Not reported	
Range of total length at test initiation (if reported):	mm : mm	Not reported	

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Was the optional size selection method used?	No	Not reported	
Details on larval selection:	Not reported		
Loading rate (rearing density):		Not reported for larvae; 10 tadpoles/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.*
Type of food:	Sera Micron®		*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:	Sera North America		
Iodide concentration in diet (if known):	53.7 ug/g		

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:	The feeding regimen was the same as guideline recommendations (Table 2).		*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):	continuous-flow diluter system		*Intermittent flow proportional diluters or continuous flow serial diluters are recommended.*[2]
Flow-through rate (if applicable):	32 mL/min	± 3 (complete volume turnover approximately every 2.7 hours for each replicate test vessel)	*Recommended flow-through rate is 25 mL/min (complete volume replacement ca. every 2.7 hrs).*
Details on toxicant mixing for flow-through systems (if applicable):	Stainless steel mixing chamber was used.	The dilution water flow and resulting test solution volume in the mixing chamber was maintained using a stainless steel float valve. The test solution was gravity fed	*Recommended toxicant mixing for flow-through systems: 1) Mixing chamber is recommended but not required; 2) Aeration is not recommended for mixing;*

[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
		from the mixing chamber and split equally to replicate test vessels (contained in a temperature controlled water trough) via a Teflon manifold with Teflon delivery tubing. The diluter system was calibrated prior to test initiation, and diluter operation was monitored at least twice daily throughout the test. If there was an indication that the diluter calibration had changed (e.g., diluter malfunction, change in water quality or measured concentrations), calibration of necessary diluter components was checked and adjusted as appropriate.	*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:	natural water	Water was obtained from the upper Saginaw Bay of Lake Huron off Whitestone Point by the City of Midland Water Treatment Plant and supplied to The Dow Chemical Company prior to municipal treatment for human consumption. Water was limed and flocculated with ferric chloride, sand-filtered, pH-adjusted with gaseous CO2, carbon-filtered, and UV-irradiated.	*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	Laboratory dilution water is routinely analyzed for pesticides, organics, metals, and other inorganics twice per year. In particular, chlorate, perchlorate, and iodide levels are also determined.	
Iodide supplementation in water?	No		*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).*
Test vessel type/materials:	Glass sealed together with clear silicone adhesive		*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).*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Test vessel size:	30 x 14.5 x 20 cm deep		
Fill volume:	5.2 L		
Additional details on exposure system:			

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

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Hardness (mg/L as $CaCO_3$)	64	76	67	7 days	*EPA recommends hardness 40 to 48 mg/L as $CaCO_3$.*
pH	7.0	7.8		7 days	*EPA recommends pH 7.5 ± 1, inter-replicate and inter-treatment differentials should not exceed 0.5.*

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Dissolved oxygen (mg/L)	5.3	7.9		7 days	*EPA recommends dissolved oxygen (DO) >3.5 mg/L (>40% air saturation). OECD recommends DO concentration >3.5 mg/L (>40% air saturation).*
Temperature	21.9	22.5		7 days	*EPA recommends temperature 22±1°C; inter-replicate and inter-treatment differentials should not exceed 0.5°C.*
Iodide	<LOQ	<LOQ			*EPA recommends aquatic iodide range 0.5 - 10 µg/L (supplemental iodide should not exceed 2 µg/L).*
Ammonia	<0.1	0.24		4 months	*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*
Fluoride	0.1	0.1			
Perchlorate	<0.2	<0.2			
Chlorate	<10	<10			

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
					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.

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?	No		
If yes, what was the method for determining the highest test concentration in the range-finder?	NA		*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:	NA		
Life stage:	NA		
Test duration:	NA		
Additional details:	NA		

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:	The solubility limit and other reference studies were used to select the highest test concentration.		*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):	Morgan et al., 1996; Palmer and Krueger, 1997a; Palmer and Kreuger, 1997b; Alexander et al., 1985		
Separation of test concentrations:	0.1-0.4		*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:	4		*EPA recommends a minimum of 3 concentrations and a control, plus solvent control if appropriate.*
Are nominal concentrations adjusted for purity?	No	"Standards were not corrected for purity." (Appendix B)	
Indicate the type of values presented for measured concentrations:	Average measured		
Limit of quantification (LOQ):	0.120 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:	7 days		*It is recommended that test item concentration be measured in one tank at each treatment level at test initiation and every week thereafter.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Number of replicates in control:	4		*EPA recommends 4 replicates.*
Number of replicates in solvent control (if applicable):			*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?	No		
Solvent type (if applicable):			
Maximum solvent concentration (if applicable):	NA		*EPA recommends that the solvent not exceed 0.02 ml/L*[3]*. OECD recommends*

[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
			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		
Photoperiod:	12 hrs light : 12 hrs dark		*EPA recommends photoperiod 12:12 (light:dark).*
Light intensity at water's surface:	0.618-0.882 Klux		*EPA recommends light intensity 0.6 - 2 Klux (at water's surface).*

[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
Additional details:		Information on test solution appearance did not appear to be reported in the report.	

Table 8: Summary of Treatment Concentrations in the Amphibian Metamorphosis Assay with 2,4-D.

Treatment ID	Nominal Concentration (mg a.i./L)	Measured Concentration (mg a.i./L)	Mean CV (%)	Details or Remarks	Guideline Recommendations
Negative Control	0	<LOQ			*EPA and OECD recommend that test item concentrations be maintained at a coefficient of variation (CV) ≤20%.*
Treatment 1	0.4	0.273	55.67		
Treatment 2	4	3.24	21.60		
Treatment 3	40	38.0	16.29		
Treatment 4	100	113	16.90		

Abbreviations: CV Coefficient of variation.

LOQ = 0.120 mg a.i./L

E. Observations

Biological Endpoints: Mortality (throughout study); NF stage and asynchronous development; wet weight, snout-vent length, hind limb length (Day 7 and 21); thyroid histopathology (Day 21)

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

Only one larvae in the 3.24 mg a.i./L treatment level died during the 21-day study.

Table 9: Larval Mortality in South African Clawed Frog.

Treatment (mg a.i./L) [mean-measured]	Larval Mortality					
	Day 7[1]			Day 21		
	n	Mortality #	Mortality %	n	Mortality #	Mortality %
Negative Control	80	0	0	60	0	0
0.273	80	0	0	60	0	0
3.24	80	0	0	60	1	1.7
38.0	80	0	0	60	0	0
113	80	0	0	60	0	0

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

Day 7 median NF stage was 54 for the negative control and all treatment levels. Day 21 median NF stage was 58 for the highest treatment level and 59 for the negative control and all other treatment levels. There were no asynchronous tadpoles.

Table 10: Larval Development in South African Clawed Frog- Developmental Stage and Asynchronous Development.

Treatment (mg a.i./L) [mean-measured]	Developmental Stage					
	Day 7			Day 21		
	n	Median Stage[1]	# Asynchronous	n	Median Stage[1]	# Asynchronous
Negative Control	4	54	0	4	59	0
0.273	4	54	0	4	59	0
3.24	4	54	0	4	59	0
38.0	4	54	0	4	59	0
113	4	54	0	4	58	0

Day 7 HLL ranged from 2.2 to 2.4 mm across the control and all treatment levels. Day 21 HLL ranged from 16.2 mm in the high treatment group to 18.0 mm in the low treatment group.

Table 11: Larval Development in South African Clawed Frog - Hind Limb Length.

Treatment (mg a.i./L) [mean-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	4	2.4	0.2	0.1	4	17.2	0.8	0.7
0.273	4	2.2	0.1	0.1	4	18.0	1.0	0.7
3.24	4	2.3	0.3	0.1	4	17.1	0.9	0.7
38.0	4	2.2	0.1	0.1	4	17.5	1.0	0.7
113	4	2.2	0.1	0.1	4	16.2	1.0	0.6

Abbreviations: SD Standard deviation.

In this table, "n" represents the number of independent replicates per treatment level.

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

Day 7 SVL ranged from 18.00 to 18.5 mm and Day 21 SVL ranged from 26.9 to 28.1 mmm across the negative control and all treatment levels. Day 7 body weight ranged from 0.48 g to 0.53 g and Day 21 body weight ranged from 1.63 g to 1.91 g acorss the negative control and all treatment levels.

Table 12: Larval Growth in South African Clawed Frog.

Treatment (mg a.i./L) [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	4	18.5	0.5	4	26.8	1.4	4	0.53	0.04	4	1.63	0.17
0.273	4	18.0	0.5	4	27.4	0.6	4	0.48	0.04	4	1.73	0.12
3.24	4	18.3	0.6	4	26.9	1.6	4	0.49	0.05	4	1.63	0.24
38.0	4	18.1	0.2	4	27.7	0.31	4	0.49	0.02	4	1.75	0.06
113	4	18.4	0.3	4	28.1	0.40	4	0.50	0.04	4	1.91	0.22

Abbreviations: SD Standard deviation.

In this table, “n” represents the number of independent replicates per treatment level.

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

There were no treatment-related histopathologic changes in the thyroid gland in any of the treatment groups. There was no evidence of glandular atrophy or hypertrophy or follicular cell hyperplasia in any of the thyroid glands examined across all treatment groups. The incidence of tall columnar cells lining the follicles (follicular cell hypertrophy) did not show any treatment-related differences and was interpreted to be within normal limits at all concentrations of 2,4-D.

Table 13: Gross Pathology and Histopathology of the Thyroid Gland in South African Clawed Frog.

Treatment (mg a.i./L) [mean-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	5	20	20
	1	20	0	20	0	20	15	20	0
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
0.273	0	20	20	20	20	20	3	20	20
	1	20	0	20	0	20	17	20	0
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
3.24	0	20	20	20	20	20	6	20	20
	1	20	0	20	0	20	14	20	0
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
38.0	0	20	20	20	20	20	5	20	20
	1	20	0	20	0	20	15	20	0
	2	20	0	20	0	20	0	20	0
	3	20	0	20	0	20	0	20	0
113	0	20	20	20	20	20	4	20	20
	1	20	0	20	0	20	16	20	0
	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 South African Clawed Frog.

Treatment (mg a.i./L) [time-weighted, mean-measured]	Additional Qualitative Observations[1]											
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase)		Follicular Cell Height (Decrease)		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.273	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3.24	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Treatment (mg a.i./L) [time-weighted, mean-measured]	Additional Qualitative Observations[1]											
	Severity	Follicular Lumen Area (Increase)		Follicular Lumen Area (Decrease)		Follicular Cell Height (Increase)		Follicular Cell Height (Decrease)		Follicular Cell Shape	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
38.0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
113	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviations: NA Not applicable.

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.

No abnormal behaviors or clinical signs of toxicity were noted among control or 2,4-D exposed tadpoles.

Table 15: Clinical Signs in *Xenopus laevis*.

Treatment (mg a.i./L) [TWA- measured]	Clinical Signs[1]		
	Type	n	Incidence
Negative Control	None	NA	NA
Solvent Control	None	NA	NA
0.00264	None	NA	NA
0.0262	None	NA	NA
0.273	None	NA	NA

Abbreviations: NA Not applicable.

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

B. Study Author's Analysis and Conclusions

There were no signs of overt toxicity among exposed tadpoles in the present study. Throughout the entire exposure period, there was only one incidence of tadpole mortality. The study author identified no indications of developmental delay or abnormal behavior and therefore concluded that concentrations of 2,4-D used in the present study (up to 113 mg/L 2,4-D) were not overtly toxic to developing *X. laevis*. There were also no signs of advanced development (as measured by developmental stage and hind limb length) or asynchronous development among 2,4-D exposed tadpoles relative to control tadpoles on either Day 7 or Day 21 of exposure. Finally, compared to thyroid glands from controls, there were no significant histopathological effects observed among thyroid glands from 2,4-D exposed tadpoles.

C. Reviewer's Analysis and Conclusions

Statistical Methods: Day 21 values for NF developmental stage and normalized HLL were consistent with a monotonic response and were analyzed using the Jonckheere-Terpstra test. Values for Day 7 SVL and Days 7 and 21 body weight showed no apparent monotonic response and met the assumptions of normality and homogeneity of variance; therefore, these endpoints were analyzed using the parametric Dunnett's test. Day 7 NF stage and Day 21 SVL were inconsistent with a monotonic response and were analyzed with the non-parametric Mann-Whitney test. All analyses were performed using the FROG program with Statistical Analysis Software (SAS v. 9.3).

Late stage (> NF 60) tadpoles were excluded from analyses of SVL, body weight, and normalized HLL. Late stage tadpoles were excluded from every treatment level: control-6 tadpoles, 0.273 mg a.i./L-10 tadpoles, 3.25 mg a.i./L-6 tadpoles, 38.0 mg a.i./L-7 tadpoles, 113 mg a.i./L-6 tadpoles.

Histopathology results were evaluated visually based on severity and incidence data, within the context of the narrative pathology report.

Conclusions:

The only statistically-significant effect was for Day 21 HLL which was decreased by 15% at the high treatment level compared to the negative control after excluding "late stage" tadpoles from the analysis.

Table 16: Developmental and Thyroid Gross Pathology/Histopathology Endpoints[1,2] in the AMA with 2,4-D.

Treatment (mg a.i./L) [mean-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	NA
0.273	54	0.48	59	0.75	0	NA	-3.7	0.25	0	NA	0	NA	No
3.24	54	>0.99	59	0.94	0	NA	-3.7	0.25	0	NA	0	NA	No
38.0	54	>0.99	59	0.59	0	NA	-3.7	0.26	0	NA	0	NA	No
113	54	>0.99	58	0.17	0	NA	-14.8	**0.02**	0	NA	0	NA	No
Statistical Test	Mann-Whitney		Jonckheere-Terpstra		NA		Jonckheere-Terpstra		NA		NA		NA

Abbreviations: Diff. Difference. NA Not applicable.

1 Unless otherwise indicated, effects are reported based on comparison to the clean water control. Negative values indicate reductions or decreases relative to the negative 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).

Table 17: Growth Endpoints[1,2] in the AMA with 2,4-D.

Treatment (mg a.i./L) [mean-measured]	Snout-Vent Length				Body Weight			
	Day 7		Day 21		Day 7		Day 21	
	% Diff.	p	% Diff.	p	% Diff.	p	% Diff.	p
Negative Control	NA	NA	NA	NA	NA	NA	NA	NA
0.273	-2.8	0.31	2.3	0.78	-9.0	0.32	6.3	0.82
3.24	-1.1	0.92	0.3	>0.99	-6.3	0.61	-0.8	>0.99
38.0	-2.2	0.53	3.4	0.78	-7.2	0.50	7.9	0.68
113	-0.7	0.98	4.9	0.24	-5.8	0.68	17.5	0.10
Statistical Test	Dunnett's		Mann-Whitney		Dunnett's		Dunnett's	

Abbreviations: Diff. Difference. NA Not applicable.

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 $p<0.05$.

E. Study Deficiencies

The coefficients of variation for the measured concentrations of the 0.273 and 3.24 mg a.i./L treatment groups exceeded the guideline performance criterion of 20%. This was likely due to biodegradation of the test material in the test vessels. All other validity and performance criteria were met.

F. Reviewer's Comments

Unlike the study author, the reviewer detected a statistically significant decrease in Day 21 normalized HLL ($p<0.05$) at the highest treatment level after excluding "late stage" tadpoles from the analysis. No other signs of toxicity were observed at this or any other treatment level. Late stage tadpoles were excluded from every treatment level: control-6 tadpoles, 0.273 mg a.i./L-10 tadpoles, 3.25 mg a.i./L-6 tadpoles, 38.0 mg a.i./L-7 tadpoles, 113 mg a.i./L-6 tadpoles. Late stage tadpoles were included in the analysis of potential effects on developmental stage.

The individuals selected for histopathology were not stage-matched to the median stage in the controls. However, there were no noteworthy differences between histopathology observations in control and treatment specimens; therefore, this guideline deviation does not appear to have substantively affected the interpretation of results.

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Appendix I: Output of Reviewer's Statistical Analysis

```
test for amphib metamorph screen study - 2,4-d
ANALYSIS RESULTS FOR VARIABLE VAR01  ( 7-d wet weight (g) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.909         0.061          0.655      0.632    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     0.53     0.04       0.02        8.03           0.46,    0.59
  Dose1  4     0.48     0.04       0.02        8.86           0.41,    0.55
  Dose2  4     0.49     0.05       0.02        9.97           0.41,    0.57
  Dose3  4     0.49     0.02       0.01        4.57           0.45,    0.52
  Dose4  4     0.50     0.04       0.02        8.41           0.43,    0.56

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl         0.52     0.48       0.58         .                   .
  Dose1        0.47     0.44       0.54       90.97               9.03
  Dose2        0.47     0.46       0.57       93.68               6.32
  Dose3        0.49     0.46       0.51       92.78               7.22
  Dose4        0.49     0.46       0.54       94.25               5.75

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.78        0.556

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     0.53    .         0.53      .        .       .       .       .       .
 Dose1    0.48   0.320      0.49     0.127     .       .       .       .       .
 Dose2    0.49   0.611      0.49     0.135    0.986    .       .       .       .
 Dose3    0.49   0.504      0.49     0.139    0.997   1.000    .       .       .
 Dose4    0.50   0.680      0.49     0.142    0.973   1.000   0.999    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
```

```
     Degrees of Freedom   TestStat    P-value
           4                 3.62       0.460

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       0.52                   .                        .
  Dose1      0.47                  0.235                    0.074
  Dose2      0.47                  0.233                    0.120
  Dose3      0.49                  0.230                    0.200
  Dose4      0.49                  0.494                    0.275

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.78        0.556

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -0.53    .        -0.50      .        .       .       .       .       .
 Dose1   -0.48   0.320     -0.50     0.906     .       .       .       .       .
 Dose2   -0.49   0.611     -0.50     0.927    0.986    .       .       .       .
 Dose3   -0.49   0.504     -0.50     0.937    0.997   1.000    .       .       .
 Dose4   -0.50   0.680     -0.50     0.943    0.973   1.000   0.999    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 3.62       0.460

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -0.52                   .                        .
  Dose1     -0.47                  0.235                    0.926
  Dose2     -0.47                  0.233                    0.880
  Dose3     -0.49                  0.230                    0.800
  Dose4     -0.49                  0.494                    0.725
```

```
 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for amphib metamorph screen study - 2,4-d
ANALYSIS RESULTS FOR VARIABLE VAR02  ( 7-d stage (median) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.509         <.001          9.000      <.001    USE NON-PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    54.00     0.00       0.00        0.00            .  ,     .
  Dose1  4    53.75     0.50       0.25        0.93          52.95,   54.55
  Dose2  4    54.00     0.00       0.00        0.00            .  ,     .
  Dose3  4    54.00     0.00       0.00        0.00            .  ,     .
  Dose4  4    54.00     0.00       0.00        0.00            .  ,     .

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        54.00    54.00      54.00         .                   .
  Dose1       54.00    53.00      54.00       99.54               0.46
  Dose2       54.00    54.00      54.00      100.00               0.00
  Dose3       54.00    54.00      54.00      100.00               0.00
  Dose4       54.00    54.00      54.00      100.00               0.00

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.00        0.438

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    54.00    .        54.00      .        .       .       .       .       .
 Dose1   53.75   0.357     53.94     0.415     .       .       .       .       .
 Dose2   54.00   1.000     53.94     0.443    0.530    .       .       .       .
 Dose3   54.00   1.000     53.94     0.458    0.530   1.000    .       .       .
 Dose4   54.00   1.000     53.94     0.468    0.530   1.000   1.000    .       .

*******************************************************************************
```

```
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 4.00       0.406

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      54.00                   .                        .
  Dose1     54.00                  0.478                    0.159
  Dose2     54.00                  1.000                    0.500
  Dose3     54.00                  1.000                    0.673
  Dose4     54.00                  1.000                    0.760

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.00        0.438

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -54.00    .       -53.88      .        .       .       .       .       .
 Dose1  -53.75   0.357    -53.88     0.855     .       .       .       .       .
 Dose2  -54.00   1.000    -54.00     0.618    0.530    .       .       .       .
 Dose3  -54.00   1.000    -54.00     0.636    0.530   1.000    .       .       .
 Dose4  -54.00   1.000    -54.00     0.648    0.530   1.000   1.000    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 4.00       0.406

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -54.00                   .                        .
  Dose1    -54.00                  0.478                    0.841
  Dose2    -54.00                  1.000                    0.500
  Dose3    -54.00                  1.000                    0.327
  Dose4    -54.00                  1.000                    0.240
```

```
 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for amphib metamorph screen study - 2,4-d
ANALYSIS RESULTS FOR VARIABLE VAR03  ( 7-d sn-vent length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.977         0.890          0.425      0.788    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    18.53     0.54       0.27        2.90          17.67,   19.38
  Dose1  4    18.00     0.49       0.24        2.72          17.22,   18.78
  Dose2  4    18.33     0.56       0.28        3.03          17.44,   19.21
  Dose3  4    18.13     0.21       0.10        1.14          17.80,   18.45
  Dose4  4    18.40     0.29       0.15        1.60          17.93,   18.87

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        18.60    17.80      19.10         .                    .
  Dose1       18.00    17.40      18.60       97.17                2.83
  Dose2       18.20    17.80      19.10       98.92                1.08
  Dose3       18.15    17.90      18.30       97.84                2.16
  Dose4       18.40    18.10      18.70       99.33                0.67

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.92        0.476

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    18.53    .        18.53      .        .       .       .       .       .
 Dose1   18.00   0.305     18.21     0.198     .       .       .       .       .
 Dose2   18.33   0.915     18.21     0.211    0.831    .       .       .       .
 Dose3   18.13   0.528     18.21     0.218    0.994   0.965    .       .       .
 Dose4   18.40   0.983     18.21     0.223    0.703   0.999   0.898    .       .
```

```
*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 3.39       0.494

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      18.60                   .                        .
  Dose1     18.00                  0.332                    0.117
  Dose2     18.20                  0.673                    0.328
  Dose3     18.15                  0.341                    0.254
  Dose4     18.40                  0.887                    0.580

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.92        0.476

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -18.53    .       -18.24      .        .       .       .       .       .
 Dose1  -18.00   0.305    -18.24     0.881     .       .       .       .       .
 Dose2  -18.33   0.915    -18.24     0.905    0.831    .       .       .       .
 Dose3  -18.13   0.528    -18.24     0.917    0.994   0.965    .       .       .
 Dose4  -18.40   0.983    -18.40     0.803    0.703   0.999   0.898    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 3.39       0.494

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -18.60                   .                        .
  Dose1    -18.00                  0.332                    0.883
  Dose2    -18.20                  0.673                    0.672
  Dose3    -18.15                  0.341                    0.746
```

```
  Dose4    -18.40                    0.887                    0.420

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for amphib metamorph screen study - 2,4-d
ANALYSIS RESULTS FOR VARIABLE VAR04  ( 7-d hind-limb length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.949         0.355          4.063      0.020    USE NON-PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     2.35     0.19       0.10        8.15           2.05,    2.65
  Dose1  4     2.18     0.05       0.03        2.30           2.10,    2.25
  Dose2  4     2.25     0.26       0.13       11.76           1.83,    2.67
  Dose3  4     2.20     0.08       0.04        3.71           2.07,    2.33
  Dose4  4     2.23     0.05       0.02        2.25           2.15,    2.30

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl         2.40     2.10       2.50         .                    .
  Dose1        2.20     2.10       2.20       92.55                7.45
  Dose2        2.20     2.00       2.60       95.74                4.26
  Dose3        2.20     2.10       2.30       93.62                6.38
  Dose4        2.20     2.20       2.30       94.68                5.32

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.77        0.561

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     2.35    .         2.35      .        .       .       .       .       .
 Dose1    2.18   0.343      2.21     0.134     .       .       .       .       .
 Dose2    2.25   0.768      2.21     0.143    0.956    .       .       .       .
 Dose3    2.20   0.471      2.21     0.147    0.999   0.990    .       .       .
 Dose4    2.23   0.618      2.21     0.150    0.990   0.999   0.999    .       .
```

```
*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 2.48       0.648

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       2.40                   .                        .
  Dose1      2.20                0.270                    0.089
  Dose2      2.20                0.673                    0.185
  Dose3      2.20                0.335                    0.207
  Dose4      2.20                0.399                    0.326

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.77        0.561

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -2.35    .        -2.24      .        .       .       .       .       .
 Dose1   -2.18   0.343     -2.24     0.901     .       .       .       .       .
 Dose2   -2.25   0.768     -2.24     0.923    0.956    .       .       .       .
 Dose3   -2.20   0.471     -2.24     0.933    0.999   0.990    .       .       .
 Dose4   -2.23   0.618     -2.24     0.939    0.990   0.999   0.999    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 2.48       0.648

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -2.40                   .                        .
  Dose1     -2.20                0.270                    0.911
  Dose2     -2.20                0.673                    0.815
```

```
  Dose3     -2.20                    0.335                    0.793
  Dose4     -2.20                    0.399                    0.674

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for amphib metamorph screen study - 2,4-d
ANALYSIS RESULTS FOR VARIABLE VAR05  ( 7-d norm hind-limb )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
       .             .              .          .       NO DATA FOR TEST

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     0.10     0.00       0.00        0.00            .  ,     .
  Dose1  4     0.10     0.00       0.00        0.00            .  ,     .
  Dose2  4     0.10     0.00       0.00        0.00            .  ,     .
  Dose3  4     0.10     0.00       0.00        0.00            .  ,     .
  Dose4  4     0.10     0.00       0.00        0.00            .  ,     .

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl         0.10     0.10       0.10         .                  .
  Dose1        0.10     0.10       0.10      100.00               0.00
  Dose2        0.10     0.10       0.10      100.00               0.00
  Dose3        0.10     0.10       0.10      100.00               0.00
  Dose4        0.10     0.10       0.10      100.00               0.00

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
 .         .      .    1     .        .        .       .       .       .       .


Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     0.10    .          .        .        .       .       .       .       .
 Dose1    0.10    .          .        .        .       .       .       .       .
 Dose2    0.10    .          .        .        .       .       .       .       .
```

```
 Dose3    0.10    .          .        .        .       .       .       .       .
 Dose4    0.10    .          .        .        .       .       .       .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 0.00       1.000

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       0.10                   .                        .
  Dose1      0.10                1.000                       .
  Dose2      0.10                1.000                       .
  Dose3      0.10                1.000                       .
  Dose4      0.10                1.000                       .

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                   Dose1
   Jonckheere                                 Dose1

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
 .         .      .    1     .        .        .       .       .       .       .


Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -0.10    .          .        .        .       .       .       .       .
 Dose1   -0.10    .          .        .        .       .       .       .       .
 Dose2   -0.10    .          .        .        .       .       .       .       .
 Dose3   -0.10    .          .        .        .       .       .       .       .
 Dose4   -0.10    .          .        .        .       .       .       .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 0.00       1.000

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
```

```
  Ctrl      -0.10                    .                      .
  Dose1     -0.10                  1.000                    .
  Dose2     -0.10                  1.000                    .
  Dose3     -0.10                  1.000                    .
  Dose4     -0.10                  1.000                    .

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                   Dose1
   Jonckheere                                 Dose1

test for amphib metamorph screen study - 2,4-d
ANALYSIS RESULTS FOR VARIABLE VAR06  ( 21-d stage (median) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.944         0.284          6.750      0.003    USE NON-PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    58.50     0.58       0.29        0.99          57.58,   59.42
  Dose1  4    58.75     0.50       0.25        0.85          57.95,   59.55
  Dose2  4    59.00     0.00       0.00        0.00            .  ,     .
  Dose3  4    58.50     0.58       0.29        0.99          57.58,   59.42
  Dose4  4    58.25     0.50       0.25        0.86          57.45,   59.05

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        58.50    58.00      59.00         .                   .
  Dose1       59.00    58.00      59.00      100.43              -0.43
  Dose2       59.00    59.00      59.00      100.85              -0.85
  Dose3       58.50    58.00      59.00      100.00               0.00
  Dose4       58.00    58.00      59.00       99.57               0.43

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.39        0.284

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    58.50    .        58.75      .        .       .       .       .       .
```

```
 Dose1   58.75   0.874     58.75     0.841     .       .       .       .       .
 Dose2   59.00   0.421     58.75     0.869    0.946    .       .       .       .
 Dose3   58.50   1.000     58.50     0.636    0.946   0.599    .       .       .
 Dose4   58.25   0.874     58.25     0.322    0.599   0.234   0.946    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 5.15       0.273

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      58.50                    .                       .
  Dose1     59.00                   0.624                   0.753
  Dose2     59.00                   0.223                   0.941
  Dose3     58.50                   1.000                   0.592
  Dose4     58.00                   0.624                   0.173

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.39        0.284

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -58.50    .       -58.50      .        .       .       .       .       .
 Dose1  -58.75   0.874    -58.63     0.427     .       .       .       .       .
 Dose2  -59.00   0.421    -58.63     0.455    0.946    .       .       .       .
 Dose3  -58.50   1.000    -58.63     0.471    0.946   0.599    .       .       .
 Dose4  -58.25   0.874    -58.63     0.481    0.599   0.234   0.946    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 5.15       0.273

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend
```

```
 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -58.50                    .                        .
  Dose1    -59.00                  0.624                    0.247
  Dose2    -59.00                  0.223                    0.059
  Dose3    -58.50                  1.000                    0.408
  Dose4    -58.00                  0.624                    0.827

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for amphib metamorph screen study - 2,4-d
ANALYSIS RESULTS FOR VARIABLE VAR07  ( 21-d wet weight (g) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.948         0.345          1.312      0.310    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     1.63     0.17       0.08       10.32           1.36,    1.89
  Dose1  4     1.73     0.12       0.06        6.75           1.54,    1.91
  Dose2  4     1.61     0.24       0.12       14.76           1.23,    1.99
  Dose3  4     1.75     0.06       0.03        3.23           1.66,    1.84
  Dose4  4     1.91     0.22       0.11       11.42           1.56,    2.26

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl         1.64     1.43       1.78         .                    .
  Dose1        1.76     1.57       1.82      106.27               -6.27
  Dose2        1.69     1.26       1.80       99.17                0.83
  Dose3        1.75     1.70       1.82      107.92               -7.92
  Dose4        1.93     1.63       2.15      117.51              -17.51

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.94        0.156

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5
```

```
 Ctrl     1.63    .         1.73      .        .       .       .       .       .
 Dose1    1.73   0.819      1.73     0.863     .       .       .       .       .
 Dose2    1.61   1.000      1.73     0.889    0.875    .       .       .       .
 Dose3    1.75   0.681      1.73     0.902    0.999   0.770    .       .       .
 Dose4    1.91   0.104      1.73     0.909    0.580   0.157   0.708    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 5.41       0.247

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       1.64                    .                        .
  Dose1      1.76                   0.346                    0.876
  Dose2      1.69                   1.000                    0.500
  Dose3      1.75                   0.494                    0.772
  Dose4      1.93                   0.156                    0.969

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.94        0.156

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -1.63    .        -1.63      .        .       .       .       .       .
 Dose1   -1.73   0.819     -1.67     0.428     .       .       .       .       .
 Dose2   -1.61   1.000     -1.67     0.457    0.875    .       .       .       .
 Dose3   -1.75   0.681     -1.75     0.203    0.999   0.770    .       .       .
 Dose4   -1.91   0.104     -1.91     0.021    0.580   0.157   0.708    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 5.41       0.247

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend
```

```
 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -1.64                    .                        .
  Dose1     -1.76                  0.346                    0.124
  Dose2     -1.69                  1.000                    0.500
  Dose3     -1.75                  0.494                    0.228
  Dose4     -1.93                  0.156                    0.031

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                   Dose4
   Jonckheere                                 Dose4

test for amphib metamorph screen study - 2,4-d
ANALYSIS RESULTS FOR VARIABLE VAR08  ( 21-d sn-vent length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.981         0.945          5.995      0.004    USE NON-PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    26.78     1.39       0.70        5.21          24.56,   28.99
  Dose1  4    27.40     0.56       0.28        2.04          26.51,   28.29
  Dose2  4    26.85     1.63       0.81        6.06          24.26,   29.44
  Dose3  4    27.68     0.31       0.15        1.12          27.18,   28.17
  Dose4  4    28.08     0.40       0.20        1.44          27.43,   28.72

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        26.80    25.30      28.20         .                    .
  Dose1       27.25    26.90      28.20      102.33               -2.33
  Dose2       26.90    24.90      28.70      100.28               -0.28
  Dose3       27.60    27.40      28.10      103.36               -3.36
  Dose4       28.10    27.60      28.50      104.86               -4.86

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.18        0.361

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5
```

```
 Ctrl    26.78    .        27.36      .        .       .       .       .       .
 Dose1   27.40   0.798     27.36     0.859     .       .       .       .       .
 Dose2   26.85   1.000     27.36     0.885    0.937    .       .       .       .
 Dose3   27.68   0.550     27.36     0.899    0.995   0.779    .       .       .
 Dose4   28.08   0.253     27.36     0.907    0.877   0.460   0.979    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 4.19       0.381

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      26.80                    .                        .
  Dose1     27.25                   0.780                    0.668
  Dose2     26.90                   1.000                    0.529
  Dose3     27.60                   0.780                    0.787
  Dose4     28.10                   0.235                    0.971

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.18        0.361

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -26.78    .       -26.78      .        .       .       .       .       .
 Dose1  -27.40   0.798    -27.13     0.377     .       .       .       .       .
 Dose2  -26.85   1.000    -27.13     0.403    0.937    .       .       .       .
 Dose3  -27.68   0.550    -27.68     0.150    0.995   0.779    .       .       .
 Dose4  -28.08   0.253    -28.08     0.058    0.877   0.460   0.979    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 4.19       0.381

MannWhit - testing each trt median signif. different from control
```

```
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -26.80                    .                        .
  Dose1    -27.25                  0.780                    0.332
  Dose2    -26.90                  1.000                    0.471
  Dose3    -27.60                  0.780                    0.213
  Dose4    -28.10                  0.235                    0.029

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                Dose4

test for amphib metamorph screen study - 2,4-d
ANALYSIS RESULTS FOR VARIABLE VAR09  ( 21-d hind-limb length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.955         0.443          0.118      0.974    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    17.23     0.77       0.38        4.46          16.00,   18.45
  Dose1  4    18.00     1.02       0.51        5.65          16.38,   19.62
  Dose2  4    17.08     0.91       0.46        5.35          15.62,   18.53
  Dose3  4    17.48     1.00       0.50        5.71          15.89,   19.06
  Dose4  4    16.20     0.98       0.49        6.07          14.64,   17.76

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        17.20    16.40      18.10         .                    .
  Dose1       17.70    17.20      19.40      104.50               -4.50
  Dose2       16.90    16.20      18.30       99.13                0.87
  Dose3       17.60    16.30      18.40      101.45               -1.45
  Dose4       16.45    14.80      17.10       94.05                5.95

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.96        0.153

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
```

```
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    17.23    .        17.61      .        .       .       .       .       .
 Dose1   18.00   0.607     17.61     0.799     .       .       .       .       .
 Dose2   17.08   0.998     17.28     0.650    0.642    .       .       .       .
 Dose3   17.48   0.986     17.28     0.669    0.930   0.973    .       .       .
 Dose4   16.20   0.378     16.20     0.094    0.100   0.686   0.350    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 5.67       0.225

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      17.20                    .                        .
  Dose1     17.70                   0.413                    0.845
  Dose2     16.90                   0.889                    0.384
  Dose3     17.60                   0.678                    0.500
  Dose4     16.45                   0.283                    0.086

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.96        0.153

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -17.23    .       -17.20      .        .       .       .       .       .
 Dose1  -18.00   0.607    -17.20     0.602     .       .       .       .       .
 Dose2  -17.08   0.998    -17.20     0.637    0.642    .       .       .       .
 Dose3  -17.48   0.986    -17.20     0.656    0.930   0.973    .       .       .
 Dose4  -16.20   0.378    -17.20     0.668    0.100   0.686   0.350    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 5.67       0.225
```

```
MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -17.20                    .                        .
  Dose1    -17.70                  0.413                    0.155
  Dose2    -16.90                  0.889                    0.616
  Dose3    -17.60                  0.678                    0.500
  Dose4    -16.45                  0.283                    0.914

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for amphib metamorph screen study - 2,4-d
ANALYSIS RESULTS FOR VARIABLE VAR10  ( 21-d norm hind-limb )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.794         <.001          0.750      0.573    USE NON-PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     0.68     0.05       0.03        7.41           0.60,    0.75
  Dose1  4     0.65     0.06       0.03        8.88           0.56,    0.74
  Dose2  4     0.65     0.06       0.03        8.88           0.56,    0.74
  Dose3  4     0.65     0.06       0.03        8.88           0.56,    0.74
  Dose4  4     0.58     0.05       0.02        8.70           0.50,    0.65

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl         0.70     0.60       0.70         .                    .
  Dose1        0.65     0.60       0.70       96.30                3.70
  Dose2        0.65     0.60       0.70       96.30                3.70
  Dose3        0.65     0.60       0.70       96.30                3.70
  Dose4        0.60     0.50       0.60       85.19               14.81

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.92        0.160

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC
```

```
Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     0.68    .         0.68      .        .       .       .       .       .
 Dose1    0.65   0.914      0.65     0.315     .       .       .       .       .
 Dose2    0.65   0.914      0.65     0.337    1.000    .       .       .       .
 Dose3    0.65   0.914      0.65     0.349    1.000   1.000    .       .       .
 Dose4    0.58   0.066      0.58     0.013    0.341   0.341   0.341    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 5.87       0.209

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       0.70                    .                       .
  Dose1      0.65                   0.624                   0.247
  Dose2      0.65                   0.624                   0.246
  Dose3      0.65                   0.624                   0.256
  Dose4      0.60                   0.100                   0.019

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                 Dose4
   Jonckheere                               Dose4

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.92        0.160

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -0.68    .        -0.64      .        .       .       .       .       .
 Dose1   -0.65   0.914     -0.64     0.880     .       .       .       .       .
 Dose2   -0.65   0.914     -0.64     0.905    1.000    .       .       .       .
 Dose3   -0.65   0.914     -0.64     0.916    1.000   1.000    .       .       .
 Dose4   -0.58   0.066     -0.64     0.924    0.341   0.341   0.341    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 5.87       0.209
```

```
MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -0.70                    .                       .
  Dose1     -0.65                  0.624                   0.753
  Dose2     -0.65                  0.624                   0.754
  Dose3     -0.65                  0.624                   0.744
  Dose4     -0.60                  0.100                   0.981

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)
```

DATA EVALUATION RECORD

2,4-DICHLOROPHENOXYACETIC ACID (2,4-D)

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

EPA Contract No. EP10H001452
Task Assignment No. 2-26-2012 (MRID 48614301)

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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1910 Sedwick Road,
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Primary Reviewer:	Signature:	Sandra Hastings
Sandra Hastings	Date:	3/19/2012
Secondary Reviewer:	Signature:	Scott Studenberg
Scott D. Studenberg, Ph. D., D.A.B.T.	Date:	3/26/2012
Program Manager:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	3/26/2012
Quality Assurance:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	3/26/2012

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: Chester Rodriguez, Ph.D. **Signature:** (for)
Health Effects Division **Date:** 6/5/15
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:**
Health Effects Division **Date:** 6/15/15

Template version 08/2011

DATA EVALUATION RECORD

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

PC CODE: 030001 **DP BARCODE:** D398638

TXR#: 0052104 **CAS No.:** 94-75-7

TEST MATERIAL (PURITY): 2,4-D (98.5% a.i.)

SYNONYMS: (2,4-dichlorophenoxy) acetic acid

CITATION: LeBaron, M.J., Schisler, M.R., and Visconti, N.R. (2011). Evaluation of 2,4-Dichlorophenoxy Acetic Acid (2,4-D) In An *In Vitro* Androgen Receptor Binding Assay. The Dow Chemical Company, Toxicology & Environmental Research and Consulting, Midland, MI. Laboratory Project Study ID: 111111, October 27, 2011. MRID #48614301. Unpublished.

SPONSOR: Industry Task Force II on 2,4-D Research Data, c/o McKenna Long & Aldridge LLP, 1900 K Street NW, Washington, D.C.

TEST ORDER #: CON-030001-1

EXECUTIVE SUMMARY: In an androgen receptor (AR) binding assay (MRID 48614301), ventral prostate cytosol from Sprague Dawley rats was used as the source of AR to conduct a competitive binding experiment to measure the binding of a single concentration of [^{3}H]-R1881 (1 nM) in the presence of increasing concentrations (10^{-11} to 10^{-4} M) of 2,4-D (98.5% purity). The test guideline recommends testing up to 10^{-3} M; however, the sponsor selected 10^{-4} M as the highest concentration based on *in vivo* toxicokinetic analyses in the rat; concentrations higher than 10^{-4} M were not relevant for testing in this assay as they are substantially above the inflection point for linear toxicokinetics (see Appendix B of study report). Ethanol was used as a solvent at a final concentration of <3%. A total of three independent runs were performed, and the assay included dexamethasone as a weak positive control, and R1881 as the ligand reference standard.

Saturation binding experiments were conducted to demonstrate that the AR in the rat prostate cytosol was present at adequate levels and functioning with appropriate affinity for the radiolabeled ligand. The saturation binding experiment resulted in a maximum binding capacity (B_{max}) of 3.245 fmol/100 μg protein and the dissociation constant (K_d) was 0.4641 nM. Although these values were slightly below the range of values from the validation studies, the results were highly reproducible and all other performance criteria and the competitive binding

assays indicated acceptable performance of the assay. The Scatchard plot indicated a linear response across the concentrations of ligand added. Nonspecific binding as a percent of total binding was less than 20% across the entire concentration range in the saturation binding assays (range 6.2-19.8%, with the exception of the high concentration (10 nM) in one assay, which was 24.6%).

There were no appreciable alterations in R1881 AR binding activity at 2,4-D concentrations ranging from 10^{-11} to 10^{-4} M in the competitive binding experiments, therefore, the log IC_{50} and relative binding affinity (RBA) for 2,4-D could not be calculated. The log IC_{50} values for R-1881 alone and the positive control, dexamethasone, were −9.0 and −4.4 M, respectively. Compared to R1881, the RBA for dexamethasone was 0.0027 %. In all instances, R1881 and the positive control met the QC performance criteria established in the test guideline.

Based on the results from the three runs, 2,4-D is classified as a Non-binder in the Androgen Receptor Binding Assay.

The study **satisfies** the EDSP Tier 1 Test Order requirements for an Androgen Receptor Binding Assay (OCSPP 890.1150).

COMPLIANCE: Signed and dated GLP and Quality Assurance statements were provided in the study report.

I. MATERIALS AND METHODS

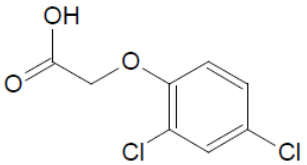
A. MATERIALS

1. Test Facility: The Dow Chemical Company, Toxicology & Environmental Research and Consulting

Location: Midland, MI
Study Director: Schisler, M.R.
Other Personnel: LeBaron, M.J. (Lead Scientist); Visconti, N.R. (Research Biologist); Gollapudi, B.B. (Technical reviewer)
Study Period: July 11, 2011 – October 27, 2011

2. Test substance: 2,4-D

Description: Off-white powder
Source: NuFarm Americas, Inc. (Burr Ridge, IL)
Lot/Batch #: 2006 2433 8006-USA (expiry date: March 3, 2013)
Purity: 98.5% a.i.
Solubility: Up to 315 mg/L in water; soluble in ethanol up to 30 mM
Volatility: 1.9 x 10^{-5} Pa at 25°C
Stability: 2-yr shelf life
Storage conditions: Ambient
CAS #: 94-75-7
Molecular weight: 221.0
Structure:



3. Non-labeled ligand: R1881

Supplier: Perkin-Elmer (Boston, MA)
Catalog #: R0908
Lot #: 614156
Purity: >97%
CAS #: 965-93-5

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

Supplier: Perkin-Elmer (Boston, MA)
Catalog and Batch #: NET590250UC, Lot# 614814
Date of production: July 1, 2010
Date of use: July 11, 2011 to July 28, 2011
Radiochemical purity: >97%
Specific activity: 85.1 Ci/mmol
Concentration of stock: 1.0 mCi/mL

5. Positive control: Dexamethasone

Supplier: Sigma (St. Louis, MO)
Catalog # D4902
Lot #: BCBC9269
Purity: 98.9%
CAS # : 50-02-2

6. Solvent/vehicle control: Ethanol
Justification for choice of solvent: None provided
Final Concentration: <3%

B. METHODS

1. Preparation of Rat Ventral Prostate Cytosol: The rat ventral prostate tissue was purchased from Charles River Laboratories (Wilmington, MA). Male Sprague Dawley rats (number not reported) were castrated at approximately 90 days of age and euthanized approximately 24 hours later. The ventral prostate tissues were collected and stored at approximately −80°C until use, and were processed as a batch and used for multiple studies.

The cytosol was prepared by adding low-salt TEDG buffer [0.01 M Tris, 1 mM sodium molybdate, 1.5 mM EDTA, 10% glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF) with dithiothreitol (DTT)] at pH 7.4 to the ventral prostate tissues at 10 mL/g of tissue. The tissues were minced, homogenized on ice, and centrifuged for 30 min at 30,000 × *g* at 4°C. The supernatant was collected, pooled from all tissues, aliquoted (amounts not reported) and stored at −80°C until used. Protein concentration of the cytosol prepared for this study was determined to be 6.566 mg/mL using the Pierce BCA method (Thermo Scientific Pierce Research Lab, Rockford, IL).

2. Saturation Radioligand Binding Experiment: The summary of conditions for the saturation binding experiment is provided in Table 1 below.

TABLE 1. Summary of Conditions for Saturation Binding Experiment [a]		
Source of receptor		Rat prostate cytosol
Concentration of radioligand (as serial dilutions)		0.25-10 nM
Concentration of non-labeled ligand (100X [radioligand])		25-1000 nM
Optimization of receptor concentration		Sufficient to bind 8.6-9.0%[b] of radioligand at 0.25 nM
Temperature		~2-8 ºC
Incubation time		~16 hours
Composition of assay buffer (TEDG)	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride	1.0 mM
	DTT	1.0 mM

a Data were not included in the study report, but are reported as a separate validation report.
b As indicated in the guideline for acceptable assay performance the receptor concentration bound less than 25 to 35% of the radiolabeled R1881.

On the day of the assay, the specific activity of the stock solution [^{3}H]-R1881 was not adjusted for decay over time, and serial dilutions in TEDG buffer were prepared to achieve the final concentrations in cytosol of 0.25, 0.5, 0.7, 1.0, 1.5, 2.5, 5.0, and 10.0 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 1.97 mg protein/assay tube, which resulted in the binding of 8.6-9.0% of the total radioactivity added. As indicated in the guideline for acceptable assay performance the receptor concentration bound less than 25 to 35% of the radiolabeled R1881. 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 run contained three concurrent 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
1.00	1.00	100	10	30
1.50	1.50	150	10	45
2.50	2.50	250	100	7.5
5.00	5.00	500	100	15
10.00	10.00	1000	100	30

a Data were not included in the study report, but are reported as a separate validation report.
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 [3H]-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 [3H]-R1881. R1881 was added in a 100-fold molar excess of [3H]-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 [3H]-R1881 or 7.5, 15, 21, or 30 µL of 100 nM [3H]-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 for approximately 16 hours at 2-8°C. Samples were maintained at temperatures of ~4°C except during whole rack vortexing. To separate bound from free R1881, hydroxyapatite (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 aspirated and discarded. The samples were washed 3 times in 50 mM TRIS buffer. Following the last wash and decanting of the Tris buffer, pellets were then extracted by addition of 2 ml ethanol. The samples were vortexed 3 times at 5 minute intervals. Samples were maintained on ice at all times between vortexing. Each ethanol supernatant was then decanted into a scintillation vial, and the radiation was quantified by liquid scintillation counting. A total of 4 runs were performed on 2 batches of cytosol with similar results. For the batch of cytosol used for the competitive assay, 2 runs were performed, which had highly similar binding profiles. Final determination of acceptable AR binding assay performance was primarily based on guideline suggested standards for the competitive binding assay, although the saturation binding parameters were evaluated.

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 4.3-5.2% of 1.0 nM radioligand [b]
Concentration of test substance (as serial dilutions)		10^{-11} to 10^{-4} M
Incubation Temperature		4-8 ºC
Incubation time		Overnight (~16 hours)
Composition of assay buffer	Tris	0.01 M (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10% (v/v)
	Phenylmethylsulfonyl fluoride with DTT	1 mM
	Sodium molybdate	1 mM
	Protease inhibitor	60 µM

a Data were obtained from pages 15, 18, and 19 of the study report.
b Data were obtained from pages 37, 39 and 41 of the study report; protein concentrations (µg/tube) were not reported.

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 (specific activity of 85.1 Ci/mmol) to the AR in the presence of increasing concentrations of a test substance. The amount of cytosolic protein used in the assay contained enough receptor to bind 4.3-5.2% of the [^{3}H]-R1881.

Ethanol was used as the solvent vehicle, and the solubility of the test material in the vehicle and assay buffer was evaluated visually. No precipitation was noted.

Dilutions of the test substance, reference standard (R1881), weak positive control (dexamethasone), and solvent control (ethanol) were prepared to achieve the concentrations shown in Table 4. Each assay consisted of three independent runs on three different days. For each run, a set of duplicate blanks and triplicate tubes with 1 µM R1881 (non-specific binding, NSB) were run at the beginning and end of each run. Each run also included triplicate samples of each centration of the reference standard, the weak positive control, and 2,4-D, resulting in a total of 77 samples per run. In addition, duplicate blanks followed by six replicates [^{3}H]-R1881 only (for total binding calculations) were run the day before each analysis run (the day of preparation of sample tubes).

TABLE 4. Competitor Final Molar (M) Concentrations in Competitive Binding Assay [a, b]				
Solvent Control	**Reference standard**	**Weak positive control**	**Test Chemical**	None
Ethanol	R1881	Dexamethasone	2,4-D	
Tubes 3-5 and 72-74	Tubes 6-23 and 75-77 [c]	Tubes 24-47	Tubes 48-71	Tubes 1-2
	1×10^{-6}	1×10^{-3}	1×10^{-4}	
	1×10^{-7}	1×10^{-4}	1×10^{-5}	
	1×10^{-8}	1×10^{-5}	1×10^{-6}	
	1×10^{-9}	1×10^{-6}	1×10^{-7}	
	1×10^{-10}	1×10^{-7}	1×10^{-8}	
	1×10^{-11}	1×10^{-8}	1×10^{-9}	
	--	1×10^{-9}	1×10^{-10}	
	--	1×10^{-10}	1×10^{-11}	

a Data were obtained from pages 37-42 of the study report.
b Each concentration of each chemical was run in triplicate, plus duplicate blanks for a total of 77 tubes per run. Tubes 3-77 contained 50 µL of triamcinolone acetonide and 30 µL [^{3}H]-R1881. Samples were dried, and 300 µL of prostate cytosol were added. Tubes 3-77 also contained 10 µL of the solvent control, reference standard (non-radiolabeled R1881), weak positive control, or test substance, with the exception of Tubes 6-8 and 75-77 that contained 30 µL of non-radiolabeled R1881 (used to evaluate non-specific binding). Six tubes analyzed the day prior to each run analysis contained only 30 µL of [^{3}H]-R1881 to determine ligand activity.
c Tubes 6-8 and 75-77 were used to evaluate non-specific binding by adding 100x of cold (non-radiolabeled) R1881.

Sample tubes were stored overnight at 4-8°C in the dark to allow the reaction to reach equilibrium, bound R1881 was separated from free R1881 by washing with HAP buffer and extraction with ethanol, followed by scintillation counting of bound [^{3}H]-R1881.

4. <u>Data Analysis</u>: The top and bottom of the curve, Hill slope, inhibition concentration (IC_{50}), and standard deviations were assessed using GraphPad Prism v. 5, and the data were fitted to a "one site binding" non-linear regression model (GraphPad Prism v. 5).

5. Definitions

a. Classification of test material

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

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.

Table 5. Data Interpretation Criteria Specified in OCSPP 890.1150			
Criteria		Classification	Values
Data fit 4-parameter nonlinear regression model	Average curve across runs crosses 50%[a]	Binder	2
	Average lowest portion of curves across runs is between 50% and 75% activity[b]	Equivocal	1
	Average lowest portion of curves across runs is greater than 75% activity[b]	Non-Binder	0
Data do not fit the model	----		

a If the curve fell outside the range for the weak positive control (see test acceptability criteria), the run as classified as equivocal.
b If the test compound was not soluble above 10^{-6} M and the binding curve did not cross 50%, the chemical was judged to be untestable.

b. Descriptors for receptor binding

B_{max}: maximal binding capacity
K_d: dissociation constant
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

II. RESULTS

A. <u>SATURATION BINDING EXPERIMENT</u>: Saturation binding experiment parameters are presented in Table 6. The dissociation constant (K_d) for [^{3}H]-R1881 was 0.4641, and the estimated B_{max} (nM) was 0.06392 for the batch of prostate cytosol that was used for this study. The K_d was below the recommended range reported in the test guideline (0.685-1.57 nM). Confidence in these numbers is high according to the goodness of fit (R^2 = 0.9871-0.9937) and the small variation among runs.

TABLE 6. Saturation Binding Experiment of R-1881 with Androgen Receptor from Rat Prostate Cytosol[a]

Parameter	Run 1[b]	Run 2[b]	Run 3[b]	Mean Runs 1-2[c]
R^2 (unweighted)	0.9937	0.9871	ND	0.9871-0.9937
B_{max} (nM)	0.06194	0.06590	ND	0.06392
B_{max} (fmol/100µg protein)	3.146	3.343	ND	3.245
K_d (nM)	0.4359	0.4922	ND	0.4641

a Data were not included in the study report, but are reported as a separate validation report.
b Two saturation runs were performed for this batch of cytosol.
c The range of R^2 is reported and the mean is reported for the other parameters.
R^2 Goodness of fit for curve calculated for specific binding,
ND Not determined

Figure 1 illustrates the non-specific, specific, and total binding curves for [^{3}H]-R1881 to the androgen receptor. The specific binding reached a plateau and the non-specific binding was generally less than 20% of total binding at all concentrations (range 6.2%-19.8%) except the highest concentration in Run 1 (24.6%). All other values indicated acceptable performance of

the assay. Figure 2 is a Scatchard plot that illustrates the binding of [^{3}H]-R1881 to the androgen receptor. The data fit results in a linear plot.

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

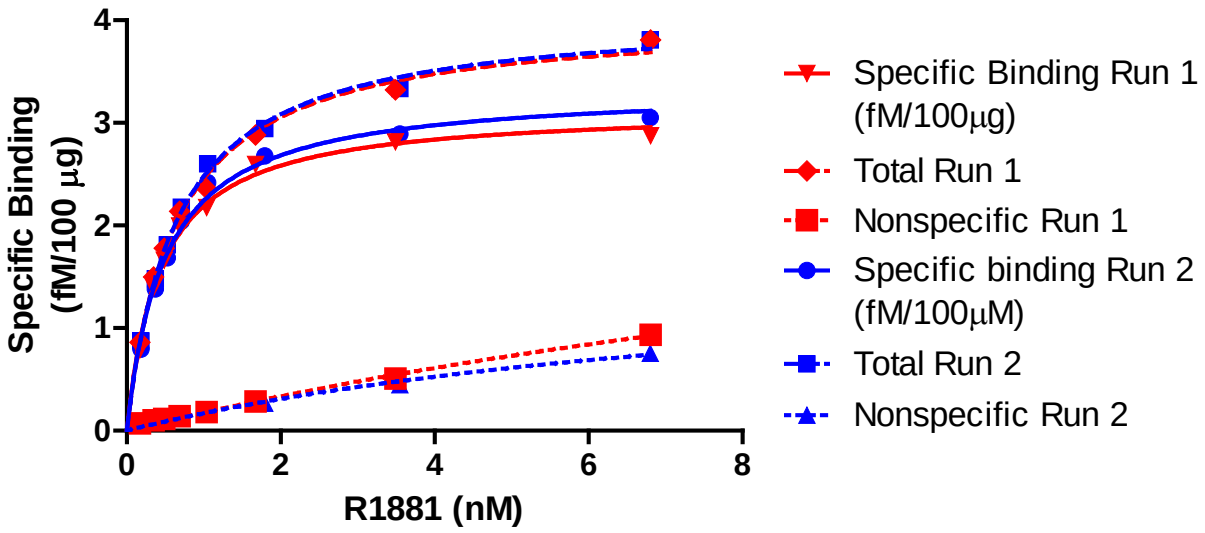
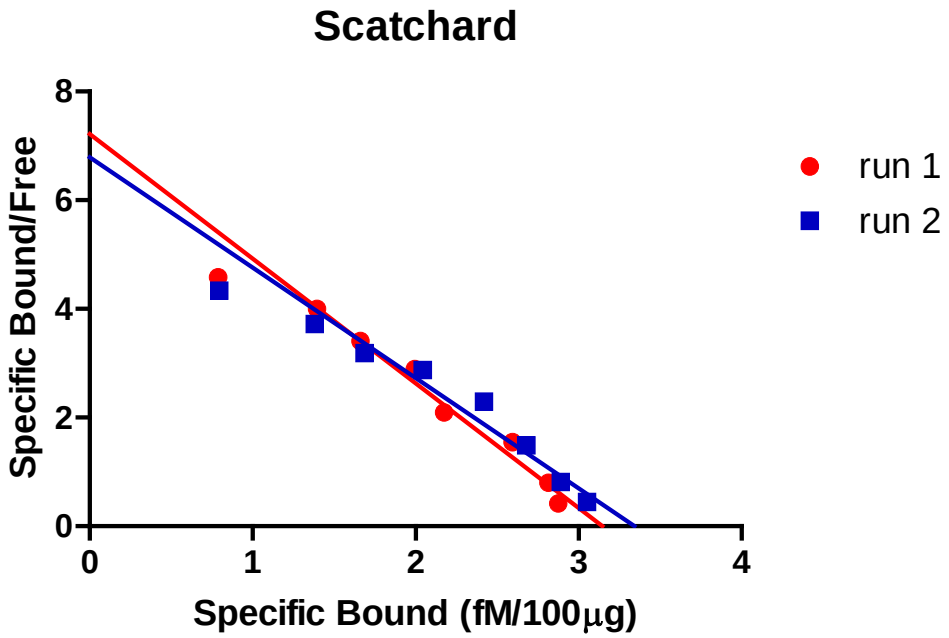


FIGURE 2. Scatchard Plot of the Binding of [3H]-R1881 to the Androgen Receptor.



B. COMPETITIVE BINDING EXPERIMENT: The results from the three competitive binding experiments are summarized in Tables 7 and 8 and shown graphically in Figures 3 and 4. The estimated mean log IC_{50} for 2,4-D could not be determined as it did not result in 50% displacement of the radioligand at any concentration. The estimated average log IC_{50}s for R1881 and the weak positive control (dexamethasone) were −9.0 and −4.4 M, respectively. The mean RBA for the positive control was 0.0027%. Confidence in these numbers is high due to the small variation. No precipitation of the test compound was visually observed at any concentration ($\leq 10^{-4}$ M). The solvent control responses indicated no drift in the study assay.

TABLE 7. Competitive Binding Experiment Results for Strong and Weak Positive with AR from Rat Prostate Cytosol [a,b]

	Assay #1	Assay #2	Assay #3	Mean ± SEM	Performance Criteria
R1881	Percent (%) of Total Binding				
10^{-11}	99.8	96.3	101.7	99.0±1.6	---
10^{-10}	92.1	87.3	93.5	90.9 ±1.9	---
10^{-9}	50.0	51.1	52.5	51.2 ±0.7	---
10^{-8}	10.0	9.5	9.7	9.7 ±0.2	---
10^{-7}	0.2	0.2	0.1	0.2 ±0.0	---
Bottom (%)	-0.6	-1.6	-1.1	-1.1	-2.0 to 2.0
Top (%)	101	97	103	101	82 to 114
Log IC_{50} (M)	-8.993	-8.986	-8.956	-8.978	---
IC_{50} (M)	1.017e-009	1.032e-009	1.106e-009	1.052e-009	
Hill slope	-1.0	-0.9	-1.0	-1.0	-1.2 to -0.8
R^2	0.9998	0.9999	1.000	1.000	---
RBA (%)	100	100	100	100	100
Dexamethasone	Percent (%) of Total Binding				
10^{-10}	98.2	97.9	104.2	100.1±2.0	---
10^{-9}	98.1	97.5	104.0	99.9 ±2.1	---
10^{-8}	97.9	99.0	102.1	99.7 ±1.3	---
10^{-7}	96.5	98.0	104.7	99.7±2.5	---
10^{-6}	94.7	96.3	99.3	96.8 ±1.3	---
10^{-5}	79.6	79.1	80.5	79.7 ±0.4	---
10^{-4}	29.7	25.3	27.7	27.6 ±1.3	---
10^{-3}	3.9	2.3	3.6	3.2 ±0.5	---
Bottom (%)	-0.5	-0.5	-0.6	-0.6	-12 to 12
Top (%)	98	98	104	100	87 to 106
Log IC_{50} (M)	-4.379	-4.440	-4.416	-4.412	---
IC_{50} (M)	4.177e-005	3.630e-005	3.840e-005	3.871e-005	
Hill slope	-1.0	-1.1	-1.0	-1.0	-1.4 to -0.6
R^2	0.9998	0.9999	0.9995	1.000	---
RBA (%)	0.0024	0.0028	0.0029	0.0027	---

a Data were obtained from page 30 of the study report.
b The mean and standard deviations are reported for the combined runs.
NA Not applicable.
R^2 Goodness of fit
IC_{50} Concentration of the test substance at which 50% of radioligand is displaced from the AR by the competitor
RBA (%) Relative binding affinity

TABLE 8. Competitive Binding Experiment Results for 2,4-D with AR from Rat Prostate Cytosol [a,b]

	Assay #1	Assay #2	Assay #3	Mean ± SEM
2,4-D	Percent (%) of Total Binding			
10^{-11}	98.79	98.24	99.89	99.0 ± 0.5
10^{-10}	102.44	99.72	99.55	100.6 ± 0.9
10^{-9}	103.53	97.07	99.95	100.2 ± 1.9
10^{-8}	103.22	99.22	104.25	102.2 ± 1.5
10^{-7}	101.49	99.04	103.98	101.5 ± 1.4
10^{-6}	103.17	98.32	104.51	102.0 ± 1.9
10^{-5}	102.33	97.65	105.58	101.9 ± 2.3
10^{-4}	105.64	99.8	106.32	103.9 ± 2.1
Bottom (%) (95% CI)	103.2 (101.1 to 105.4)	n/a	99.70 (97.9 to 101.5)	n/a
Top (%) (95% CI)	~-146.6 (very wide)	n/a	105.1 (103.8 to 106.4)	n/a
Log IC_{50} (M) (95% CI)	n/a	n/a	n/a	n/a
IC_{50} (M) (95% CI)	n/a	n/a	n/a	n/a
Log EC_{50} (M) (95% CI)	~-13.15 (very wide)	n/a	-8.363 (-9.431 to -7.294)	n/a
EC_{50} (M) (95% CI)	~7.139e-014 (very wide)	n/a	4.339e-009 (3.707e-010 to 5.078e-008)	n/a
Hill slope (95% CI)	~-0.8113 (very wide)	n/a	1.939 (-3.018 to 6.895)	n/a
R^2	0.6318	n/a	0.9371	n/a
Data Interpretation	negative	negative	negative	n/a
RBA (%)	n/a	n/a	n/a	n/a

a Data were obtained from page 31 of the study report.
b The mean and standard deviations are reported for the combined runs.
n/a Not applicable.
R^2 Goodness of fit
IC_{50} Concentration of the test substance at which 50% of radioligand is displaced from the AR by the competitor
RBA (%) Relative binding affinity

Collective Responses of the Independent AR Binding Assays

The collective responses of the three independent AR binding assays for 2,4-D indicates no apparent alterations in radiolabeled R1881 binding at any of the concentrations tested. The mean results of the three independent assays are shown in Figure 4 (R1881 and dexamethasone controls) and Figure 5 (2,4-D) of the study report. The final classification of 2,4-D was based on the average of the three valid assays with a mean value of "0" (*i.e.*, (0+0+0)/3) as described in Text Table 5 of the study report. Based on these data, 2,4-D was classified as non-binding at concentrations up to 10^{-4} M.

Data Interpretation

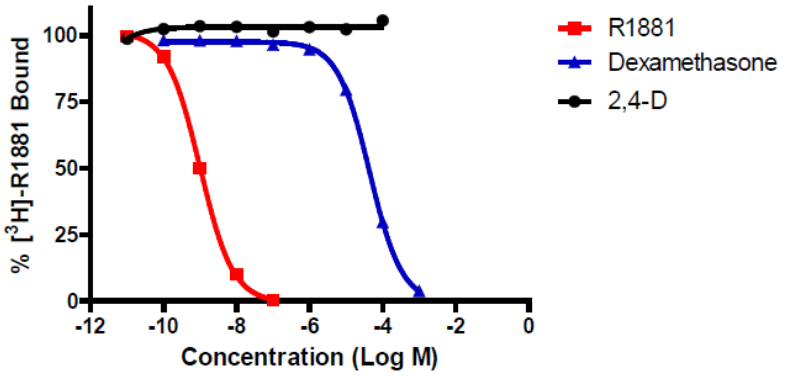
Test Validity

The QC criteria for the reference chemicals in the AR binding assay indicated that the assay performed according to the specified criteria. As with any biological system, there was slight

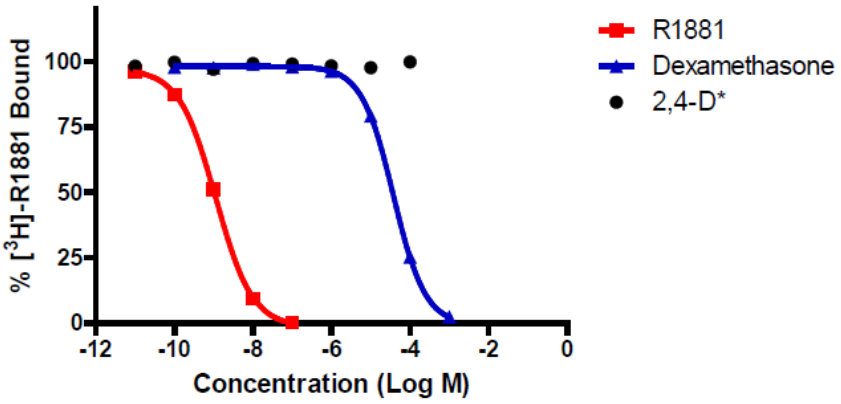
variability between assays, but the overall robustness of the responses for R1881 (strong positive control/standard curve) and dexamethasone (weak positive control) indicated that each assay included in this assessment performed as expected. Thus, assay #1, #2, and #3 of the AR binding assay with 2,4-D were considered valid.

FIGURE 3. Percentage R1881 Bound to the Androgen Receptor in the Presence of Radioinert R1881, Dexamethasone, and 2,4-D (Assays 1 – 3).

Assay #1



Assay #2



*Non-linear regression could not be calculated.

Assay #3

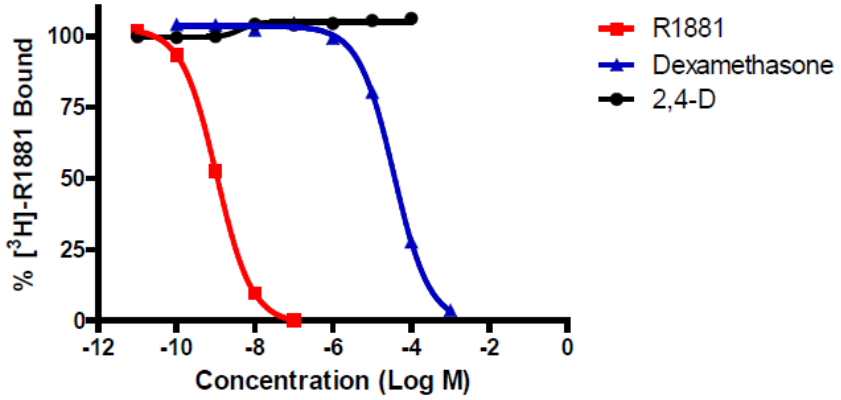
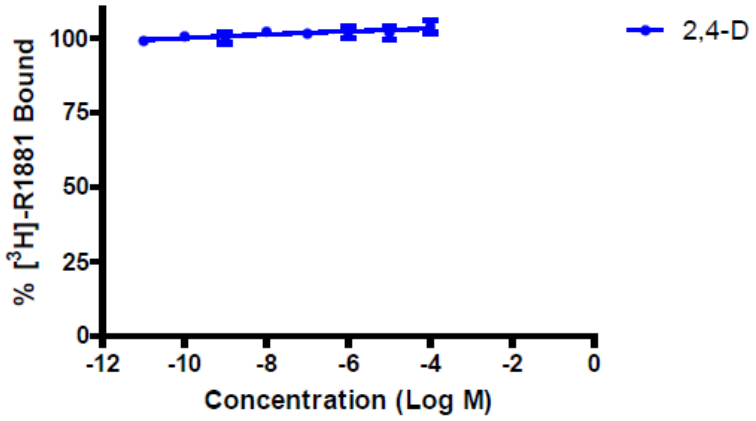


FIGURE 4. Mean of Percentage R1881 Bound to the Androgen Receptor in the Presence of 2,4-D from Three Assays.



C. <u>PERFORMANCE CRITERIA</u>: To ensure that the competitive binding assay was functioning properly, each run was evaluated using the criteria in Table 9. 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 Hill slope of −1.0 to −0.9. Examination across the runs indicated consistency of the Hill slope, placement along the X-axis, and top and bottom plateaus.

TABLE 9. 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%	4.3-5.2%	X	
2,4-D Top (% binding)	80 to 115	100 to 106	X	
R1881 fitted curve parameters				
Top (% binding)	82 to 114	97 to 103	X	
Bottom (% binding)	−2.0 to 2.0	−1.6 to −0.6	X	
Hill Slope	−1.2 to −0.8	−1.0 to −0.9	X	
Weak positive control (dexamethasone) fitted curve parameters				
Top (% binding)	87 to 106	98 to 104	X	
Bottom (% binding)	−12 to 12	−0.6 to −0.5	X	
Hill Slope	−1.4 to −0.6	−1.1 to −1.0	X	
Saturation Binding Experiment K_d (nM)	(0.685-1.57 nM)	0.4641		X
Non-specific binding (%)	≤10.0	7.30 to 8.23 [c]	X	

a Data were obtained from pages 30, 31 and 37-42 of the study report.
b These values represent ranges from the validation study.
c Values reported for the three NSB tubes at the beginning of each run; does not include the three NSB tubes at the end of the run.
NR Not reported

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATOR'S CONCLUSIONS: Based on the combined responses in each of three independent androgen receptor binding assays, it was determined that 2,4-D had no appreciable effect in the binding of the reference androgen ([3H]-R1881) at any concentration (up to 10^{-4} M). The results of the *in vitro* AR binding assay using rat prostate cytosol indicate that, under the conditions of this study, 2,4-D was negative for AR binding at concentrations up to 10^{-4} M.

B. AGENCY COMMENTS: The saturation binding experiment resulted in a maximum binding capacity (B_{max}) of 3.245 fmol/100 µg protein and the dissociation constant (K_d) was 0.4641 nM. Although these values were slightly below the range of values from the validation studies, the results were highly reproducible and all other performance criteria and the competitive binding assays indicated acceptable performance of the assay

The test guideline recommends testing up to 10^{-3} M; however, the sponsor selected 10^{-4} M as the highest concentration, based on *in vivo* toxicokinetic analyses in the rat. Specific binding was >75% at all concentrations tested (10^{-11} to 10^{-4} M). An IC_{50} and RBA could not be calculated for 2,4-D as it did not result in 50% displacement at any concentration.

The estimated average log IC_{50}s for R1881 and the weak positive control (dexamethasone) were −9.0 and −4.4 M, respectively. The mean RBA for the positive control was 0.0027%. Confidence in these numbers is high due to the small variation. No precipitation of the test compound was visually observed at any concentration ($\leq 10^{-4}$ M). The solvent control responses indicated no drift in the study assay, and all performance criteria were met in all three runs.

Based on the results of the three runs, 2,4-D is classified as a non-binder for the androgen receptor at concentrations up to 10^{-4} M.

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:

- Only two saturation binding runs were conducted rather than the three runs recommended in the test guideline.

DATA EVALUATION RECORD

2,4-DICHLOROPHENOXYACETIC ACID (2,4-D)

Study Type: OCSPP 890.1200, Aromatase Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-26-2012 (MRID 48614302)

Prepared for
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Quality Assurance:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	3/26/2012

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: Patience Browne, Ph.D. **Signature:**
Office of Science Coordination and Policy **Date:** 5/26/15
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:**
Health Effects Division, Office of Pesticide Programs **Date:** 6/19/15

Template version 08/2011

DATA EVALUATION RECORD

STUDY TYPE: Aromatase (Human Recombinant); OCSPP 890.1200

PC CODE: 030001 **DP BARCODE:** D398640

TXR#: 0052104 **CAS No.:** 94-75-7

TEST MATERIAL (PURITY): 2,4-D (98.5% a.i.)

SYNONYMS: 2,4-Dichlorophenoxyacetic acid

CITATION: Coady, K.K. and Sosinski, L.K. (2011) 2,4-Dichlorophenoxyacetic acid: Evaluation of 2,4-dichlorophenoxyacetic acid in the human recombinant aromatase assay. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, MI. Laboratory Project Study ID: 111036, September 27, 2011. MRID 48614302. Unpublished.

SPONSOR: Industry Task Force II on 2,4-D Research Data, c/o McKenna Long & Aldridge LLP, 1900 K Street NW, Washington, D.C.

TEST ORDER #: CON-030001-01

EXECUTIVE SUMMARY: In an *in vitro* aromatase (CYP 19) assay (MRID 48614302), 2,4-D (98.5% a.i., lot#: 2006 2433 8006-USA) in ethanol (1%) was incubated with human recombinant aromatase and tritiated androstenedione (1-β [^{3}H(N)]-androst-4-ene-3,17-dione; [^{3}H]ASDN) for 15 minutes at 37 °C to assess the potential of 2,4-D to inhibit aromatase activity. 2,4-D was tested at logarithmic concentrations from 10^{-10} M to 10^{-4} M in three independent runs. The test guideline recommends testing up to 10^{-3} M; however, the sponsor selected 10^{-4} M as the highest concentration based on *in vivo* toxicokinetic analyses in the rat. The sponsor considered concentrations higher than 10^{-4} M to be not relevant for testing in this assay as they are substantially above the inflection point for linear toxicokinetics (See Appendix B of the AR binding assay study report, MRID 48614301).

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 independent 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 test chemical series (10^{-10} to 10^{-4} M) with 3 repetitions per concentration.

Aromatase activity in the full activity controls ranged from 0.131 to 0.244 nmol·mg-protein^{-1}·min^{-1} for the three test runs, with a mean and standard deviation of 0.186±0.036 nmol·mg-protein^{-1}·min^{-1}. Activity in the background controls ranged from 7.31 to 11.51% of the full activity controls. The responses of full activity controls were outside of the 90 to 110% range in the 2nd and 3rd replicates in the Run 1 (86.6 and 113.8%, respectively).

Results for the positive control were generally within the recommended ranges for the top of the curve, bottom curve, Hill slope, log IC_{50}, and coefficient of variation for replicates of each concentration within runs, with the exception that the bottom of the curve in Runs 2 and 3 (-8.2 and -7.2, respectively) exceeded the acceptable range (-5 to +6). Also, the coefficients of variations (%CVs) for replicates of each concentration of 2,4-D within a run were generally within the 15% guideline, with the exception that the overall %CV for the highest two concentrations (-45.2 and 415.9%) exceeded the acceptable limit. For 4-OH ASDN, the estimated log IC_{50} averaged −7.23 M and the slope was −0.92.

For 2,4-D, aromatase activity averaged 0.195±0.045 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration (10^{-10} M) and 0.168±0.025 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration (10^{-4} M). The data for 2,4-D were modeled for Run 1, and the goodness of fit (R^2) value was 0.83; however, the 2,4-D data from Runs 2 and 3 could not be modeled. The average dose-response curve indicated that the aromatase activity of the test material at concentrations ranging from 10^{-10} M to 10^{-4} M was essentially equivalent to the activity observed in the full activity controls. At 10^{-4} M, aromatase activity was approximately 91%. Since the average lowest portion of the activity response curve was greater than 75% activity, 2,4-D is classified as a non-inhibitor of aromatase activity up to the highest concentration tested (10^{-4} M). High CVs were observed for 4-OH ASDN at the two highest concentrations and at a single concentration for 2,4-D. Individual values were occasionally outside of the performance criteria ranges (with the mean value within range).

Based on the data from the average response curve, 2,4-D is classified as a Non-inhibitor of aromatase activity in this assay.

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

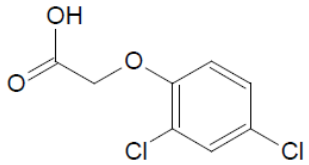
COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Substance: 2,4-D

Description: Off-white powder
Source: Nufarm Americas, Inc. (Burr Ridge, IL)
Lot/Batch #: 2006 2433 8006-USA
Purity: 98.5% a.i.
Volatility: Not reported
Storage conditions: Ambient
Stability: Not reported
Solvent: Ethanol
Solubility (in test solvent): Soluble up to 0.01 M
Highest Concentration Tested: 10^{-4} M in all runs
Stock Solution Preparation Methodology: Dissolved the test material in ethanol.
Molecular weight: 221.0
CAS #: 94-75-7
Structure:



2. Non-Labeled Substrate: Androstenedione (ASDN)

CAS # : 63-05-8
Source: Steraloids, Inc. (Cat. # A6030100)
Lot/Batch #: L1627
Purity: 98.4%

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

Source: Perkin Elmer Life and Analytical Sciences (Cat. # NET 926)
Lot/Batch #: 619344
Radiochemical Purity (Supplier): >97%
Specific activity: 26.3 Ci/mmol
Radiochemical Purity (In-lab determination): Not reported

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

CAS # : 566-48-3
Source: Sigma-Aldrich (Cat. # F2552)
Lot/Batch #: 081K2133
Purity: 99.6%

5. Solvent (Vehicle Control): Ethanol

Source: Sigma Aldrich (Cat. # E7023)
Lot/Batch #: 72596PMV
Justification for choice of solvent: Not provided. Ethanol is a Guideline preferred solvent.
Concentration (% of total volume in assays): 1% v/v

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

Source: Gentest (Woburn, MA; Cat. # 456260)
Lot/Batch #: 03897
Protein concentration: 7.4 mg/mL
Cytochrome C reductase activity: 290 nmol/min/mg
Aromatase activity: 6.0 pmol/min/pmol P450

B. METHODS

1. Assay Components and Preparations: A mixture of non-labeled and radiolabeled [^{3}H]-ASDN was prepared to result in a 2 µM ASDN solution with a predicted radioactive content of 1.0 µCi/mL.

Test chemical stock solutions were prepared such that the total volume of each test chemical formulation used per assay was no more than 1% v/v of the total assay volume. The report specified that ethanol was chosen because it was mentioned in the guideline as a preferred solvent.

A stock solution of the positive control substance, 4-OH ASDN, was formulated in ethanol. Fresh serial dilutions of the stock solution were prepared each time the aromatase inhibition assay was conducted. 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 Gentest, and aliquoted into individual vials based on protein content. Microsomes were stored at approximately −80º C until use.

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	6 mM
[^{3}H]ASDN	2 µM
Propylene Glycol	100 µL
Temperature	37°C
Incubation Time	15 min

2. Suitability Assessments: The protein concentration in an aliquot of the microsomes was determined each day of use, and microsomes were diluted with phosphate buffer such that approximately 0.004 mg/mL protein was present in the final reaction solution. Aromatase activity of the microsomes was provided by the vendor as 6.0 pmol/min/pmol P450. The minimum aromatase activity in the full activity control samples was determined to be 0.131 nmol/mg-protein/min, which was greater than the minimum acceptable aromatase activity of 0.10 nmol/mg-protein/min.

3. Aromatase Assay: Each assay run contained four tubes for the full enzyme activity and four 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 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 nmol·mg-protein^{-1}·min^{-1}.

4. Demonstration of Proficiency: It was stated that all assays were performed by personnel with demonstrated proficiency performing the assay as outlined in test guideline 890.1200. Proficiency records are archived in training records at the test facility and a copy of these data were archived with the 2,4-D specific report.

a. Positive Control

(1) Initial Demonstration of Laboratory Proficiency: Raw data were not provided, however, summary data of proficiency exercised were equivocal. Relative aromatase activity in the presence of prochloraz, a known aromatase inhibitor indicated dose-dependent inhibition. Aromatase activity appeared to be unaffected by ronidazole, a non-inhibitor at concentrations ranging from 10^{-10} to $10^{-2.5}$ M. Dose-dependent inhibition of aromatase was demonstrated in the presence of fenarimol and nitrofen, two recognized inhibitors of aromatase; however, the data were highly variable.

(2) Demonstration of Proficiency of New Technician for Conducting Assay (when applicable): Demonstration of proficiency by a new technician, if applicable, was not reported. The positive control data for slope, top and bottom percent, and log IC_{50} met the criteria as listed in section (i) of OCSPP 890.1200, with the exception of the bottom of the curve, which was below the recommended value for Run 2 (–8.2) and Run 3 (–7.2).

TABLE 2. Performance Criteria for the Positive Control

Parameter	Lower Limit Criteria	Upper Limit Criteria	Actual Lower Limit[a]	Actual Upper Limit[a]
Slope	−1.2	−0.8	–1.1	–0.78
Top (%)	90	110	91	100
Bottom (%)	−5	+6	–8.2	–2.6
Log IC_{50} (M)	−7.3	−7.0	–7.3	–7.2

a Data were obtained from page 32 of the study report.

b. Proficiency Chemicals: Standard curves were provided for the reference chemicals prochloraz, ronidazole, fenarimol, and nitrofen (Appendix A of the protocol on pages 84-86 of the study report).

TABLE 3. Proficiency Chemicals

Compound	CAS#	Class	Concentrations[a]
Prochloraz	Not provided	Inhibitor	10^{-10} to 10^{-3} M
Fenarimol	60168-88-9	Inhibitor	10^{-10} to 10^{-3} M
Nitrofen	1836-75-5	Inhibitor	10^{-10} to 10^{-3} M
Ronidazole	Not provided	Non-inhibitor	10^{-10} to 10^{-3} M

a Concentration ranges taken from figures.

5. **Determination of Aromatase Activity with Test Chemical(s):** The response of aromatase activity to the presence of eight concentrations of 2,4-D per run, in triplicate, was tested during three independent runs (Table 4). Solubility was visually assessed (presence of cloudiness or a precipitate). No precipitation was observed at any concentration in any run. The response of each full activity control within a run was between 91 to 108% of the average full activity, with the exception of two full activity control responses (87 and 114%) from the beginning and end, respectively, of Run 1 that were outside of the guideline recommended range of 90 to 110%.

TABLE 4. Test Chemical Study Design for each Test Run

Sample Type	Repetitions (Tubes)	Description	Reference or Chemical (M)
Full Activity Control	4	All test components[a] 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}
2,4-D Conc 1	3	All test components plus 2,4-D	1×10^{-4}
2,4-D Conc 2	3	All test components plus 2,4-D	$1\times10^{-4.5}$
2,4-D Conc 3	3	All test components plus 2,4-D	1×10^{-5}
2,4-D Conc 4	3	All test components plus 2,4-D	1×10^{-6}
2,4-D Conc 5	3	All test components plus 2,4-D	1×10^{-7}
2,4-D Conc 6	3	All test components plus 2,4-D	1×10^{-8}
2,4-D Conc 7	3	All test components plus 2,4-D	1×10^{-9}
2,4-D Conc 8	3	All test components plus 2,4-D	1×10^{-10}

a The complete assay contained buffer, propylene glycol, microsomal protein, [^{3}H]ASDN, and NADPH.

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:** Statistical analyses and graphical displays were conducted using Graph Pad Prism (Version 4.0, La Jolla, CA). Basic statistical analyses were performed on the data, which included means of replicates, standard deviation of the mean, relative standard deviation, and coefficient of variation. The Hill slope and log IC_{50} values across three independent runs were compared based on a one-way random effects analysis of variance, treating runs as random effects.

   The response curve was fitted by nonlinear regression analysis. Model fits were carried out using a 4-parameter regression model. For each run, percent of full activity control were plotted versus logarithm (base 10) of the test chemical concentration or 4-OH ASDN concentration. Each run was plotted with the data's best fit curve. Additionally, the average inhibition response curve across all runs was also plotted.

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 un-testable in the aromatase assay.

II. RESULTS

A. CONTROL ACTIVITY: Aromatase activity in the full activity controls ranged from 0.131 to 0.244 nmol·mg-protein^{-1}·min^{-1} for the three test runs, with a mean and standard deviation of 0.186±0.036 nmol·mg-protein^{-1}·min^{-1}. Activity in the background controls ranged from 7.31 to 11.51% of the full activity controls. The response of each full activity control was generally between 90 to 110% of the average full activity, with the exception of the 2nd and 3rd replicates in the Run 1 (86.6 and 113.8%, respectively). The response of the full activity controls and background controls were acceptable.

B. POSITIVE CONTROL: For the positive control substance (4-OH ASDN), aromatase activity averaged 0.180±0.027 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration (10^{-10} M) and -0.008±0.004 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 three runs is presented in Table 7, along with the overall standard deviation and %CV. Inhibition response curves for the positive control from each run and the average of all runs are shown in Figure 1. These results were generally within the recommended ranges for the top of the curve, bottom curve, Hill slope, log IC_{50}, and coefficient of variation for replicates of each concentration, with the following

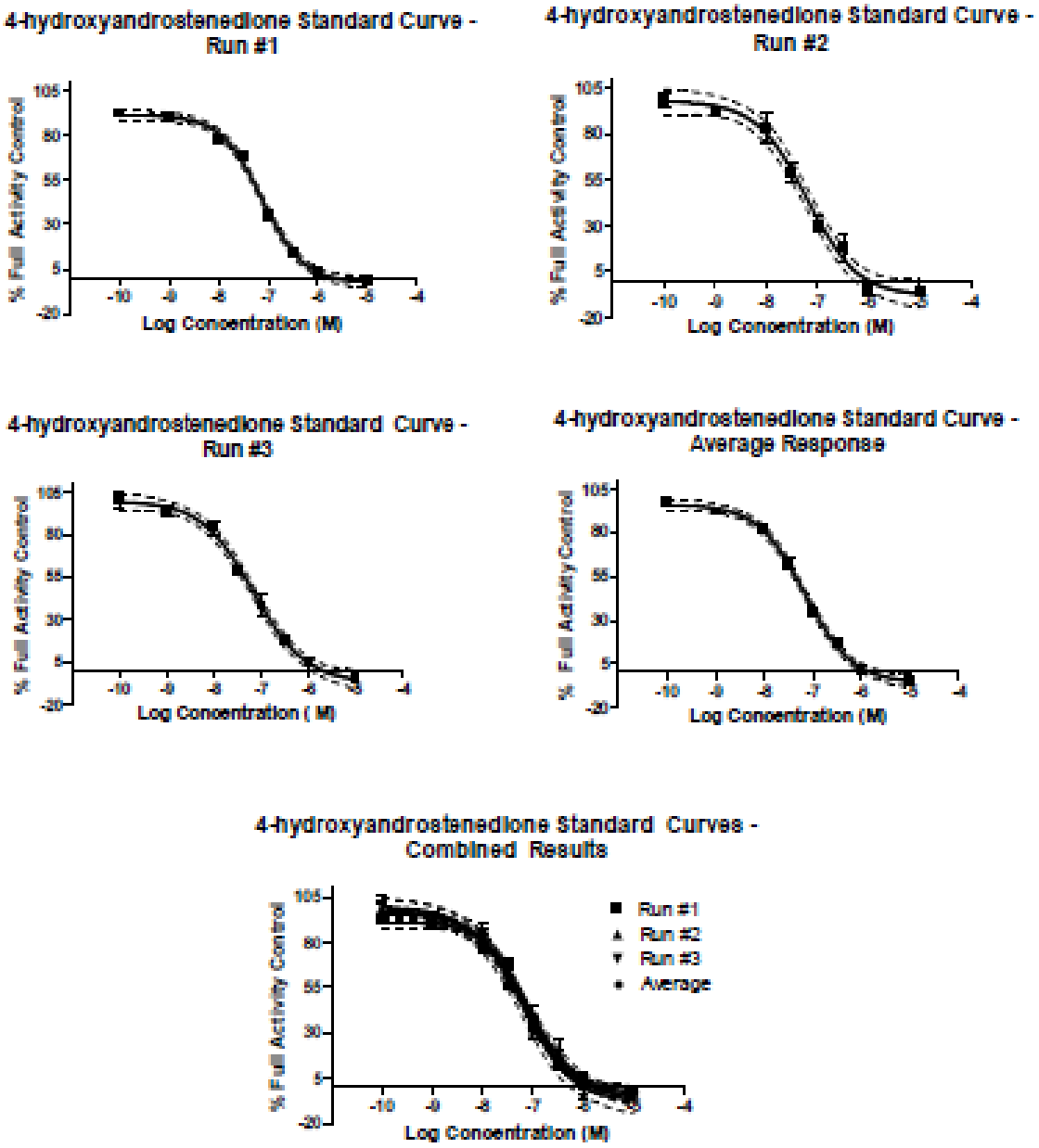
exceptions: the overall %CV for the highest two concentrations (-45.2 and 415.9%) exceeded the acceptable limit of 15%, and the bottom of the curve in Runs 2 and 3 (-8.2 and -7.2, respectively) exceeded the acceptable range (-5 to +6).

TABLE 7. Effect of 4-OH ASDN and 2,4-D on Aromatase Activity (as percent of control) from Independent Runs [a]

Chemical	Concen. (Log M)	# Runs	Overall Mean	Overall SD	Overall SEM	Overall %CV
4-OH ASDN (positive control)	−5	3	-4.49	2.03	1.17	-45.2
	−6	3	1.10	4.59	2.65	415.9
	−6.5	3	16.86	2.40	1.39	14.2
	−7	3	34.80	3.96	2.28	11.4
	−7.5	3	61.98	5.07	2.93	8.2
	−8	3	81.46	3.35	1.93	4.1
	−9	3	92.09	1.65	0.95	1.8
	−10	3	97.17	4.21	2.43	4.3
2,4-D	−4	1	91.15	7.05	4.07	7.7
	−4.5	3	92.20	7.34	4.24	8.0
	−5	3	88.05	5.65	3.26	6.4
	−6	3	91.77	4.17	2.41	4.5
	−7	3	98.02	13.05	7.54	13.3
	−8	3	105.55	9.09	5.25	8.6
	−9	3	100.33	22.05	12.73	22.0
	−10	3	104.10	11.19	6.46	10.7

a Values were calculated by the reviewers based on data provided on pages 66-68.
SD Standard Deviation
SEM Standard Error of the Mean
CV Coefficient of Variance

FIGURE 1. Inhibition Response Curves for 4-OH ASDN.



C. **TEST SUBSTANCE:** For 2,4-D, aromatase activity averaged 0.195±0.045 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration (10^{-10} M) and 0.168±0.025 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration (10^{-4}). The mean aromatase activity of 2,4-D (expressed as %full control activity) for each concentration tested across all three runs is presented in Table 7 (above), along with the overall standard deviation and %CV. Inhibition response curves for 2,4-D from each run are shown in Figure 2, and the average inhibition response curve across all runs is shown in Figure 3. The overall %CV for the 10^{-9} M concentration (22%) exceeded the acceptable limit of 15%.

FIGURE 2. Inhibition Response Curves for 2,4-D From Each Test Run.

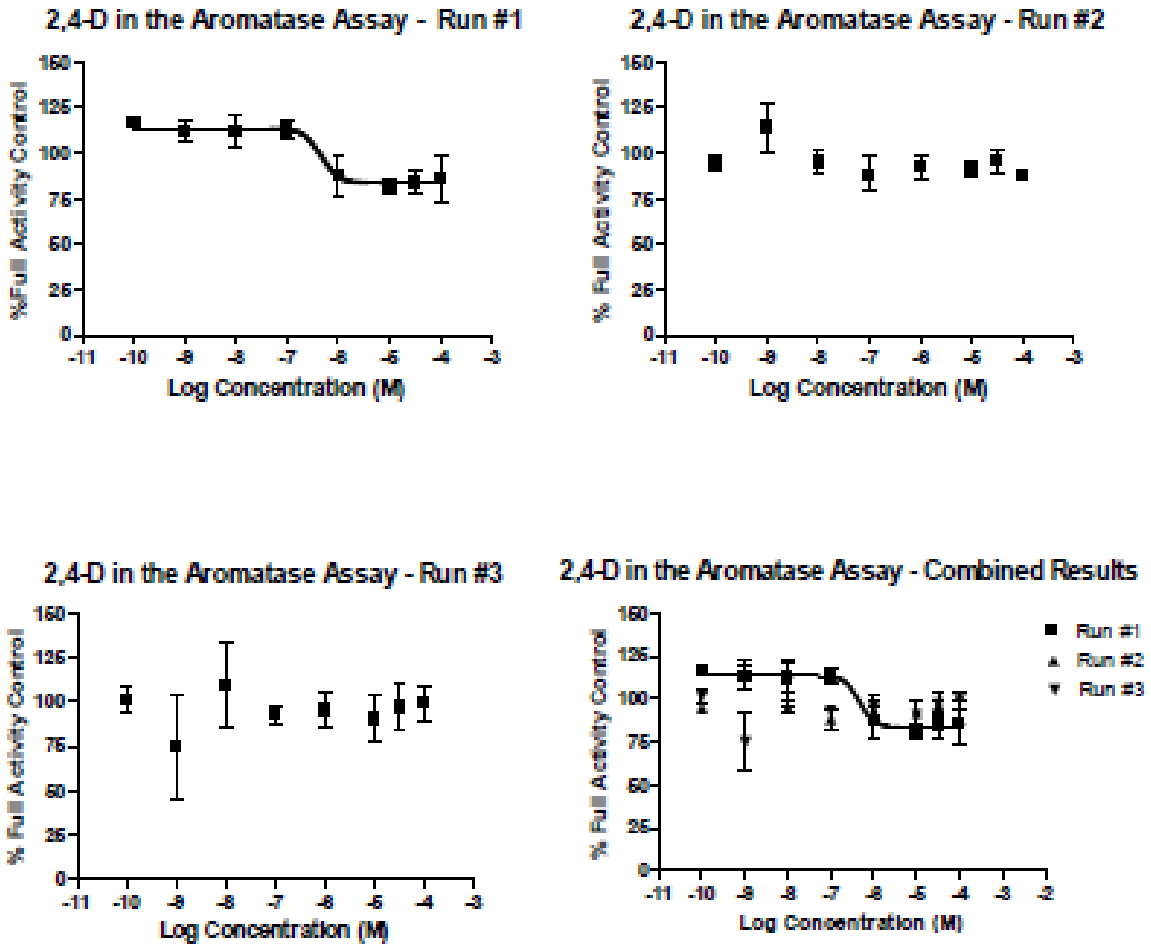
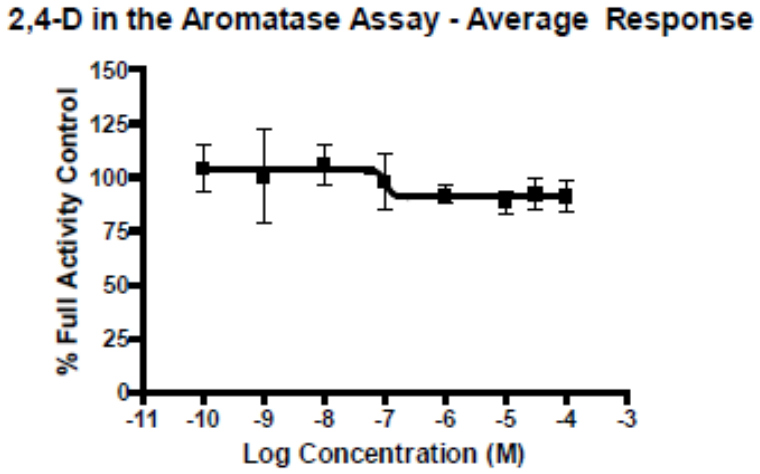


FIGURE 3. Mean Inhibition Response Curve for 2,4-D.



The data for 2,4-D were modeled for Run 1, and the goodness of fit (R^2) value was 0.83; however, data from Runs 2 and 3 were not amenable to modeling. The average dose-

response curve indicated that the aromatase activity of the test material at concentrations ranging from 10^{-10} M to 10^{-4} M was essentially equivalent to the activity observed in the full activity controls.

For positive control curves, the best fit Hill slope and log IC_{50} values across three independent runs were similar.

The effect of the positive control on inhibition of aromatase activity is presented in Table 8. For 4-OH ASDN, the estimated log IC_{50} averaged −7.23 M and the slope was −0.92. Confidence in the mean log IC_{50} for the positive control is high due to the small variation (<1% CV). Confidence in the mean slope is low due to the large variation (17.8% CV).

TABLE 8. Effect of 2,4-D on Aromatase Activity (as Percent of Control) From Independent Runs [a]

Chemical	Run 1	Run 2	Run 3	Mean	SD	%CV
Log IC_{50} (M)						
2,4-D	NA	NA	NA	NA	NA	NA
4-OH ASDN	−7.2	−7.3	−7.2	−7.23	0.06	-0.80
Slope						
2,4-D	NA	NA	NA	NA	NA	NA
4-OH ASDN	−1.1	−0.88	−0.78	−0.92	0.16	-17.79

a Data were provided on page 32 of the study report. Mean, SD and %CV were calculated by the reviewers based on these data.
SD Standard Deviation
CV Coefficient of Variance
NA Not applicable. Values for 2,4-D were not suitable for modeling.

Based on the data from the average response curve and the criteria listed above in Table 6, the results support the conclusion that 2,4-D is a non-inhibitor of aromatase activity.

III. DISCUSSION AND CONCLUSIONS

A. <u>INVESTIGATORS CONCLUSIONS</u>: The average response from the three independent runs with 2,4-D did not fit the four parameter regression model. Additionally, average aromatase activity for 2,4-D was similar to full activity controls at all concentrations tested. Therefore, 2,4-D is classified as a non-inhibitor of aromatase activity.

B. <u>AGENCY COMMENTS</u>: Aromatase activity in the full activity controls ranged from 0.131 to 0.244 nmol·mg-protein^{-1}·min^{-1} for the three test runs, with a mean and standard deviation of 0.186±0.036 nmol·mg-protein^{-1}·min^{-1}. Activity in the background controls ranged from 7.31 to 11.51% of the full activity controls. The responses of the full activity controls were outside of the 90 to 110% range in the 2nd and 3rd replicates in the Run 1 (86.6 and 113.8%, respectively).

For the positive control substance (4-OH ASDN), aromatase activity averaged 0.180±0.027 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration (10^{-10} M) and -0.008±0.004 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration (10^{-5} M). These results were within the recommended ranges for the top of the curve, bottom curve, Hill slope, log IC_{50}, and coefficient of variation for replicates of each concentration, with the following

exceptions: the overall %CV for the highest two concentrations (-45.2 and 415.9%) exceeded the acceptable limit of 15%, and the bottom of the curve in Runs 2 and 3 (-8.2 and -7.2, respectively) exceeded the acceptable range (-5 to +6).

For 2,4-D, aromatase activity averaged 0.195±0.045 nmol·mg-protein^{-1}·min^{-1} at the lowest tested concentration (10^{-10} M) and 0.168±0.025 nmol·mg-protein^{-1}·min^{-1} at the highest tested concentration (10^{-4}). The overall %CV for the 10^{-9} M concentration (22%) exceeded the acceptable limit of 15%. The data for 2,4-D were modeled for Run 1, and the goodness of fit (R^2) value was 0.83. The average dose-response curve indicated that the aromatase activity of the test material at concentrations ranging from 10^{-10} M to 10^{-4} M was essentially equivalent to the activity observed in the full activity controls.

For 4-OH ASDN, the estimated log IC_{50} averaged −7.23 M and the slope was −0.92. Confidence in the mean log IC_{50} for the positive control is high due to the small variation (<1% CV), but confidence in the mean Hill slope is low due to the large variation (17.8% CV).

At 10^{-4} M aromatase activity was 91% compared to the full activity controls. Since the average lowest portion of the activity response curve was greater than 75% activity, 2,4-D is classified as a non-inhibitor of aromatase activity up to the highest concentration tested (10^{-4} M).

C. STUDY DEFICIENCIES: None

DATA EVALUATION RECORD

2,4-DICHLOROPHENOXY ACETIC ACID (2,4-D)

Study Type: OCSPP 890.1250, Estrogen Receptor Binding Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-26-2012 (MRID 48614303)

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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: Patience Browne, Ph.D. **Signature:**
Office of Science Coordination and Policy **Date:** 5/26/15
Secondary Reviewer: Greg Akerman, Ph.D. **Signature:**
Health Effects Division **Date:** 6/19/15

Template version 08/2011

DATA EVALUATION RECORD

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

PC CODE: 030001 **DP BARCODE:** D398640

TXR#: 0052104 **CAS No.:** 94-75-7

TEST MATERIAL (PURITY): 2,4-D (98.5% a.i.)

SYNONYMS: 2,4-dichlorophenoxy acetic acid

CITATION: LeBaron, M.J., Shisler, M.R., Visconti, N.R. (2011). Evaluation of 2,4-Dichlorophenoxy Acetic Acid (2,4-D) in an *in vitro* Estrogen Receptor Binding Assay. Toxicology & Environmental Research and Consulting, Dow Chemical Company, Midland, MI. Laboratory Study ID.: 111121, October 27, 2011. MRID 48614303. Unpublished.

SPONSOR: Industry Task Force II on 2,4-D Research Data, c/o McKenna Long & Aldridge LLP, 1900 K Street NW, Washington, DC.

TEST ORDER #: CON-030001-1

EXECUTIVE SUMMARY: In an estrogen receptor (ER) binding assay (MRID 48614303) for 2,4-D (98.5%, Lot# 2006 2433 8006-USA) , uterine cytosol from Sprague Dawley rats was used as the source of ER to conduct saturation binding and competitive binding experiments in this assay. The competitive binding experiment was conducted to measure the binding of a single concentration of [^{3}H]-17β-estradiol (1 nM) in the presence of logarithmic increasing concentrations of 2,4-D from 10^{-11} to 10^{-4} M, rather than 10^{-10} to 10^{-3} recommended in the test guideline. The justification for lowering the top test concentration was based on toxicokinetic data in the rat; concentrations higher than 10^{-4} M were not relevant for testing in this assay as they are substantially above the inflection point for linear toxicokinetics. Ethanol was used as a solvent at a final concentration of <3%. The assay included 19-norethindrone as a weak positive control, octyltriethoxysilane as a negative control, and 17β-estradiol as the natural ligand reference material, and three independent runs were performed on separate days.

Summary data pertaining to the saturation binding experiment were reported separately in the study profile submitted by the test order recipient. The K_d for [^{3}H]-17β-estradiol was 0.1032 nM and the B_{max} (nM) was 0.07097 for the prepared rat uterine cytosol used in these experiments. The K_d for the run was within the expected range of 0.03 to 1.5 nM, and the B_{max} was within the expected range of 10-150 fmol/100 μg protein. The data produced a linear Scatchard plot.

In the competitive binding experiment, no precipitation was observed at any concentration tested. The mean specific binding in the presence of 2,4-D was >95% at 2,4-D concentrations of $\leq 10^{-4}$ M in all three runs. The estimated mean log IC_{50} and RBA was not calculated for 2,4-D as the percent binding inhibition did not reach 50% for any run.

The estimated mean log IC_{50} for the natural ligand, 17β-estradiol, and the weak positive control (19-norethindrone) was −9.0 and −5.5 M, respectively. The mean RBA was 0.034% for 19-norethindrone. All performance criteria were met for 17β-estradiol, 19-norethindrone and octyltriethoxysilane.

2,4-D was tested over a concentration range that fully defined the top of the curve. The mean specific radioligand binding in the presence of 2,4-D was >95% at 2,4-D concentrations of $\leq 10^{-4}$ M. Based on the results from the three runs, 2,4-D 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, Data Confidentiality, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

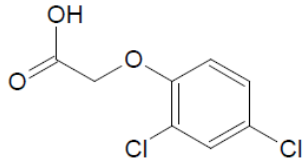
A. MATERIALS

1. Test Facility: Toxicology & Environmental Research and Consulting; Dow Chemical

Location: Midland, MI
Study Director: M.R. Schisler
Other Personnel: M.J. LeBaron (Lead Scientist), N.R. Visconti (Research Biologist), B.B. Gollapudi, (Technical Reviewer)
Study Period: September 14, 2011 - October 27, 2011

2. Test substance: 2,4-D

Description: Technical, Off-white Powder
Source: NuFarm Americas, Inc.
Lot/Batch #: 2006 2433 8006-USA
Purity: 98.5%
Solubility: 315 mg/L in water, 30 mM in ethanol
Volatility: 1.9 x 10^{-5} Pa at 25°C
Stability: 2 year shelf life
Storage conditions: Ambient
CAS #: 94-75-7
Molecular weight: 221.0
Structure:



3. Non-labeled ligand: 17β-estradiol

Supplier: Sigma, St. Louis MO
Catalog #: E8875
Batch #: 098K1372
Purity: 100%
CAS #: 50-28-2

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

Supplier: Perkin-Elmer, Boston MA
Catalog #: NET517001MC
Batch#: 639068
Radiochemical purity: >97%
Specific activity: 162.9 Ci/mmol
Concentration of stock: 1.0 mCI/mL

5. Positive control: 19-norethindrone

Supplier: Sigma, St Louis, MO
Catalog #: N4128
Batch #: 030M1359
Purity: 99%
CAS #: 68-22-4

6. Negative control: Octyltriethoxysilane

Supplier: Sigma, St Louis, MO
Catalog #: 440213
Batch #: 72596AMV
Purity: 98.58%
CAS #: 2943-75-1

7. Solvent/vehicle control: Ethanol

Justification for choice of solvent: None reported; 2,4-D is more soluble in water than ethanol
Final Concentration: <3%

B. METHODS

1. **Preparation of Rat Uterine Cytosol (RUC):** Trimmed uterine tissue from 85-100 day old female (Crl:CD(SD)) rats that were ovariectomized approximately 7-10 days prior to tissue harvest was purchased from Charles River (Wilmington, MA). Tissues were stored at −10° C until use (up to 6 months). The uteri were weighed, placed in ice-cold TEDG (Tris, EDTA, DTT, glycerol) + PMSF (phenylmethylsulfonyl fluoride) buffer and homogenized, followed by centrifugation for 10 min at 2500 × *g* at 4º C. 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 3.573 mg/mL with the Pierce BCA method (Thermo Scientific Research Lab, Rockford, IL) using a protein kit compatible with DTT in the TEDG buffer. Cytosol was divided into aliquots (volume not reported) for immediate use or storage at −80º C for up to 90 days. The cytosol preparation was identified as Batch 2, prepared on 8/10/11.

2. **Saturation (radioligand) Binding Experiment**: A saturation binding experiment was conducted to demonstrate that the ER was present in adequate concentrations and had the appropriate affinity for the native ligand. A summary of the conditions for the saturation binding experiment are provided 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)		0.03-3.0 nM
Concentration of non-labeled ligand (100X [radioligand])		3.0-300 nM
Concentration of receptor		Sufficient to bind 40.77% of radioligand at 0.03 nM[b]
Temperature		~2-8 ºC
Incubation time		~16 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM

a Data were not included in the study report, but were reported in the study profile submitted separately.
b This value was slightly higher than the suggested range in the guideline; however, all other values, including minimal ligand depletion, indicated acceptable performance in the assay.

The specific activity of the stock [^{3}H]-17β-estradiol was not adjusted for decay over time on the day of the assay. Serial dilutions of radiolabeled estradiol in TEDG + PMSF buffer were prepared to achieve a final concentration 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. The optimal protein concentration was determined to be 0.1191 mg protein/assay tube, which resulted in the binding of 40.77% of the total radioactivity added. This value was slightly higher than the suggested range in the guideline. 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 run contained three concurrent replicates 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 conc.)
500 µL	500 µL	50 µL	Total volume in each assay tube

a Data were not included in the study report, but were reported in the study profile submitted separately.
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 for ~16 hours at ~4°C. To separate bound from free estradiol, hydroxyapatite (HAP) slurry was added to each tube and vortexed (4 times with 5-minute intervals). Subsequently, the contents of each tube were washed three times as follows: TEDG +PMSF buffer was added, vortexed, centrifuged for 10 min at 1000 x g, and the supernatant decanted and discarded. After washing, ethanol was added to the HAP pellet remaining in each tube to extract the [3H]-17β-estradiol, followed by vortexing, and centrifugation for 10 min at 1000 x g. An aliquot of supernatant was radioassayed by scintillation counting. The temperature was maintained at approximately 4ºC throughout the assay prior to extraction with ethanol. A total of two saturation binding runs on two batches of cytosol were performed with similar results. For the batch of cytosol used for the competitive binding assay, a single saturation run was performed.

3. **Competitive Binding Experiment:** A summary of the experimental conditions for the competitive binding experiment is presented 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 6.75-7.15% of radioligand
Concentration of test substance (as serial dilutions)		10^{-11} to 10^{-4} mM
Temperature		4-8 °C
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH not reported)
	EDTA	1.5 mM
	Glycerol	10% (v/v)
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM

a Data were obtained from pages 15, 21, 40, 42 and 44 of the study report.

The solubility of 2,4-D in ethanol was evaluated visually. On the day of the assay, the specific activity of the stock solution [^{3}H]-17β-estradiol (not adjusted for decay over time) was diluted in TEDG + PMSF buffer to achieve a final concentration of 1 nM. For each batch of cytosol, the optimal protein concentration was determined by testing serial amounts of protein per tube, using 1.0 nM radiolabeled estradiol, until a concentration was reached that bound 6.75-7.15% of the total radioactivity added. 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 runs performed on separate days. Each run included three replicates of each test substance at each concentration, plus four blank and six samples of the master mix to determine full radioactivity, resulting in a total of 112 samples.

TABLE 4. Molar (M) concentrations in Competitive Binding Assay Run [a, b]			
2,4-D	Positive control	Negative control	Reference Chemical
	19-norethindrone	Octyltriethoxysilane	Non-labeled 17β-estradiol
Tubes 81-104 [c]	Tubes 33-56 [c]	Tubes 57-80 [c]	Tubes 11-32 and 105-112 [c]
10^{-10}	$10^{-8.5}$	10^{-10}	Solvent control [d]
10^{-9}	$10^{-7.5}$	10^{-9}	10^{-11}
10^{-8}	10^{-7}	10^{-8}	10^{-10}
10^{-7}	$10^{-6.5}$	10^{-7}	$10^{-9.5}$
10^{-6}	10^{-6}	10^{-6}	10^{-9}
10^{-5}	$10^{-5.5}$	10^{-5}	$10^{-8.5}$
10^{-4}	$10^{-4.5}$	10^{-4}	10^{-8}
	10^{-4}	10^{-3}	10^{-7}

a Data were obtained from pages 40-41 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 + PMSF 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 96 tubes per run.
d Solvent is ethanol

Tubes were incubated with gentle vortexing for 16-20 hours at 4±2 °C. To separate bound from free estradiol, hydroxyapatite (HAP) slurry was added to each tube and the tubes were vortexed (4 times with 5-minute intervals). Subsequently, the contents of each tube were washed three times as follows: TEDG+PMSF buffer was added, vortexed, centrifuged for 10 min at 1000 × *g*, and the supernatant decanted and discarded. Ethanol was then added to the HAP pellet remaining in each tube to extract the [^{3}H]-17β-estradiol, allowed to sit at room temperature for 15-20 min with vortexing (4 times with 5-minute intervals), and centrifugation for 10 min at 1000 × *g*. A portion of supernatant was radioassayed by scintillation counting. The temperature was maintained at 4±2 °C throughout the assay prior to extraction with ethanol.

C. <u>DATA ANALYSIS</u>: For the competitive binding experiment, total binding and non-specific binding data were modeled with a non-linear regression program [Graph Pad Prism v. 5.0 (GraphPad Software, Inc., La Jolla, CA)]. Nonlinear regression methods were used to fit a curve for 17β-estradiol, 19-norethindrone, octyltriethoxysilane, and 2,4-D data to the Hill equation with log IC_{50} as a parameter to be estimated. Estimates of model parameters [e.g., log IC_{50}, IC_{50}, Hillslope, R^2, and relative binding affinity (RBA)] were determined with Graph Pad Prism.

1. <u>Definitions</u>

a. <u>Classification of test material</u>: 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 = 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. <u>Descriptors for receptor binding</u>:

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

II. RESULTS

A. <u>SATURATION BINDING EXPERIMENT</u>: Summary data pertaining to the saturation binding experiment were reported separately in the study profile submitted by the test order recipient. Individual data on saturation binding were not reported. Saturation binding experiment parameters are presented in Table 5. The K_d for [^{3}H]-17β-estradiol was 0.1032 nM and the B_{max} (nM) was 0.07097 for the prepared rat uterine cytosol used in these experiments. The K_d for the run was within the expected range of 0.03 to 1.5 nM. The B_{max} was also within the expected range of 10-150 fmol/100 µg protein. Non-specific, specific

and total binding curves for [^{3}H]-estradiol to the ER are shown in Figure 1. The data produced a linear Scatchard plot (Figure 2).

TABLE 5. Saturation Binding Experiment of 17β-estradiol with Estrogen Receptor from Rat Uterine Cytosol

Parameter	Run 1	Run 2	Run 3	Runs 1-3
R^2 (unweighted)	0.967	NR	NR	NR
B_{max} (nM)	0.07097	NR	NR	NR
B_{max} (fmol/100 µg protein)	59.28	NR	NR	NR
K_d (nM)	0.1032	NR	NR	NR

a Data were not included in the study report, but were reported in the study profile submitted separately.
b Only a single run of the saturation binding experiment was conducted on the batch of cytosol used for this competitive binding experiment.
R^2 Goodness of fit for curve calculated for specific binding

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

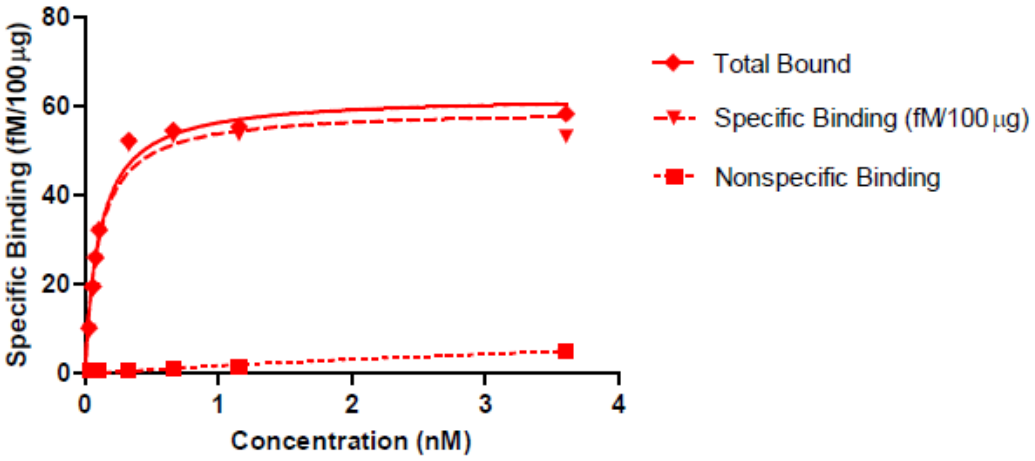
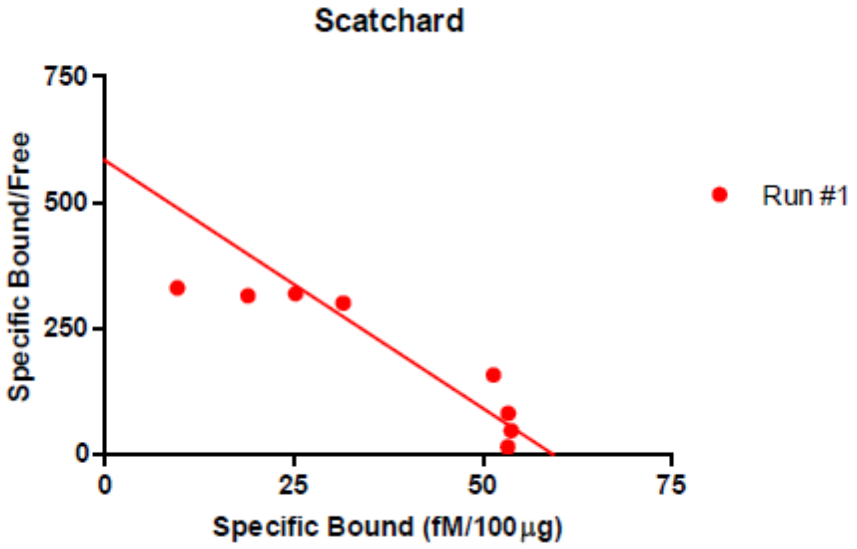


FIGURE 2. Scatchard Plot of the Binding of [^{3}H]-17β-Estradiol to the ER.



B. <u>COMPETITIVE BINDING EXPERIMENT</u>: The results from the three competitive binding experiments are summarized in Table 6 and presented graphically in Figures 3-5. No precipitation was observed at any concentration tested. The mean specific binding in the presence of 2,4-D was >95% at concentrations $\leq 10^{-4}$ M in all three runs. The estimated mean log IC_{50} and RBA was not calculated for 2,4-D as the percent binding inhibition did not reach 50% for any run.

The estimated mean log IC_{50} for the natural ligand, 17β-estradiol, and the weak positive control (19-norethindrone) was −9.0 and −5.5 M, respectively. The mean RBA was 0.034% for 19-norethindrone. As the lowest average percent binding in the presence of 2,4-D was ≥95% at concentrations up to 10^{-4} M, 2,4-D is classified as not interactive (0) in this assay (Table 7).

TABLE 6. Competitive Binding Assay of 2,4-D with Estrogen Receptor from Rat Uterine Cytosol [a]

Parameter		Run 1	Run 2	Run 3	Mean ± SE[b]
r^2 (unweighted),	17β-estradiol	0.9990	0.9997	0.9995	0.9990-0.9997
	19-norethindrone	0.9999	0.9997	0.9962	0.9962-0.9999
	2,4-D	0.5875	NC	0.7841	0.5875-0.7841
Log IC_{50} (M),	17β-estradiol	−8.948	−8.942	−8.961	−8.951 ± 0.006
	19-norethindrone	−5.462	−5.435	−5.534	−5.477 ± 0.030
	2,4-D	NC	NC	NC	NC
IC_{50} (M),	17β-estradiol	1.13×10^{-9}	1.14×10^{-9}	1.10×10^{-9}	$1.12\times10^{-9} \pm 0.01\times10^{-9}$
	19-norethindrone	3.45×10^{-6}	3.76×10^{-6}	2.92×10^{-6}	$3.33\times10^{-6} \pm 0.25\times10^{-6}$
	2,4-D	NC	NC	NC	NC
Log RBA (%),	19-norethindrone	−3.5	−3.5	−3.5	−3.5 ± 0.0
	2,4-D	NC	NC	NC	NC
RBA (%),	19-norethindrone	0.033	0.031	0.032	0.032 ± 0.001
	2,4-D	NC	NC	NC	NC

a Data were obtained from page 33 of the study report.
b The range is reported for r^2; the mean ± SE is reported for all other parameters.
r^2 Goodness of fit
RBA (%) Relative binding affinity
NA Not applicable. r^2 is more appropriately expressed as a range, as opposed to a mean.

TABLE 7. Binding Classification of 2,4-D 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 28-30 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 E2 Bound to the Estrogen Receptor in the Presence of Test Compound (Run 1).

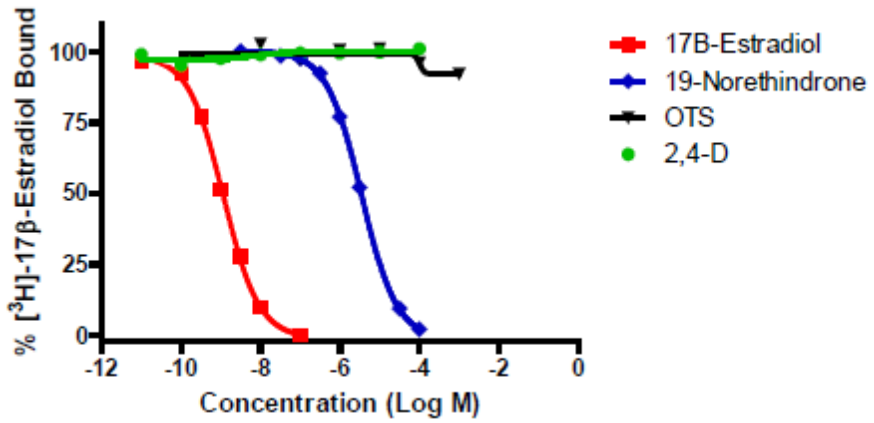


FIGURE 4. Percentage E2 Bound to the Estrogen Receptor in the Presence of Test Compound (Run 2).

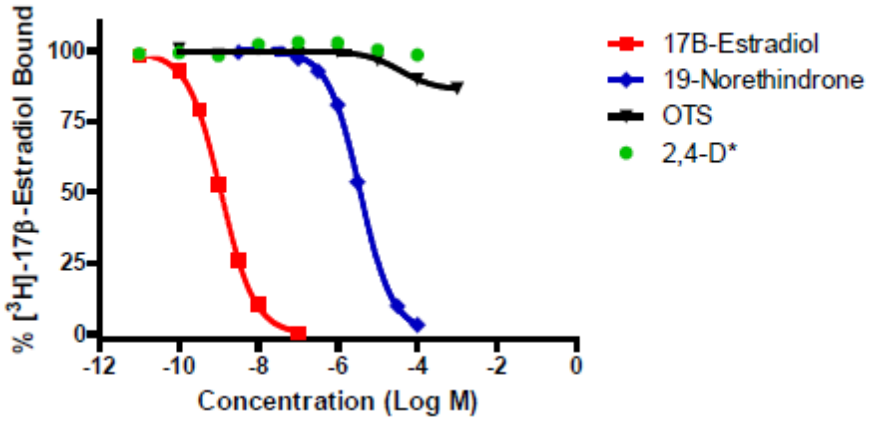
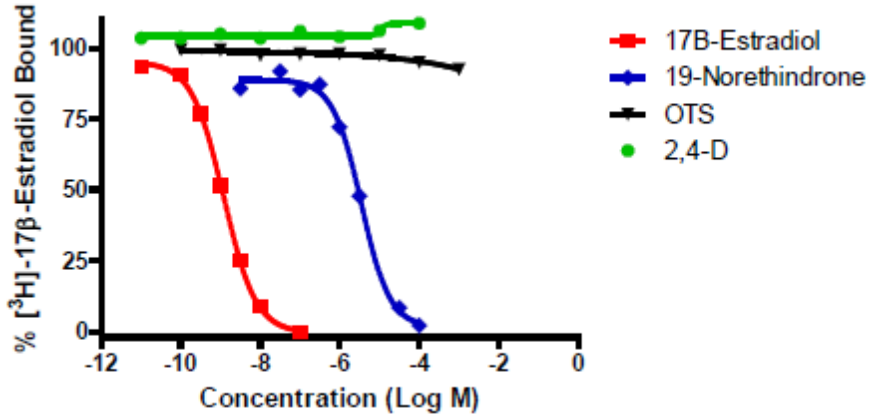


FIGURE 5. Percentage E2 Bound to the Estrogen Receptor in the Presence of Test Compound (Run 3).



C. **PERFORMANCE CRITERIA:** To ensure that the competitive binding assay functioned properly, each run was evaluated using the following criteria (Table 8):

TABLE 8. Criterion [a]	Tolerance Limit(s)	Value	Yes	No
17β-estradiol fitted curve parameters				
Log_e residual SD [Log_e (SyX)]	≤2.35	−0.11 to 0.54	X	
Top (% binding)	94 to 111	95 to 99	X	
Bottom (% binding)	−4 to 1	−0.9 to −0.4	X	
Hill Slope ($log_{10}(M)^{-1}$)	−1.1 to −0.7	−1.08 to −0.98	X	
Weak Positive control (19-norethindrone) fitted curve parameters[b]				
Log_e residual SD	<2.60	−0.52 to 1.10	X	
Top (% binding)	110 to 90	89 to 100		X
Bottom (% binding)	1 to -5	−1.2 to 1.7		X
Hill Slope ($log_{10}(M)^{-1}$)	-1.1 to -0.7	−1.2 to −0.99	X	
Solvent concentration				
Ethanol	≤3%	<3%	X	
Negative control (octyltriethoxysilane) does not displace more than 25% of [^{3}H]-17β-estradiol from the ER on average across all concentrations	≤25%	≤13.5	X	

a Data were obtained from page 33 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.
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 Hill slope of approximately −1.0. The recommended ranges of the weak positive control 19-norethindrone were occasional outside of the ranges for the top and bottom of curves established for norethynodrel. The fitted curve parameters demonstrate that 19-norethindrone is an acceptable weak positive control in the subject assay.

The percent binding of 2,4-D at this top plateau 98.8 – 103.5% was within 25 percentage points of the value for the lowest concentration of the estradiol standard 93.5 – 98.1%. 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. <u>INVESTIGATOR'S CONCLUSIONS</u>: Based on the combined responses in each of three independent estrogen receptor binding assays, it was determined that 2,4-D had no appreciable effect on the binding of the reference estrogen at any concentration, up to 10^{-4}M. The results of the *in vitro* estrogen receptor binding assay using rat uterine cytosol indicate that, under the conditions of this study, 2,4-D was negative (not interactive) for estrogen receptor at concentrations up to 10^{-4}M.

B. <u>AGENCY COMMENTS</u>: The highest concentration of 2,4-D tested in this assay was 10^{-4} M. The Test Guideline recommends testing up to 10^{-3} M unless there is evidence of insolubility. The justification provided in the study report for lowering the top concentration was based on toxicokinetic data generated in the rat. The Test Guideline recommends testing up to 10^{-3} M to adequately assess the potential for the test chemical to interact with the ER in mammalian as well as non-mammalian taxa. The justification for lowering the top concentration is inadequate since it does not apply to non-mammalian taxa.

Summary data pertaining to the saturation binding experiment were reported separately in the study profile submitted by the test order recipient; individual data on saturation binding were not reported. The K_d for [^{3}H]-17β-estradiol was 0.1032 nM and the B_{max} (nM) was 0.07097 for the prepared rat uterine cytosol used in these experiments. The K_d for the run was within the expected range of 0.03 to 1.5 nM, and the B_{max} was within the expected range of 10-150 fmol/100 µg protein. The data produced a linear Scatchard plot (Figure 2).

The mean specific binding in the presence of 2,4-D was ≥95% at concentrations ≤10^{-4} M in all three runs. The estimated mean log IC_{50} and RBA was not calculated for 2,4-D as the percent binding inhibition did not reach 50% for any run

The estimated mean log IC_{50} for the natural ligand, 17β-estradiol, and the weak positive control (19-norethindrone) was −9.0 and −5.5 M, respectively. The mean RBA was 0.034% for 19-norethindrone. All performance criteria were met for 17β-estradiol and octyltriethoxysilane. Examination of the data for the reference ligand and the weak positive control across the runs indicated consistency of the Hill slope, placement along the X-axis, and top and bottom plateaus.

2,4-D was tested over a concentration range that fully defined the top of the curve. The percent binding at this top plateau 98.8 – 103.5% was within 25 percentage points of the value for the lowest concentration of the estradiol standard 93.5 – 98.1%. Therefore, based on the combined responses in each of three independent estrogen receptor binding assay runs, it was determined that 2,4-D was not interactive with the estrogen receptor at concentrations up to 10^{-4} M.

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 highest concentration of 2,4-D tested in this assay was 10^{-4} M. The Test Guideline recommends testing up to 10^{-3} M unless there is evidence of insolubility. The justification provided in the study report for lowering the top concentration was based on toxicokinetic data generated in the rat. The Test Guideline recommends testing up to 10^{-3} M to adequately assess the potential for the test chemical to interact with the ER in mammalian as well as non-mammalian taxa.

DATA EVALUATION RECORD

2,4-DICHLOROPHENOXYACETIC ACID (2,4-D)

Study Type: OCSPP 890.1300, Estrogen Receptor Transcriptional Activation

EPA Contract No. EP10H001452
Task Assignment No. 2-26-2012 (MRID 48614304)

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:	2/19/2012
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Program Manager:	Signature:	
Jack D. Early, M.S.	Date:	3/27/2012
Quality Assurance:	Signature:	
Jack D. Early, M.S.	Date:	3/27/2012

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: Ray Kent, Ph.D. **Signature:** [illegible] for (RK)
Health Effects Division **Date:** 6/30/15
Secondary Reviewer: Minerva Mercado, Ph.D. **Signature:** [illegible]
Health Effects Division **Date:** 6-30-15
Template version 08/2011

DATA EVALUATION RECORD

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

PC CODE: 030001 **DP BARCODE:** D398640

TXR#: 0052104 **CAS No.:** 94-75-7

TEST MATERIAL (PURITY): 2,4-D (98.5% a.i.)

SYNONYMS: (2,4-Dichlorophenoxy) acetic acid

CITATION: LeBaron, M.J., Kah, H.L. (2011). Evaluation of 2,4-Dichlorophenoxy Acetic Acid (2.4-D) in an *In Vitro* Estrogen Receptor Transcriptional Activation Assay in Human Cell Line HeLa-9903. Toxicology & Environmental Research and Consulting, Midland, MI. Laboratory Report No.: 111043, October 17, 2011. MRID 48614304. Unpublished.

SPONSOR: Industry Task Force II on 2,4-D Research Data, c/o McKenna Long & Aldridge LLP, 1900 K Street NW, Washington, D.C.

TEST ORDER #: CON-03001-01

EXECUTIVE SUMMARY: In an estrogen receptor transcriptional activation assay (MRID 48614304), hERα-HeLa-9903 cells cultured *in vitro* were exposed to 2,4-D (98.5% a.i., Lot #2006 2433 8006 USA) at logarithmically increasing concentrations from 10^{-10} to 10^{-4} M in DMSO (0.1%) for 24 hours. A total of four separate runs were performed. Each run was performed using 96-well plates and each 2,4-D concentration was tested in triplicate (3 wells/plate). Cells were exposed to the test agent for approximately 24 hours to induce reporter (luciferase) gene products. Luciferase expression in response to activation of the estrogen receptor was measured upon addition of a luciferase substrate and detection with a luminometer with acceptable sensitivity.

2,4-D was tested up 10^{-4} M based on solubility, cytotoxicity and *in vivo* toxicokinetic analysis. There were deviations from expected performance criteria for all of the four reference chemicals, but these deviations do not affect the interpretation of this study. The RPC_{Max} was <0% for the first run, 8.8% for the second run, 5.3% for the third run, and 7.0% for the fourth run; the associated PC_{Max} was 10^{-4} M for runs 1-3 and 10^{-5} M for run 4. Because the $RPC_{Max} < PC_{10}$ in all assay runs, 2,4-D was considered negative for estrogen receptor transcriptional activation in this test system.

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

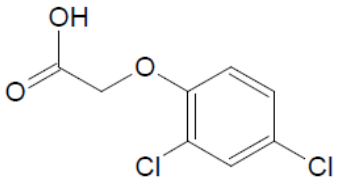
COMPLIANCE: Signed and dated GLP, Quality Assurance and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Substance: 2,4-D

Description: Technical, off-white powder
Source (Catalog #): Nufarm Americas (Not Reported)
Lot/Batch #: 2006 2433 8006 USA
Purity: 98.5%
Solubility: Up to 315 mg/L in water, soluble in DMSO up to 0.1M
Volatility: 1.9×10^{-5} Pa at 25 °C
Stability: 2 year shelf life
Storage conditions: Ambient
Vapor pressure: 1.9×10^{-5} Pa at 25°C
CAS #: 94-75-7
Structure:



2. Reference substances

17β-estradiol (strong estrogen; positive control)
Supplier: Sigma, St. Louis, MO
Catalogue and Batch #: Cat# E-8875, Lot# 079K0131
Purity: 100%
CAS # : 50-28-2

17α-estradiol (weak estrogen)
Supplier: Sigma, St. Louis, MO
Catalogue and Batch #: Cat # E-8750, Lot # 029K4116
Purity: ≥99.5%
CAS # : 57-91-0

Corticosterone (negative compound)
Supplier: Sigma, St. Louis, MO
Catalogue and Batch #: Cat # C-2505, Lot # 010M2010
Purity: 100%
CAS # : 50-22-6

17α-methyltestosterone (very weak agonist)
Supplier: Sigma, St. Louis, MO
Catalogue and Batch #: Cat # M-7252, Lot # 060M1543V
Purity: 99%
CAS # : 58-18-4

3. Vehicle(s)

Solvent: DMSO, Sigma-Aldrich, Cat # 276855
Solvent control (final concentration): 0.1%

B. METHODS

1. **Cell Culture:** Stably-transfected hERα-HeLa-9903 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank and were verified to be free of mycoplasma infection by ATCC (method not reported). 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 (DCC-FBS; Hyclone Laboratories, Inc., Logan, Utah Lot# not reported), in an incubator under 5% CO_2 at 37º C. Upon reaching 75-90% confluence, cells were subcultured at least twice prior to exposure to the test material.

2. **Transcriptional Activation Assays:** For each test, cells were plated at a density of 1 - 1.5×10^4 cells/100 µL medium/well in a 96-well plate and allowed to attach for at least 3 hours. Growth media was replaced with media containing serial log dilutions of 2,4-D in DMSO (0.1% total final concentration). Cells were incubated for approximately 24 hours at approximately 37° C. Cytotoxicity was determined by a modified MTT cell viability assay, when stock solutions were diluted with treatment media, after addition to the cell culture plate and after the 24-hour treatment period. Transcriptional activation of the estrogen receptor (firefly luciferase activity) was determined using a standard assay kit (Promega, Madison, WI). Chemiluminescence was measured using a Packard TopCount NXT luminescence counter with a detection limit of ~5 cells in 100µL medium.

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

   b. **Proficiency Chemicals:** It was stated that laboratory validation assays with 10 proficiency chemicals were performed to confirm the responsiveness of the ER transcriptional activation assay. These non-GLP unpublished results were reported to have demonstrated laboratory proficiency.

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

Reference Chemical	CAS No.	Concentration Range	Class
17β-estradiol	50-28-2	10^{-14} to 10^{-8}	Strong estrogen
17α-estradiol	57-91-0	10^{-12} to 10^{-6}	Weak estrogen
Corticosterone	50-22-6	10^{-10} to 10^{-4}	Negative compound
17α-methyltestosterone	58-18-4	10^{-11} 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. Graph Pad Prism v. 5.0 (GraphPad Software, Inc., La Jolla, CA) was used to calculate the EC_{50}, PC_{10}, PC_{50}, RPC_{Max}, and PC_{Max} for 2,4-D when applicable. 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 (or 2 of 3) 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: 2,4-D was relatively non-toxic and freely soluble; therefore the highest concentration was set at 10^{-4} M based on the *in vivo* 2,4-D plasma concentrations that exceeded the threshold for linear toxicokinetics. These data were provided in an appendix to the report. No solubility or cytotoxicity issues were noted at the concentrations tested. Based on these results, logarithmically increasing concentrations from 10^{-10} to 10^{-4} M were selected for the assay.

TABLE 1. Preliminary Test for Solubility, Cytotoxicity, and Concentration-Selection for 2,4-D[a]

Concentration (M)	% Viability	Comments
10^{-4}	119.8	Toxicokinetic-derived limit dose
10^{-5}	123.6	
10^{-6}	133.1	
10^{-7}	132.0	
10^{-8}	115.1	
10^{-9}	130.4	
10^{-10}	117.6	
E2 at 1 nM	130.8	
VC[b]	100.0	

[a] Data were obtained from page 51 of the study report.
[b] Vehicle Control

B. Positive and Negative Reference Chemicals

1. Proficiency Chemicals: The responsiveness of cells to the required proficiency chemicals was not reported, but it was stated that the proficiency validation assays were conducted, and proficiency was demonstrated.

TABLE 2. Proficiency Chemicals [a]		
Compound	**Expected Response**	**Lab Response**
Diethylstilbestrol	Positive	Not reported
17α-Ethynyl estradiol	Positive	Not reported
Hexestrol	Positive	Not reported
Genistein	Positive	Not reported
Estrone	Positive	Not reported
Butyl paraben	Positive	Not reported
1, 3, 5-Tris(4-hydroxyphenyl)benzene	Positive	Not reported
Dibutyl phthalate	Negative	Not reported
Atrazine	Negative	Not reported
Corticosterone	Negative	Not reported

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. There were deviations from expected performance criteria for all of the four reference chemicals. The log EC_{50} was higher than the expected value and the Hill slope lower than the expected value for 17β-estradiol. The log PC_{50} and log PC_{10} for 17α-estradiol and 17α-methyltestosterone were lower than expected, as was the Hill slope for 17α-estradiol. For 17α-methyltestosterone, the mean RPC_{Max} was 90.8% for the first run, 152.8% for the second run, 83.3% for the third run, and 67.4% for the fourth run. For corticosterone, the mean RPC_{Max} was 6.3% for the first run, 35.5% for the second run, 7.0% for the third run, and 5.4% for the fourth run. The deviations from the expected values do not negatively affect the interpretation of this study. Performance criteria values below the validated ranges usually indicate increased sensitivity of the assay compared to validation experiments.

TABLE 3. Performance Criteria for Reference Chemicals[a]							
Reference Chemical Parameter	**Acceptable Range**	**Values**				**Acceptable**	
		Run 1	**Run 2**	**Run 3**	**Run 4**	**Yes**	**No**
17β-estradiol							
Log PC_{50}	−11.4 to −10.1	−10.8	−11.1	−10.9	−10.7	X	
Log PC_{10}	<−11	−11.9	−12.6	−12.7	−12.5	X	
Log EC_{50}	−11.3 to −10.1	−9.4	−10.4	−10.7	−10.6		Run 1
Hill Slope	0.7 to 1.5	0.3	0.3	0.7	0.8	X	
Test range	10^{-14} to 10^{-8} M	10^{-14} to 10^{-8} M				X	
17α−estradiol							
Log PC_{50}	−9.6 to −8.1	−9.3	−10.1	−9.0	−9.0	X	
Log PC_{10}	−10.7 to −9.3	−10.6	−10.9	−10.6	−10.9	X	
Log EC_{50}	−9.6 to −8.4	−8.7	−9.6	−8.8	−8.6	X	
Hill Slope	0.9 to 2.0	0.5	1.1	0.7	0.6	X	
Test range	10^{-12} to 10^{-6} M	10^{-12} to 10^{-6} M				X	
Corticosterone							
Test range	10^{-10} to 10^{-4} M	10^{-10} to 10^{-4} M				X	
17α−methyltestosterone							
Log PC_{50}	−6.0 to −5.1	−7.0	−8.8	−7.5	−6.5	X	
Log PC_{10}	−8.0 to −6.2	−8.8	−9.8	−9.2	−9.2	X	
Test range	10^{-11} to 10^{-5} M	10^{-11} to 10^{-5} M				X	

[a] Data were obtained from page 27 of the study report.

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 1513-3035 arbitrary light

units, and the overall mean TA value for the positive control was 12128-19639 (not reported for Run 4). The induction for the positive control ranged from 4.7- to 8.2-fold. The mean normalized value for the positive control was 10912-16604. The PC_{50} (50% of the maximum response) for E2 in this assay is 6212.5-9819.5 and the PC_{10} (10% of the maximum response) is 1243-1964.

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	2743	248	15564	1604	5.7	12821	NR
2	1513	290	12425	1958	8.2	10912	NR
3	3035	271	19639	1116	6.5	16604	NR
4	2603	NR	12128 [d]	915 [d]	4.7[d]	12158	NR

[a] Data were obtained from page 47, 49 and 53 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)
[d] Calculated by the reviewer from data on page 49 of the study report
NR Not reported

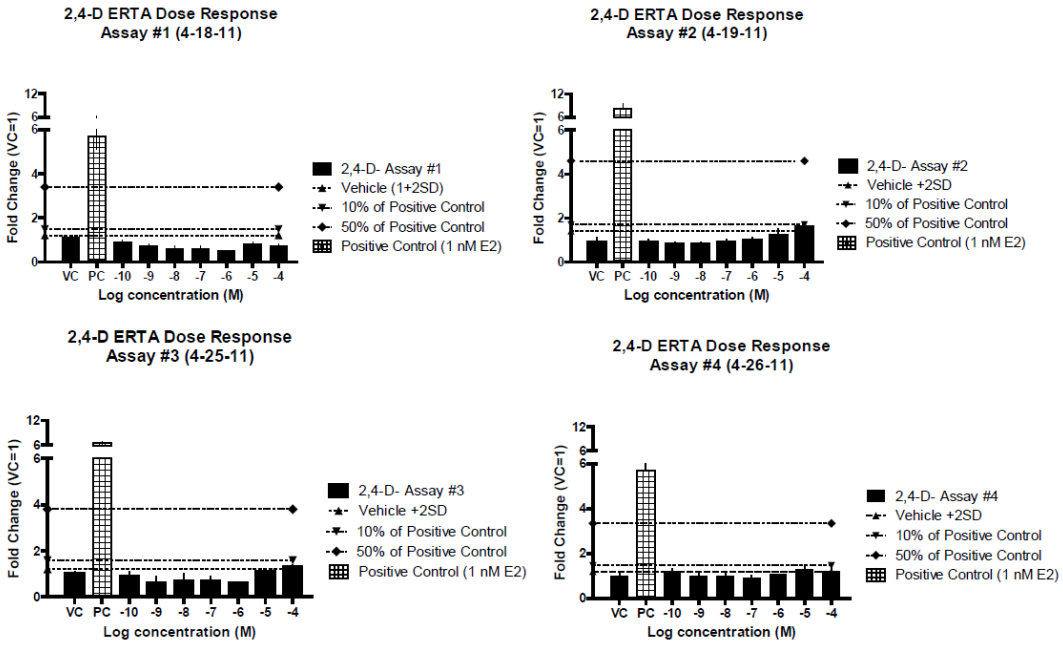
2. **Test Material:** Relative (to the PC) transcriptional activation at each concentration of the test chemical during the three assay runs is presented in Table 5. The concentration-response bar graphs depicting fold induction of relative transcriptional activation are presented in Figure 1 below. The RPC_{Max} was <0% for the first run, 8.8% for the second run 5.3% for the third run and 7.0% for the fourth run; the associated PC_{Max} was 10^{-4} M for Runs 1-3 and 10^{-5} M for Run 4. Because the $RPC_{Max} < PC_{10}$ in all four runs, 2,4-D was considered negative for estrogen receptor transcriptional activation in this test system.

TABLE 5. Relative Transcriptional Activation (RTA) of 2,4-D[a]

Parameter	RTA (mean ± SD); % of Positive Control (PC)							
	Run 1		Run 2		Run 3		Run 4	
Conc. (M)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
10^{-4}	−7.2	3.8	8.8	1.0	5.3	1.1	4.4	4.8
10^{-5}	−4.6	4.1	3.3	4.1	1.7	1.0	7.0	3.6
10^{-6}	−10.1	0.9	−0.6	1.8	−6.6	0.4	2.4	0.3
10^{-7}	−8.9	3.4	−2.1	2.0	−6.0	3.6	−1.4	2.3
10^{-8}	−8.8	5.0	−2.5	1.4	−5.8	5.4	−1.1	2.7
10^{-9}	−6.2	4.8	−3.2	0.9	−7.5	4.7	0.1	1.9
10^{-10}	−1.9	3.6	−0.8	1.2	−2.5	3.8	4.3	1.1
Log EC_{50}[b]	NA		NA		NA		NA	
Hill Slope[b]	NA		NA		NA		NA	
RPC_{Max}	0[b]		8.8		5.3		7.0	
PC_{Max}	NA		10^{-4}		10^{-4}		10^{-5}	
PC_{50}	NA		NA		NA		NA	
PC_{10}	NA		NA		NA		NA	

[a] Data were obtained from page 54 of the study report.
[b] Value of RPCMax less than or equal to 0
NA Not Applicable

Figure 1. Fold Induction of Relative Transcription Activation (RTA) of 2,4-D Compared to the Positive Control.



VC= Vehicle Control
PC= Positive Control (1 nM E2)

3. **Performance Criteria:** The results of the laboratory proficiency test were not reported. There were minor deviations from expected performance criteria for all of the four reference chemicals. The Log EC_{50} was higher than the expected value and the Hill slope lower than the expected value for 17β-estradiol. The log PC_{50} and log PC_{10} for 17α-estradiol and 17α-methyltestosterone were lower than expected, as was the Hill slope for 17α-estradiol. The minor deviations from the expected values do not negatively affect the interpretation of this study. Mean luciferase activity was 4.7 to 8.2-fold. The fold-induction corresponding to the PC_{10} of the concurrent PC was greater than 1+2 SDs of the vehicle control on all plates. Variability was minimal, and results were reproducible among runs.

III. DISCUSSION AND CONCLUSIONS

A. INVESTIGATORS' CONCLUSIONS: Based on the combined responses in each of four independent estrogen receptor transactivation assays, it was determined that 2,4-D treatment did not result in ER-mediated transcriptional activation at any concentration, including the toxicokinetic-derived limit, in this assay system (10^{-4} M). The highest concentration of 2,4 D was based on *in* vivo toxicokinetic analyses; concentrations higher than 10^{-4} M were not considered relevant for use in this assay as they are substantially above the inflection point for linear toxicokinetics.

B. AGENCY COMMENTS: 2,4-D was tested up to the toxicokinetic-derived limit, 10^{-4}M. The laboratory proficiency assays were not reported. The only deviation was a weak positive response in one run in response to corticosterone. There were minor deviations from expected performance criteria for all of the four reference chemicals, but these minor

deviations do not negatively affect the interpretation of this study. The RPC_{Max} was <0% for the first run, 8.8% for the second run 5.3% for the third run and 7.0% for the fourth run; the associated PC_{Max} was 10^{-4} M for Runs 1-3 and 10^{-5} M for Run 4. Because the RPC_{Max} < PC_{10} in all four runs, 2,4-D was considered negative for estrogen receptor transcriptional activation in this test system.

C. STUDY DEFICIENCIES: The following deficiencies were noted:

- The laboratory proficiency assays were not included in the study report, but were sent in an accompanying study profile template.
- 2,4-D was not tested up to the limit dose, but rationale was provided to support the concentrations tested.

Data Requirement:	EPA DP Barcode	388580
	OECD Data Point	
	EPA MRID	48317001
	EPA Guideline	890.1350
		Fish Short-Term Reproduction Assay

Test Material:	**2,4-Dichlorophenoxyacetic acid**		**Purity (%):** 98.6%
Common Name	2,4-D		
Chemical Name	IUPAC		
	CAS Name		
	CAS No.	94-75-7	
	Synonyms	2,4-D acid	
	EPA PC Code	030001	

Primary Reviewer: Patience Browne **Signature:** Digitally signed by PATIENCE BROWNE DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=PATIENCE BROWNE, dnQualifier=0000048202 Date: 2015.06.03 15:14:20 -04'00'

USEPA/OCSPP/OSCP **Date:** 06/25/2012

Additional Reviewer: Alicia Korol **Signature:** No longer with EPA

USEPA/OCSPP/OPP/EFED/ERB1 **Date:** 06/14/2011

Additional Reviewer: Amy Blankinship **Signature:** AMY BLANKINSHIP Digitally signed by AMY BLANKINSHIP DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=AMY BLANKINSHIP, dnQualifier=0000040917 Date: 2015.06.05 10:04:22 -04'00'

USEPA/OCSPP/OPP/EFED/ERB3 **Date:** 12/28/2012

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: 2015.06.01 13:17:23 -04'00'

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

Date Evaluation Completed: 05/27/2015

CITATION: Marino, T.A., K.K. Coady, L.K. Sosinski, J. Thomas. 2010, DICHLOROPHENOXYACETIC ACID: A FISH SHORT-TERM REPRODUCTION ASSAY USING THE FATHEAD MINNOW, *Pimephales promelas*, Toxicology and Environmental Research and Consulting, The Dow Chemical Company. and Midland, Michigan 48674, Laboratory ID: 101026, Industry Task Force II on 2,4-D Research Data c/o McKenna Long & Aldridge LLP, Washington, D.C, Completed on 06 December 2010.

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 2,4-D with fathead minnow (*Pimephales promelas*) was conducted under flow-through conditions. Adult fish (20 spawning groups; 2 males and 4 females in each group; 4 groups/treatment; *ca*. 6 months old) were exposed to 2,4-D (98.6% purity) at nominal concentrations of 0 (negative control), 0.400, 4.00, 40.0, and 100 mg a.i./L concentrations with corresponding mean-measured concentrations of <0.10 (<LOQ, negative control), 0.245, 3.14, 34.0, and 96.5 mg a.i./L, respectively. The test system was maintained at 24.5 to 25.2°C and a pH of 7.02 to 7.76.

The single mortality observed during the assay occurred in a female in the high treatment group. There were no significant differences for male or female body weight or length relative the negative control. At test termination, observations of secondary sex characteristics were observed in the negative control and treated groups; no treatment-related effects were reported. Clinical signs included the loss of an eye, ascites, and scoliosis (bent tail) which were observed in single fish in the negative control or treatment groups.

Spawning occurred in the negative control at least every 4 days in 3 of the 4 replicates, and mean fecundity was 29.8 eggs/female/day/replicate; fertility in the negative control was 96.9%. Fecundity was significantly decreased (Jonckheere-Terpstra; $p<0.05$) by 34% in the high treatment group with a non-significant ($p>0.05$) concentration-dependent trend of decreased fecundity in the lower treatment groups compared to the negative control. There were no significant differences for fertility between the 2,4-D treatments and the negative control.

There were no significant differences ($p>0.05$) between the 2,4-D treatment groups and the negative control for male or female gonado-somatic index (GSI) or plasma vitellogenin (VTG). There was also no significant difference ($p>0.05$) for male nuptial tubercle scores; no tubercles were observed for females. No apparent treatment-related histopathological effects were observed in males and females. Although not concentration-dependent, an increase in the number of female ovaries that were observed as Stage 2 (compared to Stage 3 or 4) was reported for the 2,4-D treatments compared to the negative control. Plasma sex steroid concentrations were not reported.

The performance and validity criteria were met in this study with the exception that the coefficient of variation (CV) for the mean-measured concentration of the lowest treatment group was 43%, exceeding the guideline criterion of <20%. This deviation 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: 6 months

Mean body weight at test initiation: 3.7 g for males, 1.9 g for females

Mean length at test initiation: Not reported

Test type: Flow-through

Table 1: Summary of Reproductive and HPG Effects[1,2] in the Fish Short-Term Reproduction Assay (FSTRA) with 2,4-D.

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
0.245	No	No	No	No	No	No	No	No	No	No	NA	NA	NA	NA
3.14	No	No	No	No	No	No	No	No	No	No	NA	NA	NA	NA
34.0	No	No	No	No	No	No	No	No	No	No	NA	NA	NA	NA
96.5	Yes	No	No	No	No	No	No	No	No	No	NA	NA	NA	NA

Abbreviations: Conc. Concentration. 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.

I. MATERIALS AND METHODS

Guideline Followed: This study was conducted in accordance with the Endocrine Disruptor Screening Program Guidelines, OCSPP (form. OPPTS) 890.1350: Fish Short-Term Reproduction Assay, Environmental Protection Agency (EPA) 740-C-09-007, October 2009. Supporting guidance documents used include: Organization for Economic Cooperation and Development (OECD) Guideline for Testing of Chemicals No. 229: "Fish Short Term Reproduction Assay", 2009; "A Short-term Test Method for Assessing the Reproductive Toxicity of Endocrine-Disrupting Chemicals Using the Fathead Minnow (Pimephales promelas)", EPA/600/R-01/067, 2002; and "Guidance Document on Aquatic Toxicity Testing of difficult Substances and Mixtures", OECD Series on Testing and Assessment. No. 23. 2000. Deviations from the OCSPP 890.1350 include the following:

1. The coefficient of variation (CV) for the mean-measured concentration exceeded 20% in the 0.245 mg a.i/L treatment group.
2. Ammonia levels were detected at 0.24 mg/L, exceeding guideline recommendations of ≤1 µg/L.
3. Organic carbon levels were not reported in the routine laboratory dilution water solute analysis.
4. OCSPP guidelines recommend chlorine levels at <10 µg/L; chlorine in the dilution water was not detectable at a level of 0.20 mg/L and it is unknown whether levels exceeded 10 µg/L.

These deviations do not impact the interpretation of the study.

Compliance: Signed and dated GLP, Quality Assurance, and No Data Confidentiality statements were provided. All phases of this study were conducted in accordance with the GLP Principles of the USEPA – FIFRA GLPs Title 40 CFR, Part 160 – Federal Insecticide Fungicide Rodenticide Act (FIFRA), Good Laboratory Practice Standards, Final Rule; OECD Series on principles of Good Laboratory Practice and Compliance Monitoring, Number 1. OECD Principles on Good Laboratory Practice (as revised 1997) ENV/MC/CHEM/(98)17; and European Community (EC) – European

Parliament and council Directive 2004/10/EC (O.J. No. L 50/44, 20/02/2004).

A. Test Material 2,4-Dichlorophenoxyacetic acid

Description: Water Solubility = 569 mg/L
log Kow = 2.81

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. : Lot No. 2006 2433 8006-USA

Purity: 98.6%

Impurities: None identified

Stability of Compound: The measured 2,4-D concentrations in the replicates across the treatments ranged from 28.0 to 105% of nominal concentrations. Measured concentrations were highly variable, particularly at lowest test concentrations, and the study report attributed this to possible microbial metabolism in the tanks even though there was regular cleaning of the tanks. The CVs for the replicates in the lowest treatment group ranged from 38-49%. The mean measured concentrations were 0.245, 3.14, 34.0, and 96.5 mg a.i./L 2,4-D which was 61.3, 78.5, 85.0, and 96.5% of nominal concentrations, respectively.

Storage Conditions of Test Chemicals: Not reported

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?	not reported	Test fish were obtained from the commercial supplier, New England Bioassay, Manchester, CT.	*EPA recommends that fish be from a single laboratory stock.*
Were acclimation conditions same as definitive test?	Yes	water quality, temperature, and lighting were the same as test conditions	*EPA recommends that fish be acclimated under water quality and illumination conditions that are similar to the definitive test.*
Acclimation period:	30 days		*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:		For 2 weeks out of the 30 days period, mortality was reported at a rate of 1.8%	*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:	thawed, frozen brine shrimp		*EPA recommends that fish be fed frozen brine shrimp twice per day to promote active reproduction and maintain body condition.*
Source of food:	Brine Shrimp Direct, Ogden, UT		
Frequency of feeding:	at least 2 times/day	1.25-2.0 ml shrimp/replicate vessel	

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Details on feeding:		Feeding start date not reported, but fish were fed during the holding/acclimation, pre-exposure, and exposure periods. Food was withheld 12 hours prior to test termination. Fish food tested free of relevant contaminants.	

Table 3: Fish Selection and Pre-Exposure Performance.

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Age at test initiation:	5.5 months	6 months at beginning of exposure period	*EPA recommends reproductively mature (sexually dimorphic) fish, 4.5 - 6 months old.*
Mean weight of males at test initiation (if determined):	3.7 g	Mean calculated from all males selected for the test	*EPA recommends that a subsample of fish be weighed before the test to estimate the mean weight for each sex.*
Range of individual weights (males) at test initiation (if determined):	individual data not provided	range of individual weights were kept within ±20% of the mean	*It is recommended that the individual weight of each fish selected for the test be within ±20% of the estimated mean for each sex.*
Mean weight of females at test initiation (if determined):	1.9 g	Mean calculated from all females selected for the test	

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Range of individual weights (females) at test initiation (if determined):	Individual data not provided	range of individual weights were kept within ±20% of the mean	
Mean length of males at test initiation (if determined):	Not reported		
Mean length of females at test initiation (if determined):	Not reported		
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:	32	The 32 test vessels included 12 test vessels more than the 20 that would be needed for the definitive test.	*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		

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Pre-exposure fecundity:	≥15 eggs/female/ reproductive day/ replicate	mean = 28.8 eggs/female/day; range from 5.6 to 53.2 eggs/female/day across all replicates	*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:	≥2 times in 7 days	The top 20 performing spawning groups (≥15 eggs/female/reproductive day) were used in the exposure period and were assigned to concentration levels via complete randomized block design.	*EPA recommends that spawning occur at least twice in the 7 days prior to the definitive test.*
Details on pre-exposure:		Spawning substrate were inspected and the number of eggs laid and eggs found to be infertile were recorded daily	

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:	Fflow-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:	Continuous flow diluter	A continuous-flow diluter (syringe pump delivery) system delivered stock solution to a mixing chamber where the solution was mixed with dilution water. The test solution was split equally to replicate test vessels.	*Intermittent flow proportional diluters or continuous flow serial diluters are recommended.[2]*
Flow-through rate:	45 ± 4.5 mL/min		*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:		Diluter system was calibrated prior to exposure period. For concentrations measured in test vessels, mixing cells, and stock solutions, the percent of target concentration ranged from 108% to 121%. Within that range, the flow splitting accuracy of the concentrations for each stock solution-test vessel pairs were within 10% of each other. Homogeneity of test concentration was analyzed in one vessel at the 0.40 and 100 mg a.i./L treatment levels. Of the 4 samples taken at each treatment level, the %CV for the 0.40 and 100 mg a.i./L levels was 0.646% and 0.348%, respectively.	*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?	not reported		*EPA recommends aeration if dissolved oxygen reaches ≤4.9 mg/L (≤60% saturation).*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Source of dilution water:	Natural water	Dilution water originated from Lake Huron which was supplied to the Dow Chemical Company via the City of Midland Water Treatment Plant. The water from the lake was limed and flocculated with ferric chloride and then pumped to the laboratory. Before use in the lab, the water was sand-filtered, pH-adjusted with gaseous CO_2, carbon-filtered, and UV-irradiated.	*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.*
Was dilution water analyzed for pesticides, heavy metals, and other contaminants?	Yes	Dilution water is bi-annually analyzed for pesticides, organics, metals and other inorganics.	
Test vessel type/materials:	Glass sealed together with clear silicone adhesive		*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:	29 cm x 20 cm x 25 cm		*EPA recommends the use of 18 L test chambers (e.g., 40 x 20 x 20 cm).*
Fill volume:	10 L	approximate fill volume; water depth 13 cm	*EPA recommends 10 L solution per tank.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Spawning substrate material:	PVC pipe, cut lengthwise		*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:	9 cm	9 cm long, 10 cm diameter	
Additional details on exposure system:			

Temperature was measured in one test vessel through the exposure period. Temperature, DO, and pH were measured in each test vessel at Day 0 and weekly thereafter during the assay (Table 5). Hardness, alkalinity, and conductivity were measured from a sample in the control and highest exposure level solutions at Day 0 and weekly thereafter (Table 5).

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

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Temperature (°C)	24.5	25.2	25.0[1]	Weekly	*EPA recommends temperature 25±1°C; inter-replicate and inter-treatment differentials should not exceed 1°C.*
pH	7.02	7.76	7.38[1]	Weekly	*EPA recommends pH 6.5 to 9.0.*
Dissolved oxygen (mg/L)	6.20	8.29	7.39[1]	Weekly	*EPA recommends dissolved oxygen (DO) ≥4.9 mg/L (>60% air saturation)*

Parameter	Minimum	Maximum	Mean	Measurement Interval	Guideline Recommendations
Total alkalinity (mg/L as $CaCO_3$)	30	46	36.5[1]	Weekly	*EPA recommends total alkalinity >20 mg/L as $CaCO_3$.*
Hardness [mg/L as $CaCO_3$]	62	68	63.5[1]	Weekly	
Total organic carbon (mg/L)		≤2 mg/L		Once	*EPA recommends that total organic carbon in dilution water be ≤2 mg/L.*
Unionized ammonia (µg/L)		<0.10 mg/L (as N)			*EPA recommends that unionized ammonia in the dilution water be ≤1 µg/L.*
Residual chlorine (µg/L)			<200	Biannually	*EPA recommends that residual chlorine in dilution water be <10 µg/L.*
Other: Ammonia (mg/L)			240	biannually	*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.*
Other: Conductivity (µmhos/cm)	177	207	188.5[1]	weekly	

[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?	No		*EPA recommends conducting a range-finder if 96-hour LC_{50} data for the fathead minnow are unavailable.*
If yes, what was the method for determining the highest test concentration in the range-finder?	NA		*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:	NA		
Life stage:	NA		*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:	NA		*EPA recommends a 96-hour exposure.*

Additional details:	NA		*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.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Method for selecting the highest test concentration in the definitive test:	Reference studies with fathead minnow	The nominal test concentrations were based on two available 2,4-D studies with the fathead minnow with reported LC_{50} values of 320 mg a.i./L (Alexander *et al.,* 1985) and 133 mg a.i./L (Mayer and Ellersieck, 1986). From these two lethal endpoints, the experimental high concentrations could be estimated at 100 and 40 mg a.i./L, respectively, as specified in the 890.1350 guideline. Because of the uncertainty between the two concentrations, both were selected as high concentrations and 4 concentrations were used for the experimental 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., 1/3 the 96-hour LC_{50}), whichever concentration is lowest.*
Reference study citation (if applicable):	Alexander *et al.,* 1985; Mayer and Ellersieck, 1986		
Separation of test concentrations:	0, 0.4, 4.0, 40, 100 mg/L		*EPA suggests that a concentration separation of between 0.33 (or three-fold) and 0.1 (or ten-fold) is scientifically acceptable*[1].

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
Number of test concentrations:	4		*EPA recommends a minimum of 3 concentrations and a control, plus solvent control if appropriate.*
Are nominal concentrations adjusted for purity?	Not reported		
Indicate the type of values presented for measured concentrations:	Mean-measured		
Limit of quantification (LOQ):	0.1 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:	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.*

Parameter	Value(s)	Details or Remarks	Guideline Recommendations
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):	NA		*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.*
Number of female fish per replicate at test initiation:	4		*EPA recommends 4 females per replicate.*
Was a solvent used?	No		
Was a positive control used?	No		

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:	591 - 770 lux		*EPA recommends light intensity 540 – 1080 lux (at water's surface).*
Additional details:		Information regarding test solution appearance during the study did not appear to be reported.	

Table 8: Summary of Treatment Concentrations in the Fish Short-Term Reproduction Assay with 2,4-D.

Treatment ID	Nominal Concentration (mg a.i./L)	Mean Measured Concentration (mg a.i./L)	Mean CV (%)	Details or Remarks	Guideline Recommendations
Control (dilution water only)	0.00	<0.10	NA		*EPA recommends that test item concentrations be maintained at a coefficient of variation (CV) ≤20%.*
Treatment 1	0.400	0.245	43.3[1]	61.3% nominal	
Treatment 2	4.00	3.14	13.3	78.5% nominal	
Treatment 3	40.0	34.0	7.0	85.0% nominal	
Treatment 4	100	96.5	4.5	96.5% nominal	

Abbreviations: CV Coefficient of variation.

[1] Study authors reported a dramatic decrease in measured concentrations between Days 7 and 14 due to a suspected increase in microbial populations capable of metabolizing 2,4-D. Though this occurred in all concentrations, the effect was proportionately greater on the lowest nominal concentration. The %CVs were >20% for each time point in this treatment group, though authors report that the variation in concentration was consistent among the replicates and thus the overall exposure was consistent among replicates.

E. Observations

Biological Endpoints: Mortality, external abnormalities, abnormal behavior relative to controls, fecundity, fertilization, secondary sex characteristics, full body weight and length, gonadal status (GSI and histology), nuptial tubercle scoring, and vitellogenin concentrations. Observations of appearance were made at test termination, than fish were euthanized, weighed and measured. Blood was collected from the caudal peduncle using heparinized capillary tubes. Fish viscera were removed and fixed in Davidson's solution then preserved in formalin. Gonads were removed, weighed, placed into a plastic tissue cassette and then Davidson's fixative solution. Vitellogenin blood plasma levels were measured with the fathead minnow VTG ELISA kit obtained from Biosense Laboratories, Bergen, Norway. In addition to test samples, each ELISA plate contained 6 calibration standards and 2 non-specific binding assay blanks. Plasma steroid concentrations were not measured.

Were raw (individual) data provided? Yes, with the exception of the re-analysis of female VTG levels and the coloration/appearance of females. Data were also provided in spreadsheet form via email.

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

Only one fish from the 96.5 mg a.i./L treatment group died during the test. No mortalities occurred in the control fish.

Table 9: Adult Fish Survival in Fathead Minnow.

Treatment (mg a.i./L) [mean-measured]	Males			Females		
	n	# Surviving	% Survival	n	# Surviving	% Survival
Negative Control (<LOQ)	8	8	100	16	16	100
0.245	8	8	100	16	16	100
3.14	8	8	100	16	16	100
34.0	8	8	100	16	16	100
96.5	8	8	100	16	15	93.8

n = number of individuals per treatment at test initiation.

LOQ=0.10 mg a.i./L

Mean male body weight ranged from 3.57 g (negative control) to 3.93 g (34 mg a.i./L), and mean female body weight ranged from 1.59 g (negative control) to 1.62 g (3.14, 34, and 96.5 mg a.i./L). Mean male length ranged from 51.5 mm (negative control) to 54.5 mm(34 mg a.i./L), and mean female length ranged from 42.1 mm (3.14 mg a.i./L) to 42.5 mm (96.5 mg a.i./L).

Table 10: Size at Test Termination in Fathead Minnow.

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	3.57	0.141	4	1.59	0.169	4	51.5	1.54	4	42.4	1.37
0.245	4	3.72	0.308	4	1.64	0.074	4	53.0	1.37	4	42.7	0.56
3.14	4	3.69	0.144	4	1.62	0.173	4	52.2	0.89	4	42.1	0.92
34.0	4	3.93	0.488	4	1.62	0.173	4	54.5	2.20	4	42.4	1.47
96.5	4	3.73	0.408	4	1.62	0.082	4	53.8	2.45	4	42.5	0.78

SD Standard deviation.

n = number of independent replicates per treatment.

LOQ=0.10 mg a.i./L

Fecundity ranged from 12.5 to 38.6 eggs/female/reproductive data across all treatment groups, and fertilization success as a percentage of embryos to unfertilized eggs ranged from 91.7 to 99.4%.

Table 11: Fecundity and Fertilization Success in Fathead Minnow.

Treatment (mg a.i./L) [mean-measured]	Fecundity[1]		Fertilization Success (%)[2]	
	Mean	± SD	Mean	± SD
Negative Control (<LOQ))	29.8	6.4	96.9	0.50
0.245	29.0	6.47	96.3	3.08
3.14	25.9	5.27	96.1	1.54
34.0	22.9	8.72	97.7	0.78
96.5	19.7	5.97	95.8	2.68

LOQ=0.10 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.

Male media tubercle scores ranged from 31 (negative control and 0.245 mg a.i./L) to 35 (3.14 mg a.i./L). No tubercles were noted in females.

Table 12: Nuptial Tubercle Score in Fathead Minnow.

Treatment (mg a.i./L) [mean-measured]	Males		Females[1]	
	n	Median Tubercle Score[2]	n	Median Tubercle Score
Negative Control (<LOQ)	4	31	4	0
0.245	4	31	4	0
3.14	4	35	4	0
34.0	4	33	4	0
96.5	4	34	4	0

n = number of independent replicates per treatment.

LOQ=0.10 mg a.i./L

[1] The study authors reported that no tubercles were observed on female fish.

[2] Mean tubercle scores: 32, 31, 34, 33, and 35 for the negative control and mean-measured 0.245, 3.14, 34.0, and 96.5 mg a.i./L treatment levels, respectively.

Mean male GSI ranged from 1.2 to 1.3%, and mean female GSI ranged from 11.2% (3.15 mg a.i./L) to 13.3% (96.5 mg a.i./L).

Table 13: Gonado-Somatic Index (GSI) in Fathead Minnow.

Treatment (mg a.i./L) [mean-measured]	Males			Females		
	n	Mean GSI[1] (%)	±SD	n	Mean GSI[1] (%)	±SD
Negative Control (<LOQ)	4	1.2	0.1	4	11.9	1.7
0.245	4	1.2	0.3	4	12.9	2.3
3.14	4	1.2	0.1	4	11.2	2. 6
34.0	4	1.2	0.2	4	11.8	3.4
96.5	4	1.3	0.1	4	13.3	2.0

n = number of independent replicates per treatment.

LOQ=0.10 mg a.i./L

[1] Gonado-somatic index (%) is calculated as gonad weight (to the nearest 0.1 mg) / body weight (mg) x 100.

The study author reports that there were no effects of 2,4-D on the histology or germ cell distribution (staging) of either the testes or ovaries of the test fish. All histopathological findings were considered basal variation unassociated with 2,4-D exposure due to a lack of a concentration-response relationship. Although not concentration-dependent, an increase in the number of female ovaries that were observed as Stage 2 (compared to Stage 3 or 4) was noted by the reviewer for the 2,4-D treatments compared to the negative control. A summary of the reported stage in the ovaries is shown in Table 14b.

The study report did not include observations of decreased proportion of spermatagonia, increased vascular or interstitial proteinaceous fluid, asynchronous gland development, altered proportions of spermatocytes or spermatids, or granulomatous inflammation. There was a single observation of mineralization of the seminiferous tubule/duct (severity grade 1) in one control male. Macrophages (histiocytes; severity grade 1) were observed in female fish in the controls and at all treatment levels.

Table 14a: Gonadal Staging in Fathead Minnow.

Treatment (mg a.i./L) [mean-measured]	Males		Females	
	n	Median Stage[1]	n	Median Stage[2]
Negative Control (<LOQ)	8	3	4	4
0.245	8	4	4	4
3.14	8	3	4	3
34.0	4	3	4	3
96.5	4	3	4	4

n = total number of animals per treatment upon which observations were made at test termination.

LOQ=0.10 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.

Table 14b. Ovary staging (from study report)

Ovary	Control	0.245 mg a.i./L	3.14 mg a.i./L	34 mg a.i./L	96.5 mg a.i./L
Examined	16	16	16	16	15
Stage 2	0	3	4	6	3
Stage 3	6	2	6	3	4
Stage 4	10	11	6	7	8

Table 15: Gonadal Histopathology in Male Fathead Minnow.

Treatment (mg a.i./L) [mean-measured]	Diagnostic Observations[1]										
	Severity	Increased Proportion of Spermatogonia		Presence of Testis-Ova		Mineralization of seminiferous tubule		Increased Testicular Degeneration		Interstitial Cell Hypertrophy/ Hyperplasia	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control (<LOQ)	0	8	8	8	8	8	7	8	8	8	8
	1	8	0	8	0	8	1	8	0	8	0
	2	8	0	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0	8	0
0.245	0	8	8	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0	8	0
3.14	0	8	7	8	8	8	8	8	8	8	8
	1	8	1	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0	8	0
34.0	0	8	7	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0	8	0
	3	8	1	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0	8	0
96.5	0	8	8	8	8	8	8	8	8	8	8
	1	8	0	8	0	8	0	8	0	8	0
	2	8	0	8	0	8	0	8	0	8	0
	3	8	0	8	0	8	0	8	0	8	0
	4	8	0	8	0	8	0	8	0	8	0

LOQ=0.10 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.

Treatment (mg a.i./L) [mean-measured]	Severity	Additional Diagnostic Observations[2]									
		Decreased Proportion of Spermatogonia		Increased Vascular or Interstitial Proteinaceous Fluid		Asynchronous Gonad Development		Altered Proportions of Spermatocytes or Spermatids		Granulomatous Inflammation	
		n[1]	Incidence	n[1]	Incidence	n[1]	Incidence	n[1]	Incidence	n[1]	Incidence
Negative Control (<LOQ)	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.245	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[2]											
	Severity	Decreased Proportion of Spermatogonia		Increased Vascular or Interstitial Proteinaceous Fluid		Asynchronous Gonad Development		Altered Proportions of Spermatocytes or Spermatids		Granulomatous Inflammation	
		n[1]	Incidence	n[1]	Incidence	n[1]	Incidence	n[1]	Incidence	n[1]	Incidence
3.14	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
34.0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[2]											
	Severity	Decreased Proportion of Spermatogonia		Increased Vascular or Interstitial Proteinaceous Fluid		Asynchronous Gonad Development		Altered Proportions of Spermatocytes or Spermatids		Granulomatous Inflammation	
		n[1]	Incidence	n[1]	Incidence	n[1]	Incidence	n[1]	Incidence	n[1]	Incidence
96.5	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviations: NA Not applicable.

LOQ=0.10 mg a.i./L

1 Number of individual fish observed

2 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.

Treatment (mg a.i./L) [mean-measured]	Diagnostic Observations[1]						
	Severity	Increased Oocyte Atresia		Perifollicular Cell Hyperplasia/ Hypertrophy		Decreased Yolk Formation	
		n	Incidence	n	Incidence	n	Incidence
Negative Control (<LOQ)	0	16	9	16	16	16	16
	1	16	7	16	0	16	0
	2	16	0	16	0	16	0
	3	16	0	16	0	16	0
	4	16	0	16	0	16	0
0.245	0	16	8	16	16	16	16
	1	16	5	16	0	16	0
	2	16	3	16	0	16	0
	3	16	0	16	0	16	0
	4	16	0	16	0	16	0
3.14	0	16	10	16.	16	16	16
	1	16	3	16	0	16	0
	2	16	2	16	0	16	0
	3	16	1	16	0	16	0
	4	16	0	16	0	16	0
34.0	0	16	7	16	16	16	16
	1	16	5	16	0	16	0
	2	16	3	16	0	16	0
	3	16	1	16	0	16	0
	4	16	0	16	0	16	0
96.5	0	15	11	15	15	15	15
	1	15	3	15	0	15	0
	2	15	1	15	0	15	0
	3	15	0	15	0	15	0
	4	15	0	15	0	15	0

LOQ=0.10 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.

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]								
	Severity	Interstitial Fibrosis		Egg Debris in Oviduct		Granulomatous Inflammation		Decreased Post-Ovulatory Follicles	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
Negative Control (<LOQ)	0	16	16	16	16	16	16	16	16
	1	16	0	16	0	16	0	16	0
	2	16	0	16	0	16	0	16	0
	3	16	0	16	0	16	0	16	0
	4	16	0	16	0	16	0	16	0
0.245	0	16	16	16	16	16	16	16	16
	1	16	0	16	0	16	0	16	0
	2	16	0	16	0	16	0	16	0
	3	16	0	16	0	16	0	16	0
	4	16	0	16	0	16	0	16	0
3.14	0	16	16	16	16	16	15	16	16
	1	16	0	16	0	16	1	16	0
	2	16	0	16	0	16	0	16	0
	3	16	0	16	0	16	0	16	0
	4	16	0	16	0	16	0	16	0
34.0	0	16	16	16	16	16	16	16	16
	1	16	0	16	0	16	0	16	0
	2	16	0	16	0	16	0	16	0
	3	16	0	16	0	16	0	16	0
	4	16	0	16	0	16	0	16	0

Treatment (mg a.i./L) [mean-measured]	Additional Diagnostic Observations[1]								
	Severity	Interstitial Fibrosis		Egg Debris in Oviduct		Granulomatous Inflammation		Decreased Post-Ovulatory Follicles	
		n	Incidence	n	Incidence	n	Incidence	n	Incidence
96.5	0	15	15	15	15	15	15	15	15
	1	15	0	15	0	15	0	15	0
	2	15	0	15	0	15	0	15	0
	3	15	0	15	0	15	0	15	0
	4	15	0	15	0	15	0	15	0

[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=0.10 mg a.i./L

Mean male plasma vitellogenin ranged from 0.909 ng/mL (34 mg a.i./L) to 1.77 ng/mL (0.245 mg a.i./L). Mean female plasma vitellogenin ranged from 19.8×10^6 ng/mL (3.14 mg a.i./L) to 55.1×10^6 ng/mL (0.245 mg a.i./L).

Table 19: Plasma Vitellogenin in Fathead Minnow.

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	1.34	1.73	4	27.6×10^6	21.5×10^6
0.245	4	1.77	1.25	4	55.1×10^6	47.5×10^6
3.14	4	0.930	0.777	4	19.8×10^6	7.69×10^6
34.0	4	0.909	0.591	4	21.0×10^6	14.4×10^6
96.5	4	1.29	0.983	4	31.6×10^6	12.7×10^6

SD Standard deviation.

n = number of independent replicates per treatment.

LOQ=0.10 mg a.i./L

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*). Not measured.

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	ND	ND	NA	ND	ND	NA	ND	ND	NA	ND	ND
0.245	NA	ND	ND	NA	ND	ND	NA	ND	ND	NA	ND	ND
3.14	NA	ND	ND	NA	ND	ND	NA	ND	ND	NA	ND	ND
34.0	NA	ND	ND	NA	ND	ND	NA	ND	ND	NA	ND	ND
96.5	NA	ND	ND	NA	ND	ND	NA	ND	ND	NA	ND	ND

Abbreviations: [NA] Not applicable. [ND] Not determined. [SD] Standard deviation.

LOQ=0.10 mg a.i./L

No abnormal behavior was observed among control or treatment fish, such as hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, and feeding abstinence. It was reported that one female fish in the 0.245 mg a.i./L treatment group lost an eye during the exposure

period which could have been from breeding activity and/or colliding with breeding substrate. Another female fish in the 3.14 mg a.i./L treatment group exhibited ascites (*i.e.*, accumulation of fluid in the coelomic cavity). Scoliosis, or bent tail, was displayed by one fish in the 96.5 mg a.i./L treatment group and one fish in the control group. Vertical banding was observed in both control and treatment group female test fish, but no treatment-related effects were observed. The raw data for this banding was not reported.

Table 21: Secondary Sex Characteristics and Clinical Signs in Fathead Minnow at Test Termination.

Treatment (mg a.i./L) [mean-measured]	Secondary Sex Characteristics and Clinical Signs					
	Males			Females		
	Type	n	Incidence	Type	n	Incidence
Negative Control (<LOQ)	None	NA	NA	None	NA	NA
0.245	None	NA	NA	None	NA	NA
3.14	None	NA	NA	None	NA	NA
34.0	None	NA	NA	None	NA	NA
96.5	None	NA	NA	None	NA	NA

Abbreviations: [NA] Not applicable.

LOQ=0.10 mg a.i./L

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

The study author reported the following statistical methodology:

The appropriate units of statistical analyses were the measures of central tendency from the replicate test vessels. The statistical significance of all tests was judged at the 0.05 significance level, with the exception of the Shapiro-Wilk test, which was judged at the 0.01 significance level. All biological response data, apart from mortality, were analyzed and reported separately by sex.

Endpoints were statistically evaluated with the Jonckheere-Terpstra test (Hollander and Wolfe, 1973) in a step down manner if the measures of central tendency for that endpoint were consistent with a monotone concentration-response. If the endpoint response was not consistent with a monotone concentration-response, the data were assessed for normality using the Shapiro-Wilk test and variance homogeneity using Levene's test. Where non-normality or variance heterogeneity was observed, normalizing and/or variance stabilizing transformations were applied. If the data were non monotonic and normally distributed with homogeneous variances, then a significant treatment effect was determined using the one way ANOVA followed by Dunnett's test. Where non-normality or variance heterogeneity was observed normalizing and/or variance stabilizing transformations was applied. If the data were normally distributed with homogeneous variances then a significant treatment effect was determined using the one way ANOVA followed by Dunnett's test. If the data were normally distributed with heterogeneous variance, the Mann-Whitney-Wilcoxon U test was used. If no normalizing transformation was found, the Mann-Whitney-Wilcoxon U test using a Bonferroni-Holm adjustment was used.

Significant mortality was assessed, if necessary, using the Cochran-Armitage Linear Trend Test where the data was consistent with a monotonic concentration response, and otherwise from Fisher's Exact test with a Bonferroni-Holm adjustment. A treatment effect for tubercle score was determined using the multiquantal Jonckheere-Terpstra test.

Female plasma VTG was reanalyzed by the study authors in an effort to demonstrate proficiency in the method as outlined in the 890.1350 guideline. Results of the reanalysis are presented in Appendix II to this DER.

The enzyme-linked immunosorbent assay (ELISA) test was used to analyze the vitellogenin (VTG) levels in male and female test fish blood plasma. An ELISA reanalysis of VTG concentrations in female fish demonstrated the ability of using the ELISA in accordance with the methods recommended by the 890.1350 guideline; insufficient male plasma was available for reanalysis. For the primary test, the correlation coefficient (R^2) for the calibration curves were all >0.99 (meeting 890.1350 standards); the concentrations for each standard fell between 82.1 and 115% of nominal concentrations (meeting 890.1350 standards); and the average absorbance measured for the non-specific binding assay blanks was less than 0.06 absorbance units (highest recommended level by 890.1350 guidelines) although one plate produced an average absorbance unit value of 0.067.

For the reanalysis of female VTG samples, the R^2 were all >0.99 and the concentrations for each standard fell between 70-120% of nominal (850.1350 recommended range) with the exception of one standard with a recovery of 144%. Two aliquots of each sample dilution were analyzed on the same ELISA plate (well duplicates) to verify the co-efficient of variation. It was reported that 74 of the 79 samples did not differ by more than 20%, and all were less than 31%, and thus these results indicated acceptable method precision. The actual response in the well-duplicate samples was greater than the primary concentration analysis. The study author indicated that it has been shown that concentrations of VTG may increase when re-analyzed following a freeze-thaw cycle (Brodeur, *et al.*, 2006).

C. Reviewer's Analysis and Conclusions

Statistical Methods: Statistical analyses were recalculated by the reviewer following decision logic provided in test guidelines. Continuous data were tested for normality using Shapiro-Wilks test and for homogeneity of variance using Levene's test. Data that demonstrated a monotonic trend were analyzed using Jonckheere-Terpstra according the 890.1350 guideline flowchart and using Williams test according to EFED policy. Data that met the assumptions of normality and homogeneity of variance were then analyzed using Dunnett's test if the data did not demonstrate a monotonic concentration response. Data that failed the assumption of normality or homogeneity of variance were analyzed using the Mann-Whitney-Wilcoxon test. Mortality was assessed using the Fisher's exact test with a Bonferroni-Holm adjustment.

Conclusions: Fecundity was significantly decreased in the highest concentration tested, 96.5 mg a.i./L compared to the control. There was no significant difference for fertilization success (# embryos/# eggs

x 100), male tubercle score, male and female condition index (whole body length/weight), male and female GSI, or male and female VTG levels. No signs of abnormal behavior, appearance, or secondary sex characteristics in the treatment groups relative to the control. Additionally, the study author reports that there were no effects of 2,4-D on the histology or germ cell distribution (staging) of either the testes or ovaries of the test fish. While not concentration-dependent, an increase in the number of female ovaries that were observed as Stage 2 (compared to Stage 3 or 4) was reported for the 2,4-D treatments compared to the control; the number of Stage 2 ovaries was 0, 3, 4, 6, and 3 for the control, 0.245, 3.14, 34.0 and 96.5 mg a.i./L treatment group, respectively.

Table 22: Reproductive and HPG Endpoints[1,2,3] for Male Fathead Minnow in the FSTRA with 2,4-D.

Treatment (mg a.i./L) [mean-measured]	Tubercle Score		GSI		Gonadal Staging and Histo.	Plasma VTG		Plasma T		Plasma E2	
	Median	p	% Diff.	p	Effect? (Yes/No)	% Diff.	p	% Diff.	p	% Diff.	p
Negative Control (<LOQ)	31	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.245	31	0.995	2.29	0.998	No	32.1	0.954	NA	NA	NA	NA
3.14	35	0.925	-1.25	1.000	No	-30.3	0.962	NA	NA	NA	NA
34.0	33	0.978	-1.25	1.000	No	-32.1	0.954	NA	NA	NA	NA
96.5	34	0.748	8.32	0.817	No	-3.6	1.000	NA	NA	NA	NA
Statistical Test	Dunnett's[4]		Dunnett's		NA	Dunnett's		NA		NA	

NA Not applicable

LOQ=0.10 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 For percent (%) difference, positive values indicate an increase relative to the negative control, and negative values indicate a decrease relative to the negative control.

4 Mean tubercle scores: 32, 31, 34, 33, and 35 for the negative control and mean-measured 0.245, 3.14, 34.0, and 96.5 mg a.i./L treatment levels, respectively.

Table 23: Reproductive and HPG Endpoints[1,2,3] for Female Fathead Minnow in the FSTRA with 2,4-D.

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)	NA	NA	NA	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.245	-2.6	0.332	-0.64	0.975	0	NA	8.21	0.945	No	99.6	0.494	NA	NA	NA	NA
3.14	-13	0.210	-0.83	0.943	0	NA	-6.11	0.980	No	-28.4	0.889	NA	NA	NA	NA
34.0	-23	0.088	0.77	0.954	0	NA	-1.13	1.00	No	-23.5	0.678	NA	NA	NA	NA
96.5	-34	0.031	-1.16	0.839	0	NA	11.5	0.847	No	14.6	0.494	NA	NA	NA	NA
Statistical Test	Jonckheere-Terpstra		Dunnett's		NA		Dunnett's		NA	Mann-Whitney		NA		NA	

Abbreviations: Diff. Difference. E2 17β-estradiol. Fert. Fertilization. GSI Gonado-Somatic Index. Histo. Histopathology.

NA Not applicable. T Testosterone. VTG Vitellogenin.

LOQ=0.10 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 For percent (%) difference, positive values indicate an increase relative to the negative control, and negative values indicate a decrease relative to the negative control.

Table 24: Growth Endpoints[1,2,3] in the Fish Short-Term Reproduction Assay (FSTRA) with 2,4-D.

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)	NA	NA	NA	NA	NA	NA	NA	NA
0.245	4.20	0.914	2.67	0.979	2.96	0.579	0.59	0.992
3.14	3.36	0.958	2.04	0.992	1.26	0.959	-0.71	0.984
34.0	10.01	0.380	2.04	0.992	5.82	0.097	-0.12	1.000
96.5	4.48	0.895	1.57	0.997	4.32	0.273	0.18	1.000
Statistical Test	Dunnett's		Dunnett's		Dunnett's		Dunnett's	

LOQ=0.10 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 $p<0.05$.

[3] For percent (%) difference, positive values indicate an increase relative to the negative control, and negative values indicate a decrease relative to the negative control.

E. Study Deficiencies

Several deviations are listed in Section I. Materials and Methods of this DER. One test performance criterion was not met; the CV for the mean-measured concentration of the lowest treatment group was 43% which is greater than the guideline criterion of <20%. These deviations did not impact the interpretation of this study.

F. Reviewer's Comments

Two replicate spawning groups with performance ranking within the top 20 breeding groups were inadvertently excluded from assignment to the exposure period test vessels. The study author reported that this deviation did not impact the experimental test because the 20 spawning groups used for the exposure period met the minimum criteria as outlined in the 890.1350 guideline.

Ammonia and chlorine levels were measured in dilution water biannually. Ammonia levels were detected at 0.24 mg a.i./L, exceeding guideline recommendations of ≤1 µg/L. Additionally, OCSPP guidelines recommend chlorine levels <10 µg/L but chlorine in the dilution water was only quantified at a level <200 µg/L (chlorine was not detectable at 200 µg/L). Organic carbon levels in dilution water were not measured.

The study author's rationale for the decrease in test concentrations in the test vessels but not the stock solutions was the marked increase in growth and waste production from Day 7 to Day 14 which increased microbial populations. Test material microbial biodegradation had a greater percentage effect in the two lower treatment groups than the two highest treatment groups. The study author reported that biodegradation is known to occur under the conditions in the current study (Sinton *et al.,* 1986). It was noted that the variability in 2,4-D concentrations in the stock solutions might have occurred because the large stock volumes were not being continuously mixed during their use.

The reviewer reports the gonad histopathological results as they were reported by the study author (Table 6). The stage and severity grading of the gonads was conducted in accordance with the procedures described in the 890.1350 guidelines. Gonad sections were examined under a light microscope by an American College of Veterinary Pathologists board-certified veterinary pathologist.

III. REFERENCES

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APPENDIX I. OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

Endpoint	Monotonic?	Parametric?	890.1350	EFED	Comments
Survival Overall:	No	NA	Fisher's Exact Test n.s p>0.05	Fisher's Exact Test n.s p>0.05	No significant effect on overall survival
Female body weight	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Male body weight	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Female body length	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Male body length	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Female VTG	Yes	Yes	Mann Whitney; n.s. p>0.05	Mann Whitney; n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Male VTG	Yes	No	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Female GSI	Yes	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Male GSI	No	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Female tubercle score	NA	NA	NA	NA	No score

Endpoint	Monotonic?	Parametric?	890.1350	EFED	Comments
Male tubercle score	Yes	Yes	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
Fecundity	No	Yes	Jonckheere-Terpstra: dose 3 p=0.011	Williams; dose 3 p=0.031	890.1350 and EFED same conclusions, effect at dose 3
Fertility	Yes	No	Dunnett's: n.s. p>0.05	Dunnett's: n.s. p>0.05	890.1350 and EFED same conclusions, no effect
F testosterone	NA	NA	NA	NA	NA
M testosterone	NA	NA	NA	NA	NA
F estradiol	NA	NA	NA	NA	NA
M estradiol	NA	NA	NA	NA	NA

Appendix II: Reanalysis of Female Plasma VTG.

Table: Plasma Vitellogenin in Fathead Minnow (Results of Reanalysis of Female Samples).

Treatment (mg a.i./L) [measured]	Plasma Vitellogenin (VTG)		
	Females		
	n	Mean (ng/mL plasma)	±SD
Negative Control	4	59.8×10^6	26.6×10^6
0.245	4	74.8×10^6	31.0×10^6
3.14	4	55.7×10^6	18.8×10^6
34.0	4	58.7×10^6	33.7×10^6
96.5	4	92.5×10^6	27.6×10^6

SD Standard deviation.

n = number of independent replicates per treatment

Appendix III: Reviewer's Statistical Output.

Fisher's Exact Test

IDENTIFICATION	NUMBER OF ALIVE	NUMBER OF DEAD	NUMBER OF TOTAL ANIMALS
CONTROL	16	0	16
0.245	16	0	16
TOTAL	32	0	32

Critical Fisher's value (16,16,16) (alpha=0.05) is 11.0. b value is 16.
Since b is greater than 11.0 there is no significant difference
between CONTROL and TREATMENT at the 0.05 level.

Fisher's Exact Test

IDENTIFICATION	NUMBER OF ALIVE	NUMBER OF DEAD	NUMBER OF TOTAL ANIMALS
CONTROL	16	0	16
3.14	16	0	16
TOTAL	32	0	32

Critical Fisher's value (16,16,16) (alpha=0.05) is 11.0. b value is 16.
Since b is greater than 11.0 there is no significant difference
between CONTROL and TREATMENT at the 0.05 level.

Fisher's Exact Test

IDENTIFICATION	NUMBER OF ALIVE	DEAD	TOTAL ANIMALS
CONTROL	16	0	16
34.0	16	0	16
TOTAL	32	0	32

Critical Fisher's value (16,16,16) (alpha=0.05) is 11.0. b value is 16.
Since b is greater than 11.0 there is no significant difference
between CONTROL and TREATMENT at the 0.05 level.

Fisher's Exact Test

IDENTIFICATION	NUMBER OF ALIVE	DEAD	TOTAL ANIMALS
CONTROL	16	0	16
96.5	15	1	16
TOTAL	31	1	32

Critical Fisher's value (16,16,16) (alpha=0.05) is 11.0. b value is 15.
Since b is greater than 11.0 there is no significant difference
between CONTROL and TREATMENT at the 0.05 level.

Summary of Fisher's Exact Tests

GROUP	IDENTIFICATION	NUMBER EXPOSED	NUMBER DEAD	SIG 0.05
	CONTROL	16	0	
1	0.245	16	0	
2	3.14	16	0	
3	34.0	16	0	
4	96.5	16	1	

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR01 (F body weight (g))

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.952	0.390	2.884	0.059	USE PARAMETRIC TESTS

****
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval	
Ctrl	4	1.59	0.17	0.08	10.51	1.33,	1.86
Dose1	4	1.64	0.07	0.04	4.48	1.52,	1.75
Dose2	4	1.63	0.17	0.09	10.65	1.35,	1.90
Dose3	4	1.63	0.17	0.09	10.52	1.35,	1.90
Dose4	4	1.62	0.08	0.04	5.11	1.49,	1.75

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	1.53	1.47	1.84	.	.
Dose1	1.61	1.58	1.74	102.67	-2.67
Dose2	1.64	1.43	1.79	102.04	-2.04
Dose3	1.63	1.45	1.80	102.04	-2.04
Dose4	1.62	1.52	1.72	101.57	-1.57

****
PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
4	15	0.05	0.994

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5

```
 Ctrl     1.59    .         1.62      .        .       .       .       .       .
 Dose1    1.64   0.979      1.62     0.692     .       .       .       .       .
 Dose2    1.63   0.992      1.62     0.727    1.000    .       .       .       .
 Dose3    1.63   0.992      1.62     0.746    1.000   1.000    .       .       .
 Dose4    1.62   0.997      1.62     0.750    1.000   1.000   1.000    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 0.38       0.984

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       1.53                    .                        .
  Dose1      1.61                  0.343                    0.877
  Dose2      1.64                  1.000                    0.722
  Dose3      1.63                  0.889                    0.593
  Dose4      1.62                  0.676                    0.579

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.05        0.994

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -1.59    .        -1.59      .        .       .       .       .       .
 Dose1   -1.64   0.979     -1.63     0.441     .       .       .       .       .
 Dose2   -1.63   0.992     -1.63     0.470    1.000    .       .       .       .
 Dose3   -1.63   0.992     -1.63     0.487    1.000   1.000    .       .       .
 Dose4   -1.62   0.997     -1.63     0.497    1.000   1.000   1.000    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 0.38       0.984

MannWhit - testing each trt median signif. different from control
```

Jonckheere - test assumes dose-response relationship, testing INCREASING trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	-1.53	.	.
Dose1	-1.61	0.343	0.123
Dose2	-1.64	1.000	0.278
Dose3	-1.63	0.889	0.407
Dose4	-1.62	0.676	0.421

INCREASING TREND TEST SUMMARY LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL

Williams >highest dose (no sign. differences)
Jonckheere >highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR02 (M body weight (g))

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.970	0.762	2.364	0.100	USE PARAMETRIC TESTS

****
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval	
Ctrl	4	3.57	0.14	0.07	3.99	3.34,	3.80
Dose1	4	3.72	0.31	0.15	8.32	3.23,	4.21
Dose2	4	3.69	0.14	0.07	3.91	3.46,	3.92
Dose3	4	3.93	0.49	0.24	12.44	3.15,	4.70
Dose4	4	3.73	0.41	0.20	10.91	3.08,	4.38

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	3.53	3.45	3.77	.	.
Dose1	3.69	3.38	4.12	104.20	-4.20
Dose2	3.65	3.57	3.90	103.36	-3.36
Dose3	3.85	3.45	4.57	110.01	-10.01
Dose4	3.75	3.25	4.17	104.48	-4.48

****
PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
4	15	0.61	0.660

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level Mean Dunnett Isotonic Williams Tukey p-values

		p-value	mean	p-value	Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	3.57	.	3.73	.	.	.	.	.	.
Dose1	3.72	0.914	3.73	0.826	.	.	.	.	.
Dose2	3.69	0.958	3.73	0.855	1.000	.	.	.	.
Dose3	3.93	0.380	3.73	0.870	0.895	0.842	.	.	.
Dose4	3.73	0.895	3.73	0.879	1.000	1.000	0.911	.	.

```
*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 1.83       0.766

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend
```

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	3.53	.	.
Dose1	3.69	0.678	0.718
Dose2	3.65	0.283	0.830
Dose3	3.85	0.413	0.920
Dose4	3.75	0.678	0.841

```
 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.61        0.660

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC
```

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	-3.57	.	-3.57	.	.	.	.	.	.
Dose1	-3.72	0.914	-3.71	0.340	.	.	.	.	.
Dose2	-3.69	0.958	-3.71	0.364	1.000	.	.	.	.
Dose3	-3.93	0.380	-3.83	0.186	0.895	0.842	.	.	.
Dose4	-3.73	0.895	-3.83	0.190	1.000	1.000	0.911	.	.

```
*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 1.83       0.766
```

```
MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -3.53                    .                          .
  Dose1     -3.69                  0.678                      0.282
  Dose2     -3.65                  0.283                      0.170
  Dose3     -3.85                  0.413                      0.080
  Dose4     -3.75                  0.678                      0.159

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR03  ( F body length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.939         0.225          1.586      0.229    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    42.43     1.33       0.67        3.14          40.30,   44.55
  Dose1  4    42.68     0.57       0.28        1.33          41.77,   43.58
  Dose2  4    42.13     0.92       0.46        2.19          40.66,   43.59
  Dose3  4    42.38     1.49       0.74        3.51          40.01,   44.74
  Dose4  4    42.50     0.81       0.41        1.91          41.21,   43.79

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        42.20    41.10      44.20         .                    .
  Dose1       42.65    42.10      43.30      100.59               -0.59
  Dose2       42.45    40.80      42.80       99.29                0.71
  Dose3       42.25    40.90      44.10       99.88                0.12
  Dose4       42.85    41.30      43.00      100.18               -0.18

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.14        0.966

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC
```

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	42.43	.	42.55	.	.	.	.	.	.
Dose1	42.68	0.992	42.55	0.651	.	.	.	.	.
Dose2	42.13	0.984	42.33	0.565	0.948	.	.	.	.
Dose3	42.38	1.000	42.33	0.583	0.994	0.997	.	.	.
Dose4	42.50	1.000	42.33	0.595	0.999	0.987	1.000	.	.

```
*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 1.03       0.905

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend
```

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	42.20	.	.
Dose1	42.65	0.678	0.718
Dose2	42.45	1.000	0.442
Dose3	42.25	0.889	0.463
Dose4	42.85	0.676	0.566

```
 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.14        0.966

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC
```

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	-42.43	.	-42.40	.	.	.	.	.	.
Dose1	-42.68	0.992	-42.40	0.597	.	.	.	.	.
Dose2	-42.13	0.984	-42.40	0.632	0.948	.	.	.	.
Dose3	-42.38	1.000	-42.40	0.651	0.994	0.997	.	.	.
Dose4	-42.50	1.000	-42.50	0.605	0.999	0.987	1.000	.	.

```
*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 1.03       0.905
```

```
MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -42.20                    .                        .
  Dose1    -42.65                  0.678                    0.282
  Dose2    -42.45                  1.000                    0.558
  Dose3    -42.25                  0.889                    0.537
  Dose4    -42.85                  0.676                    0.434

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR04  ( M body length (mm) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.938         0.222          1.295      0.316    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    51.55     1.54       0.77        2.98          49.10,   54.00
  Dose1  4    53.08     1.35       0.67        2.53          50.93,   55.22
  Dose2  4    52.20     0.91       0.45        1.74          50.75,   53.65
  Dose3  4    54.55     2.21       1.11        4.06          51.03,   58.07
  Dose4  4    53.78     2.46       1.23        4.58          49.85,   57.70

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        51.55    49.70      53.40         .                    .
  Dose1       52.60    52.10      55.00      102.96               -2.96
  Dose2       52.00    51.40      53.40      101.26               -1.26
  Dose3       54.85    52.00      56.50      105.82               -5.82
  Dose4       54.50    50.30      55.80      104.32               -4.32

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.80        0.181

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC
```

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	51.55	.	53.03	.	.	.	.	.	.
Dose1	53.08	0.579	53.03	0.927	.	.	.	.	.
Dose2	52.20	0.959	53.03	0.944	0.955	.	.	.	.
Dose3	54.55	0.097	53.03	0.953	0.769	0.378	.	.	.
Dose4	53.78	0.273	53.03	0.958	0.980	0.726	0.971	.	.

```
*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 6.12       0.190

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend
```

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	51.55	.	.
Dose1	52.60	0.235	0.926
Dose2	52.00	0.580	0.745
Dose3	54.85	0.125	0.966
Dose4	54.50	0.235	0.975

```
 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.80        0.181

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC
```

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	-51.55	.	-51.55	.	.	.	.	.	.
Dose1	-53.08	0.579	-52.64	0.241	.	.	.	.	.
Dose2	-52.20	0.959	-52.64	0.257	0.955	.	.	.	.
Dose3	-54.55	0.097	-54.16	0.035	0.769	0.378	.	.	.
Dose4	-53.78	0.273	-54.16	0.036	0.980	0.726	0.971	.	.

```
*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
```

```
           4                 6.12       0.190

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -51.55                     .                       .
  Dose1    -52.60                   0.235                   0.074
  Dose2    -52.00                   0.580                   0.255
  Dose3    -54.85                   0.125                   0.034
  Dose4    -54.50                   0.235                   0.025

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  Dose3
   Jonckheere                                Dose3

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR05  ( F vitellogenin (ng/mL) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.932         0.172          4.120      0.019    USE NON-PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4 27595000 21470025   10735012       77.80       -6568601,61758601
  Dose1  4 55085000 47464483   23732241       86.17       -2.044E7,1.3061E8
  Dose2  4 19765000  7687585    3843793       38.89        7532336,31997664
  Dose3  4 21102500 14406782    7203391       68.27       -1821905,44026905
  Dose4  4 31632500 12736128    6368064       40.26       11366478,51898522

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl     20850000 10090000   58590000         .                    .
  Dose1    47025000  9630000   1.1666E8      199.62               -99.62
  Dose2    20845000  9690000   27680000       71.63                28.37
  Dose3    14660000 12450000   42640000       76.47                23.53
  Dose4    29195000 18870000   49270000      114.63               -14.63

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.30        0.315

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
```

Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	27595000	.	41340000	.	.	.	.	.	.
Dose1	55085000	0.373	41340000	0.852	.	.	.	.	.
Dose2	19765000	0.976	24166667	0.532	0.316	.	.	.	.
Dose3	21102500	0.988	24166667	0.550	0.350	1.000	.	.	.
Dose4	31632500	0.998	24166667	0.561	0.682	0.960	0.974	.	.

****
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
4	3.43	0.489

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	20850000	.	.
Dose1	47025000	0.494	0.807
Dose2	20845000	0.889	0.442
Dose3	14660000	0.678	0.257
Dose4	29195000	0.494	0.655

DECREASING TREND TEST SUMMARY LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
Williams >highest dose (no sign. differences)
Jonckheere >highest dose (no sign. differences)

****
PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Analysis of Variance (ANOVA) - overall F-test

Numerator df	Denominator df	F-stat	P-value
4	15	1.30	0.315

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level	Mean	Dunnett p-value	Isotonic mean	Williams p-value	Tukey p-values Dose1	Dose2	Dose3	Dose4	Dose5
Ctrl	-2.76E7	.	-2.76E7	.	.	.	.	.	.
Dose1	-5.509E7	0.373	-3.19E7	0.479	.	.	.	.	.
Dose2	-1.977E7	0.976	-3.19E7	0.510	0.316	.	.	.	.
Dose3	-2.11E7	0.988	-3.19E7	0.527	0.350	1.000	.	.	.
Dose4	-3.163E7	0.998	-3.19E7	0.538	0.682	0.960	0.974	.	.

****
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

```
     Degrees of Freedom   TestStat    P-value
           4                 3.43       0.489

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl   -2.085E7                    .                          .
  Dose1  -4.703E7                  0.494                      0.193
  Dose2  -2.085E7                  0.889                      0.558
  Dose3  -1.466E7                  0.678                      0.743
  Dose4   -2.92E7                  0.494                      0.345

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR06  ( M vitellogenin (ng/mL) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.904         0.048          2.073      0.135    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4  1337.00  1735.75     867.87      129.82       -1424.96, 4098.96
  Dose1  4  1766.25  1249.84     624.92       70.76        -222.52, 3755.02
  Dose2  4   932.00   779.35     389.67       83.62        -308.11, 2172.11
  Dose3  4   907.75   589.11     294.55       64.90         -29.65, 1845.15
  Dose4  4  1288.50   981.96     490.98       76.21        -274.01, 2851.01

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl       492.00   424.00    3940.00         .                    .
  Dose1     1769.00   447.00    3080.00      132.11               -32.11
  Dose2      807.50   153.00    1960.00       69.71                30.29
  Dose3      750.50   380.00    1750.00       67.89                32.11
  Dose4     1177.00   360.00    2440.00       96.37                 3.63

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.38        0.819

Dunnett - testing each trt mean signif. different than control
```

```
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl  1337.00    .      1551.63      .        .       .       .       .       .
 Dose1 1766.25   0.954   1551.63     0.691     .       .       .       .       .
 Dose2  932.00   0.962   1042.75     0.456    0.835    .       .       .       .
 Dose3  907.75   0.954   1042.75     0.472    0.821   1.000    .       .       .
 Dose4 1288.50   1.000   1042.75     0.482    0.974   0.991   0.989    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 1.44       0.837

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     492.00                    .                        .
  Dose1   1769.00                   0.678                    0.718
  Dose2    807.50                   0.889                    0.500
  Dose3    750.50                   0.889                    0.390
  Dose4   1177.00                   0.889                    0.447

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.38        0.819

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl -1337.00    .     -1235.75      .        .       .       .       .       .
 Dose1-1766.25   0.954  -1235.75     0.636     .       .       .       .       .
 Dose2 -932.00   0.962  -1235.75     0.671    0.835    .       .       .       .
 Dose3 -907.75   0.954  -1235.75     0.690    0.821   1.000    .       .       .
 Dose4-1288.50   1.000  -1288.50     0.674    0.974   0.991   0.989    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
```

```
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 1.44       0.837

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl    -492.00                    .                        .
  Dose1  -1769.00                   0.678                    0.282
  Dose2   -807.50                   0.889                    0.500
  Dose3   -750.50                   0.889                    0.610
  Dose4  -1177.00                   0.889                    0.553

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR07  ( F GSI )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.928         0.143          0.624      0.652    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    11.94     1.65       0.83       13.83           9.31,   14.57
  Dose1  4    12.92     2.29       1.15       17.74           9.27,   16.57
  Dose2  4    11.21     2.58       1.29       22.98           7.11,   15.31
  Dose3  4    11.81     3.44       1.72       29.16           6.33,   17.29
  Dose4  4    13.32     2.02       1.01       15.16          10.10,   16.53

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        11.59    10.48      14.12         .                    .
  Dose1       13.27    10.12      15.04      108.21               -8.21
  Dose2       11.81     7.89      13.34       93.89                6.11
  Dose3       12.77     6.88      14.81       98.87                1.13
  Dose4       13.71    10.52      15.33      111.49              -11.49

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.48        0.748
```

```
Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    11.94    .        12.43      .        .       .       .       .       .
 Dose1   12.92   0.945     12.43     0.696     .       .       .       .       .
 Dose2   11.21   0.980     12.11     0.659    0.861    .       .       .       .
 Dose3   11.81   1.000     12.11     0.678    0.966   0.997    .       .       .
 Dose4   13.32   0.847     12.11     0.690    0.999   0.750   0.906    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 2.60       0.627

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      11.59                   .                        .
  Dose1     13.27                  0.678                    0.718
  Dose2     11.81                  0.678                    0.330
  Dose3     12.77                  0.678                    0.463
  Dose4     13.71                  0.494                    0.787

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                >highest dose (no sign. differences)
   Jonckheere                              >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.48        0.748

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -11.94    .       -11.94      .        .       .       .       .       .
 Dose1  -12.92   0.945    -11.98     0.574     .       .       .       .       .
 Dose2  -11.21   0.980    -11.98     0.608    0.861    .       .       .       .
 Dose3  -11.81   1.000    -11.98     0.627    0.966   0.997    .       .       .
 Dose4  -13.32   0.847    -13.32     0.301    0.999   0.750   0.906    .       .

*******************************************************************************
```

```
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 2.60       0.627

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -11.59                    .                        .
  Dose1    -13.27                  0.678                    0.282
  Dose2    -11.81                  0.678                    0.670
  Dose3    -12.77                  0.678                    0.537
  Dose4    -13.71                  0.494                    0.213

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR08  ( M GSI )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.944         0.289          2.115      0.129    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     1.20     0.14       0.07       11.96           0.97,    1.43
  Dose1  4     1.23     0.22       0.11       18.26           0.87,    1.59
  Dose2  4     1.19     0.10       0.05        8.43           1.03,    1.35
  Dose3  4     1.19     0.23       0.11       19.11           0.83,    1.55
  Dose4  4     1.30     0.10       0.05        7.61           1.14,    1.46

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl         1.25     1.00       1.31         .                    .
  Dose1        1.30     0.92       1.41      102.29               -2.29
  Dose2        1.21     1.05       1.29       98.75                1.25
  Dose3        1.21     0.94       1.39       98.75                1.25
  Dose4        1.31     1.19       1.40      108.32               -8.32

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.33        0.856
```

```
Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     1.20    .         1.22      .        .       .       .       .       .
 Dose1    1.23   0.998      1.22     0.651     .       .       .       .       .
 Dose2    1.19   1.000      1.22     0.686    0.996    .       .       .       .
 Dose3    1.19   1.000      1.22     0.705    0.996   1.000    .       .       .
 Dose4    1.30   0.817      1.22     0.717    0.972   0.867   0.867    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 1.55       0.817

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       1.25                    .                        .
  Dose1      1.30                   0.678                    0.718
  Dose2      1.21                   0.780                    0.384
  Dose3      1.21                   0.889                    0.444
  Dose4      1.31                   0.494                    0.703

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.33        0.856

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    -1.20    .        -1.20      .        .       .       .       .       .
 Dose1   -1.23   0.998     -1.20     0.586     .       .       .       .       .
 Dose2   -1.19   1.000     -1.20     0.620    0.996    .       .       .       .
 Dose3   -1.19   1.000     -1.20     0.639    0.996   1.000    .       .       .
 Dose4   -1.30   0.817     -1.30     0.281    0.972   0.867   0.867    .       .
```

```
*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 1.55       0.817

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      -1.25                    .                        .
  Dose1     -1.30                  0.678                    0.282
  Dose2     -1.21                  0.780                    0.616
  Dose3     -1.21                  0.889                    0.556
  Dose4     -1.31                  0.494                    0.297

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR09  ( F tubercle score (median) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
       .             .              .          .       NO DATA FOR TEST

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4     0.00     0.00       0.00         .              .  ,     .
  Dose1  4     0.00     0.00       0.00         .              .  ,     .
  Dose2  4     0.00     0.00       0.00         .              .  ,     .
  Dose3  4     0.00     0.00       0.00         .              .  ,     .
  Dose4  4     0.00     0.00       0.00         .              .  ,     .

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl         0.00     0.00       0.00         .                  .
  Dose1        0.00     0.00       0.00         .                  .
  Dose2        0.00     0.00       0.00         .                  .
  Dose3        0.00     0.00       0.00         .                  .
  Dose4        0.00     0.00       0.00         .                  .

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
```

```
 .         .      .    1     .        .        .       .       .       .       .


Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     0.00    .          .        .        .       .       .       .       .
 Dose1    0.00    .          .        .        .       .       .       .       .
 Dose2    0.00    .          .        .        .       .       .       .       .
 Dose3    0.00    .          .        .        .       .       .       .       .
 Dose4    0.00    .          .        .        .       .       .       .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                0.00       1.000

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       0.00                    .                       .
  Dose1      0.00                  1.000                     .
  Dose2      0.00                  1.000                     .
  Dose3      0.00                  1.000                     .
  Dose4      0.00                  1.000                     .

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                 Dose1
   Jonckheere                               Dose1

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
 .         .      .    1     .        .        .       .       .       .       .


Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl     0.00    .          .        .        .       .       .       .       .
 Dose1    0.00    .          .        .        .       .       .       .       .
 Dose2    0.00    .          .        .        .       .       .       .       .
```

```
 Dose3    0.00    .          .        .        .       .       .       .
 Dose4    0.00    .          .        .        .       .       .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                0.00       1.000

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl       0.00                     .                       .
  Dose1      0.00                   1.000                     .
  Dose2      0.00                   1.000                     .
  Dose3      0.00                   1.000                     .
  Dose4      0.00                   1.000                     .

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  Dose1
   Jonckheere                                Dose1

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR10  ( M tubercle score (median) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.963         0.608          0.683      0.615    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    32.00     4.02       2.01       12.56          25.60,   38.40
  Dose1  4    31.25     3.88       1.94       12.43          25.07,   37.43
  Dose2  4    33.63     4.96       2.48       14.74          25.74,   41.51
  Dose3  4    33.13     1.97       0.99        5.96          29.98,   36.27
  Dose4  4    34.50     3.08       1.54        8.93          29.60,   39.40

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        31.25    28.00      37.50         .                    .
  Dose1       31.00    27.00      36.00       97.66                 2.34
  Dose2       34.50    27.00      38.50      105.08                -5.08
  Dose3       32.50    31.50      36.00      103.52                -3.52
  Dose4       34.25    31.00      38.50      107.81                -7.81

*******************************************************************************
```

```
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.48        0.749

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    32.00    .        32.90      .        .       .       .       .       .
 Dose1   31.25   0.995     32.90     0.719     .       .       .       .       .
 Dose2   33.63   0.925     32.90     0.754    0.891    .       .       .       .
 Dose3   33.13   0.978     32.90     0.772    0.950   1.000    .       .       .
 Dose4   34.50   0.748     32.90     0.783    0.732   0.997   0.984    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 2.41       0.661

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      31.25                    .                        .
  Dose1     31.00                  0.889                    0.386
  Dose2     34.50                  0.678                    0.722
  Dose3     32.50                  0.491                    0.745
  Dose4     34.25                  0.346                    0.879

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.48        0.749

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -32.00    .       -31.63      .        .       .       .       .       .
 Dose1  -31.25   0.995    -31.63     0.642     .       .       .       .       .
```

```
 Dose2  -33.63   0.925    -33.38     0.388    0.891    .       .       .       .
 Dose3  -33.13   0.978    -33.38     0.401    0.950   1.000    .       .       .
 Dose4  -34.50   0.748    -34.50     0.241    0.732   0.997   0.984    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 2.41       0.661

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -31.25                    .                        .
  Dose1    -31.00                  0.889                    0.614
  Dose2    -34.50                  0.678                    0.278
  Dose3    -32.50                  0.491                    0.255
  Dose4    -34.25                  0.346                    0.121

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR11  ( fecundity )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.964         0.633          0.391      0.812    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    29.80     6.40       3.20       21.47          19.62,   39.98
  Dose1  4    29.03     6.47       3.23       22.28          18.74,   39.31
  Dose2  4    25.93     5.27       2.64       20.34          17.53,   34.32
  Dose3  4    22.88     8.72       4.36       38.14           8.99,   36.76
  Dose4  4    19.70     5.97       2.98       30.29          10.20,   29.20

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        28.30    24.00      38.60         .                    .
  Dose1       29.05    22.90      35.10       97.40                 2.60
  Dose2       27.15    18.90      30.50       87.00                13.00
  Dose3       22.85    12.50      33.30       76.76                23.24
  Dose4       18.35    14.00      28.10       66.11                33.89
```

```
*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.61        0.223

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    29.80    .        29.80      .        .       .       .       .       .
 Dose1   29.03   0.999     29.03     0.513     .       .       .       .       .
 Dose2   25.93   0.826     25.93     0.271    0.962    .       .       .       .
 Dose3   22.88   0.418     22.88     0.105    0.693   0.965    .       .       .
 Dose4   19.70   0.146     19.70     0.031    0.322   0.683   0.959    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 5.36       0.252

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      28.30                    .                        .
  Dose1     29.05                   0.780                    0.332
  Dose2     27.15                   0.678                    0.210
  Dose3     22.85                   0.346                    0.088
  Dose4     18.35                   0.156                    0.011

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                   Dose4
   Jonckheere                                 Dose4

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.61        0.223

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl   -29.80    .       -25.47      .        .       .       .       .       .
```

```
 Dose1  -29.03   0.999    -25.47     0.884     .       .       .       .       .
 Dose2  -25.93   0.826    -25.47     0.907    0.962    .       .       .       .
 Dose3  -22.88   0.418    -25.47     0.919    0.693   0.965    .       .       .
 Dose4  -19.70   0.146    -25.47     0.926    0.322   0.683   0.959    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 5.36       0.252

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -28.30                    .                        .
  Dose1    -29.05                  0.780                    0.668
  Dose2    -27.15                  0.678                    0.790
  Dose3    -22.85                  0.346                    0.912
  Dose4    -18.35                  0.156                    0.989

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR12  ( fertility )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.944         0.281          2.641      0.075    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   4    96.93     0.50       0.25        0.52          96.13,   97.72
  Dose1  4    96.30     3.08       1.54        3.19          91.41,  101.19
  Dose2  4    96.13     1.54       0.77        1.60          93.68,   98.57
  Dose3  4    97.68     0.78       0.39        0.80          96.43,   98.92
  Dose4  4    95.80     2.68       1.34        2.79          91.54,  100.06

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl        97.10    96.20      97.30         .                    .
  Dose1       97.75    91.70      98.00       99.36                 0.64
  Dose2       96.60    93.90      97.40       99.17                 0.83
  Dose3       97.70    96.70      98.60      100.77                -0.77
  Dose4       95.30    93.20      99.40       98.84                 1.16
```

```
*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.56        0.697

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    96.93    .        96.93      .        .       .       .       .       .
 Dose1   96.30   0.975     96.70     0.515     .       .       .       .       .
 Dose2   96.13   0.943     96.70     0.547    1.000    .       .       .       .
 Dose3   97.68   0.954     96.70     0.565    0.862   0.804    .       .       .
 Dose4   95.80   0.839     95.80     0.296    0.996   0.999   0.677    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 3.93       0.415

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      97.10                    .                          .
  Dose1     97.75                  0.343                      0.877
  Dose2     96.60                  0.678                      0.385
  Dose3     97.70                  0.235                      0.825
  Dose4     95.30                  0.346                      0.474

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               0.56        0.697

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5
```

Ctrl	-96.93	.	-96.45	.	.	.	.	.	.
Dose1	-96.30	0.975	-96.45	0.717	.	.	.	.	.
Dose2	-96.13	0.943	-96.45	0.752	1.000	.	.	.	.
Dose3	-97.68	0.954	-96.74	0.693	0.862	0.804	.	.	.
Dose4	-95.80	0.839	-96.74	0.704	0.996	0.999	0.677	.	.

****
NON-PARAMETRIC ANALYSES - use alpha-level=0.05 for all tests
Kruskal-Wallis test - equality among treatment groups

Degrees of Freedom	TestStat	P-value
4	3.93	0.415

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

Level	Median	MannWhit p-value	Jonckheere p-value
Ctrl	-97.10	.	.
Dose1	-97.75	0.343	0.123
Dose2	-96.60	0.678	0.615
Dose3	-97.70	0.235	0.175
Dose4	-95.30	0.346	0.526

INCREASING TREND TEST SUMMARY LOWEST CONCENTRATION SIGNIF. GREATER THAN CONTROL

Williams	>highest dose (no sign. differences)
Jonckheere	>highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR13 (F VTG re-analysis (ng/mL))

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric analyses.

Shapiro-Wilks Test Stat	Shapiro-Wilks P-value	Levenes Test Stat	Levenes P-value	Conclusion
0.958	0.511	0.324	0.858	USE PARAMETRIC TESTS

****
BASIC SUMMARY STATISTICS

Level	N	Mean	StdDev	StdErr	Coef of Var	95% Conf.Interval
Ctrl	4	59.80	26.56	13.28	44.42	17.53, 102.06
Dose1	4	74.85	30.98	15.49	41.40	25.54, 124.15
Dose2	4	55.67	18.79	9.40	33.76	25.77, 85.57
Dose3	4	58.70	33.69	16.84	57.39	5.09, 112.31
Dose4	4	92.48	27.63	13.82	29.88	48.51, 136.45

Level	Median	Min	Max	%of Control(means)	%Reduction(means)
Ctrl	60.45	27.03	91.27	.	.
Dose1	67.38	46.50	118.13	125.16	-25.16
Dose2	59.01	32.96	71.71	93.10	6.90
Dose3	60.78	18.20	95.05	98.16	1.84

```
  Dose4       83.75    70.47     131.96      154.66             -54.66

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.21        0.346

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing negative trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5

 Ctrl    59.80    .        68.30      .        .       .       .       .       .
 Dose1   74.85   0.860     68.30     0.750     .       .       .       .       .
 Dose2   55.67   0.999     68.30     0.784    0.865    .       .       .       .
 Dose3   58.70   1.000     68.30     0.801    0.922   1.000    .       .       .
 Dose4   92.48   0.322     68.30     0.812    0.896   0.378   0.459    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 3.89       0.422

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl      60.45                    .                        .
  Dose1     67.38                   0.678                    0.718
  Dose2     59.01                   1.000                    0.442
  Dose3     60.78                   1.000                    0.426
  Dose4     83.75                   0.235                    0.871

 DECREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. LESS THAN CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.21        0.346

Dunnett - testing each trt mean signif. different than control
Williams - test assumes dose-response relationship, testing INCREASING trend
Tukey - two-sided tests, all possible comparisons, not used for NOEC or LOEC

Level    Mean   Dunnett  Isotonic  Williams                 Tukey p-values
                p-value    mean     p-value   Dose1   Dose2   Dose3   Dose4   Dose5
```

```
 Ctrl   -59.80    .       -59.80      .        .       .       .       .       .
 Dose1  -74.85   0.860    -63.07     0.513     .       .       .       .       .
 Dose2  -55.67   0.999    -63.07     0.545    0.865    .       .       .       .
 Dose3  -58.70   1.000    -63.07     0.563    0.922   1.000    .       .       .
 Dose4  -92.48   0.322    -92.48     0.078    0.896   0.378   0.459    .       .

*******************************************************************************
NON-PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Kruskal-Wallis test - equality among treatment groups
     Degrees of Freedom   TestStat    P-value
           4                 3.89       0.422

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl     -60.45                    .                        .
  Dose1    -67.38                  0.678                    0.282
  Dose2    -59.01                  1.000                    0.558
  Dose3    -60.78                  1.000                    0.574
  Dose4    -83.75                  0.235                    0.129

 INCREASING TREND TEST SUMMARY      LOWEST CONCENTRATION SIGNIF. GREATER THAN
CONTROL
   Williams                                  >highest dose (no sign. differences)
   Jonckheere                                >highest dose (no sign. differences)

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR14  ( M testosterone (ng/mL) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.958         0.511          0.324      0.858    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   0      .        .          .           .              .  ,     .
  Dose1  0      .        .          .           .              .  ,     .
  Dose2  0      .        .          .           .              .  ,     .
  Dose3  0      .        .          .           .              .  ,     .
  Dose4  0      .        .          .           .              .  ,     .

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl          .        .         .           .                   .
  Dose1         .        .         .           .                   .
  Dose2         .        .         .           .                   .
```

```
  Dose3         .        .          .           .                  .
  Dose4         .        .          .           .                  .

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.21        0.346

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl        .                      .                       .
  Dose1       .                      .                       .
  Dose2       .                      .                       .
  Dose3       .                      .                       .
  Dose4       .                      .                       .
   Jonckheere                                  Dose1

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.21        0.346

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl        .                      .                       .
  Dose1       .                      .                       .
  Dose2       .                      .                       .
  Dose3       .                      .                       .
  Dose4       .                      .                       .
   Jonckheere                                  Dose1

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR15  ( F 17b-estradiol (ng/mL) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.958         0.511          0.324      0.858    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   0      .        .          .           .              .  ,     .
```

```
  Dose1  0      .        .          .           .              .  ,     .
  Dose2  0      .        .          .           .              .  ,     .
  Dose3  0      .        .          .           .              .  ,     .
  Dose4  0      .        .          .           .              .  ,     .

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl          .        .          .           .                  .
  Dose1         .        .          .           .                  .
  Dose2         .        .          .           .                  .
  Dose3         .        .          .           .                  .
  Dose4         .        .          .           .                  .

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.21        0.346

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl        .                     .                       .
  Dose1       .                     .                       .
  Dose2       .                     .                       .
  Dose3       .                     .                       .
  Dose4       .                     .                       .
   Jonckheere                                  Dose1

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.21        0.346

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl        .                     .                       .
  Dose1       .                     .                       .
  Dose2       .                     .                       .
  Dose3       .                     .                       .
  Dose4       .                     .                       .
   Jonckheere                                  Dose1

test for fish screen study - TEST DATA 2 4 D
ANALYSIS RESULTS FOR VARIABLE VAR16  ( M 17b-estradiol (ng/mL) )

TESTS OF ASSUMPTIONS FOR PARAMETRIC ANALYSIS
Shapiro-Wilks test for Normality of Residuals -- alpha-level=0.01
Levenes test for homogeneity of variance(absolute residuals) -- alpha-level=0.05
```

```
Use parametric analyses if neither test rejected, otherwise non-parametric
analyses.
  Shapiro-Wilks  Shapiro-Wilks    Levenes    Levenes   Conclusion
    Test Stat      P-value       Test Stat   P-value
      0.958         0.511          0.324      0.858    USE PARAMETRIC TESTS

*******************************************************************************
BASIC SUMMARY STATISTICS
 Level  N     Mean    StdDev      StdErr    Coef of Var    95% Conf.Interval
  Ctrl   0      .        .          .           .              .  ,     .
  Dose1  0      .        .          .           .              .  ,     .
  Dose2  0      .        .          .           .              .  ,     .
  Dose3  0      .        .          .           .              .  ,     .
  Dose4  0      .        .          .           .              .  ,     .

 Level       Median     Min       Max    %of Control(means)   %Reduction(means)
  Ctrl          .        .          .           .                   .
  Dose1         .        .          .           .                   .
  Dose2         .        .          .           .                   .
  Dose3         .        .          .           .                   .
  Dose4         .        .          .           .                   .

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.21        0.346

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing negative trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl        .                     .                       .
  Dose1       .                     .                       .
  Dose2       .                     .                       .
  Dose3       .                     .                       .
  Dose4       .                     .                       .
   Jonckheere                                  Dose1

*******************************************************************************
PARAMETRIC ANALYSES    - use alpha-level=0.05 for all tests
    Analysis of Variance (ANOVA) - overall F-test
     Numerator df    Denominator df    F-stat       P-value
           4           15               1.21        0.346

MannWhit - testing each trt median signif. different from control
Jonckheere - test assumes dose-response relationship, testing INCREASING trend

 Level     Median          MannWhit p-value           Jonckheere p-value
  Ctrl        .                     .                       .
  Dose1       .                     .                       .
  Dose2       .                     .                       .
  Dose3       .                     .                       .
```

Dose4 . . .
Jonckheere Dose1

DATA EVALUATION RECORD

2,4-DICHLOROPHENOXY ACETIC ACID (2,4-D)

Study Type: OCSPP 890.1550, Steroidogenesis Assay

EPA Contract No. EP10H001452
Task Assignment No. 2-26-2012 (MRID 48614305)

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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Scott D. Studenberg, Ph.D., D.A.B.T.	Date:	3/22/2012
Program Manager:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	3/27/2012
Quality Assurance:	Signature:	Jack D. Early
Jack D. Early, M.S.	Date:	3/27/2012

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: Gregory Akerman **Signature:**
Health Effects Division **Date:** 6/15/15
Secondary Reviewer: John Liccione, Ph.D. **Signature:**
Health Effects Division **Date:** 5-21-15

Template version 08/2011

DATA EVALUATION RECORD

STUDY TYPE: Steroidogenesis Assay (H295R Cells); OCSPP 890.1550

PC CODE: 030001 **DP BARCODE:** D398637

TXR#: 0052104 **CAS No.:** 94-75-7

TEST MATERIAL (PURITY): 2,4-D (98.5% a.i.)

SYNONYMS: 2,4-dichlorophenoxyacetic acid; 2,4-D acid, 2-(2,4-dichlorophenoxy)acetic acid

CITATION: LeBaron, M.J.; Kan, H.L; Perala, A.W. (2011) Evaluation of 2,4-dichlorophenoxyacetic acid (2,4-D) in the *in vitro* steroidogenesis assay. Toxicology & Environmental Research and Consulting, The Dow Chemical Company, Midland, MI. Laboratory Study No.: 111038, October 20, 2011. MRID 48614305. Unpublished.

SPONSOR: Industry Task Force II on 2,4-D Research Data, 1900 K Street NW, Washington, DC

TEST ORDER #: CON-030001-1

EXECUTIVE SUMMARY: In a steroidogenesis assay (MRID 48614305), H295R cells cultured *in vitro* in 24-well plates were incubated with 2,4-D (98.5% purity, Lot # 2006 2433 8006-USA) at log concentrations of 10^{-10} to 10^{-4} M for 48 hours in triplicate in three independent experiments. Dimethyl sulfoxide (DMSO) was used as the vehicle, at a final concentration in the assay of 0.1%.

Testosterone and estradiol levels were measured using LC-APPI-MS/MS. 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. The positive controls included a known inhibitor (prochloraz) and a known inducer (forskolin) of estradiol and testosterone production.

Guideline acceptability recommendations and requirements were generally met, including lack of cytotoxicity, adequate production of testosterone and estradiol, acceptable reproducibility (low %CV), and appropriate induction and inhibition with positive controls, with two exceptions. The required concentration for estradiol production (40 pg/mL) was not met in any run for blank or solvent control, with values ranging from 26.7 to 38.3 pg/mL, but the basal concentrations were greater than 2.5-fold of the minimum detection level (10 pg/mL) as required. Secondly, all three

runs of 1 µM prochloraz only reduced estradiol to 0.6-fold that of the solvent control (instead of 0.5-fold).

2,4-D had no effect on testosterone production at concentrations up to 10^{-4} M, and no effect on estradiol production at concentrations up to 10^{-5} M. At the highest concentration tested (10^{-4} M), 2,4-D increased ($p \leq 0.05$) estradiol levels by 20% (1.2-fold) in all three runs relative to the DMSO-treated cells.

Based on hormone responses in each of the independent runs, 2,4-D treatment resulted in statistically significant and reproducible increases in estradiol production. 2,4-D treatment did not result in statistically significant and reproducible alterations in testosterone production.

The assay **satisfies** the EDSP Tier 1 Test Order requirements for a Steroidogenesis Assay (OCSPP 890.1550).

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

I. MATERIALS AND METHODS

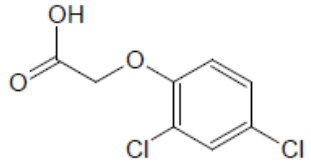
A. MATERIALS

1. Test Facility: Toxicology & Environmental Research and Consulting, The Dow Chemical Company

Location: Midland, MI
Study Director: H. L. Kan
Other Personnel: M. J. LeBaron (lead scientist), A. W. Perala (analytical chemist), B. B. Gollapudi,(technical reviewer)
Study Period: March 14, 2011 – October 20, 2011

2. Test Substance: 2,4-D

Description: Off-white powder
Lot # (expiration date): 2006 2433 8006-USA (not provided)
Purity: 98.5%
Solubility: Soluble in DMSO up to 0.1 M; up to 315 mg/L in water
Vapor pressure: 1.9×10^{-5} Pa at 25°C
Stability: 2 year shelf life; stability under test conditions not provided
Storage conditions: Ambient
CAS #: 94-75-7
Molecular weight: 221.0
Structure:



3. Positive Control (Inducer): Forskolin

Description (molecular weight): White powder (410.50)
Source: Sigma-Aldrich (St. Louis, MO)
Lot # (expiration date): 097K50653 (not provided)
Purity: 99%
Solubility (in solvent): Soluble in DMSO up to 0.01 M
Storage conditions: Ambient
CAS #: 66575-29-9

4. Positive Control (Inhibitor): Prochloraz

Description (molecular weight): Off-white powder (376.67)
Source: Sigma-Aldrich (St. Louis, MO)
Lot # (expiration date): SZE6220X (not provided)
Purity: 99.1%
Solubility (in solvent): Soluble in DMSO up to 0.01 M
Storage conditions: Ambient
CAS #: 67747-09-5

5. Solvent/Vehicle Control: Dimethyl Sulfoxide (DMSO)

Description (molecular weight): Clear liquid (78.13)
Source: Sigma-Aldrich (St. Louis, MO)
Lot # (expiration date): 16496CPV and 68996LMV (not provided)
Purity: ≥99.9%
Storage conditions: Room temperature
CAS #: 67-68-5
Justification for choice of solvent: Not provided. DMSO is a Guideline recommended solvent in which 2,4-D is reasonably soluble.
Final concentration in assay: 0.1% v/v

6. Stock Medium: Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture

Source:	Sigma-Aldrich (St. Louis, MO)
Lot # (expiration date):	RNBB 6106 (not provided)
Sodium bicarbonate:	Component of stock medium
Nu-Serum:	2.5%, BD Biosciences; Lot # 81515; tested for background hormone concentrations by performing laboratory
ITS+premix:	BD Biosciences; Lot # 88964 & 05245
Other components:	2.5 mM L-glutamine (Life Technologies, GIBCO, Grand Island, NY), and penicillin/streptomycin (25 IU/mL/25 µg/mL) (GIBCO).

7. Test Cells: H295R human adrenocortical carcinoma cells (ATCC CLR-2128; Batch # not provided) at passage 7.5 – 8.5 were incubated in the stock medium. Incubation conditions were at approximately 37° C with 5% CO_2 for approximately 24 hours prior to exposure.

The following performance criteria were met (indicated by an "x"):

X	Cell passage identifier. Cell Passage #: 7.5 – 8.5
X	Cells frozen down at passage 5
X	Frozen cells cultured for 4 additional passages
X	Total number of passages does not exceed 10

B. METHODS

1. Pre-Test Information

a. Hormone Assay Interference Test: A separate chemical interference assay was not performed, as LC/APPI-MS/MS was used along with internal spike-in controls to measure extraction and quantitation efficiency.

b. Hormone Extraction: Briefly, standards and samples were vortexed with methylene chloride containing both testosterone and estradiol internal standards, and the organic phase were transferred to clean vials. The aliquots were evaporated to dryness, and the final residue in each vial was derivatized with dansyl chloride in sodium bicarbonate buffer (100mM, pH 10.5). Samples were then subjected to LC/APPI-MS/MS analysis.

c. Laboratory Proficiency Test: The Sponsor stated that Laboratory proficiency assays were performed to optimize and validate the H295R steroidogenesis assay. These non-GLP, unpublished experimental results with positive control chemicals demonstrated laboratory proficiency and validation and are reported separately (data not presented and report not cited).

2. Test Solutions: 2,4-D, forskolin, and prochloraz were dissolved in DMSO and diluted 1:1000 in the final treatment medium. When added to the cell culture plates, these dilutions yielded final concentrations of 1 µM and 10 µM for forskolin, 0.1 µM and 1 µM for prochloraz, and 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M for 2,4-D. The selected concentrations of the test material in the dosing solutions used for the treatment in a definitive assay (generally the first assay) were verified analytically. The final concentration of DMSO in the medium was held constant at 0.1% (v/v).

3. **Cell Plating and Preincubation:** Cells were maintained in the stock medium described above. The hormone concentrations in undiluted Nu-Serum were 974 pg/mL for testosterone and 681 pg/mL for estradiol. H295R cells were grown for five passages, frozen in liquid nitrogen, then thawed and cultured for seven to eight additional passages prior to use in the assay. The cells were plated into wells of a 24-well cell culture plate at a density of approximately 200,000 – 300,000 cells in 1 mL (50 – 60% confluency). The cells were then placed into an incubator (approximately 37° C/5% CO_2) for approximately 24 hours prior to chemical exposure. After a 24-hour pre-incubation, the cells were checked microscopically for good attachment and proper morphology.

4. **Exposure:** The medium was removed from the cells and replaced with 1 mL of medium containing concentrations of 2,4-D (or solvent) in triplicate, according to the schematic presented in Table 1.

TABLE 1. Dosing Schematic for the Exposure of H295R Cells to 2,4-D (Final Concentrations in M).[a]

	1	2	3	4	5	6
A	DMSO	DMSO	DMSO	10^{-7}	10^{-7}	10^{-7}
B	10^{-4}	10^{-4}	10^{-4}	10^{-8}	10^{-8}	10^{-8}
C	10^{-5}	10^{-5}	10^{-5}	10^{-9}	10^{-9}	10^{-9}
D	10^{-6}	10^{-6}	10^{-6}	10^{-10}	10^{-10}	10^{-10}

a Data were obtained from page 16 of the study report.

A concurrent QC plate was included with each of the three 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	Blank + methanol[c]	Blank + methanol	Blank + methanol
B	DMSO	DMSO	DMSO	DMSO + methanol	DMSO + methanol	DMSO + methanol
C	Forskolin 1 µM	Forskolin 1 µM	Forskolin 1 µM	Prochloraz 0.1 µM	Prochloraz 0.1 µM	Prochloraz 0.1 µM
D	Forskolin 10 µM	Forskolin 10 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 1 µM	Prochloraz 1 µM

a Data were obtained from page 15 of the study report.
b Blank wells received medium only.
c Methanol (MeOH) was added after the exposure was terminated and the medium was removed.

Following dosing, the plates were incubated for approximately 48 hours under the conditions previously described. After the incubation period, two aliquots of medium were collected and frozen at −80°C until further processing.

5. **Cell Viability/Cytotoxicity Assay:** The cell viability/cytotoxicity testing was conducted in the QC plate and in the test chemical exposure plate immediately after termination of the exposure experiments. In addition to viability/cytotoxicity testing, cells were checked for the degree of confluence, homogeneity from well-to-well, and any signs of cytotoxicity or

altered morphology. A subjective parallel visual assessment of each well was conducted. The CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Madison Wisconsin, Cat# G3580) was used for assessment of cell viability testing, as per manufacturer's instructions. This cell proliferation assay kit is a colorimetric modified MTT cell viability assay. The Aqueous One Solution contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of viable cells in culture. The minimum cell viability required per well was ≥80%; wells with lower viability were excluded in the final data analysis.

6. **Hormone Measurement System:** The concentration of each hormone (testosterone and estradiol) was evaluated in triplicate in the culture medium. Hormones were extracted from the samples with methylene chloride, derivatized with dansyl chloride, and analyzed by LC/APPI-MS/MS. The lower limit of quantification (LLQ) for this measurement system was 25 pg/mL for testosterone and 10 pg/mL 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:** To evaluate the relative increase or decrease of hormone production after test chemical exposure, the results were normalized to the mean solvent control (SC) value for each assay and results were expressed as fold-change relative to the SC in each exposure plate. All data were expressed as mean ± Standard Deviation (SD). Relative changes were calculated as follows:

Relative Change = Hormone concentration in each well ÷ Hormone concentration of mean solvent (vehicle) control

Prior to conducting statistical analyses, the assumptions of normality and variance homogeneity were evaluated. Homogeneity of variance were evaluated by Bartlett's test and normality by Shapiro-Wilk's test at alpha = 0.01. If the data were not homogeneous or normally distributed, then the data were transformed to approximate homogeneity or a normal distribution. If the data were homogeneous and approximately normally distributed, differences between chemical treatments and SC were analyzed using a parametric analysis of variance followed by Dunnett's test, if significant. If the data were not homogeneous or normally distributed, a non-parametric test was used (Kruskal-Wallis) and if significant, was followed by the Wilcoxon rank sum test with a Bonferroni-Holm correction. Differences were considered significant at $p \leq 0.05$. These statistical analyses were considered appropriate.

II. RESULTS

A. TEST COMPOUND: Precipitation of the test compound was not reported at any concentration. Cytotoxicity was not observed at any tested 2,4-D concentration. The variability (CV) between the runs for the solvent controls was 4.6% (testosterone) and 14.6% (estradiol), both within the guideline recommendation of 30%. The variability (CV) within each run for the solvent controls in the testosterone and estradiol assays was 13.6% or less, which was within the guideline recommendation of 30%.

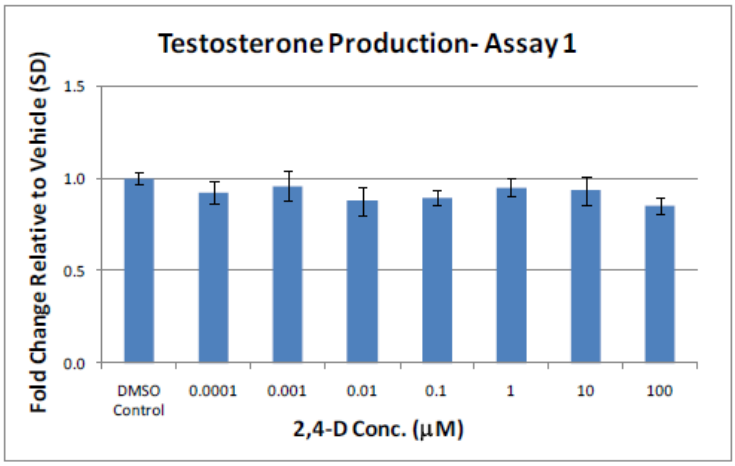
2,4-D had no effect on testosterone levels at any concentration tested (Table 3, Figure 1), and a slight effect on estradiol levels at 2,4-D concentrations of 10 µM or less. However, at a concentration of 100 µM, 2,4-D increased ($p \leq 0.05$) estradiol levels by 20% relative to DMSO-treated cells in all three runs (Figure 2).

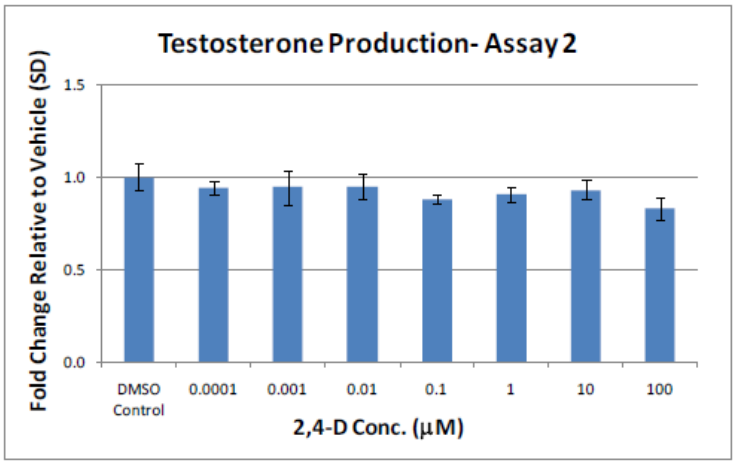
TABLE 3. Mean (±SD) Hormone Concentrations Following Treatment with 2,4-D for 48 Hours.[a]

Nominal Concentration (µM)	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Mean	± SD	Statistical Significance [b]
	Testosterone (pg/mL)			Fold Difference					
DMSO	842.0	735.0	778.0	—	—	—	—	—	—
0.0001	776.0	691.7	746.3	0.9	0.9	1.0	0.9	0.0	—
0.001	805.7	696.0	752.0	1.0	0.9	1.0	1.0	0.0	—
0.01	737.7	696.3	726.7	0.9	0.9	0.9	0.9	0.0	—
0.1	752.0	647.0	745.3	0.9	0.9	1.0	0.9	0.0	—
1	796.7	665.0	734.0	0.9	0.9	0.9	0.9	0.0	—
10	787.7	684.3	747.0	0.9	0.9	1.0	0.9	0.0	—
100	714.3	610.3	728.0	0.8	0.8	0.9	0.9	0.1	—
	Estradiol (pg/mL)			Fold Difference					
DMSO	29.8	35.7	37.0	—	—	—	—	—	—
0.0001	24.7	37.2	36.0	0.8	1.0	1.0	0.9	0.1	—
0.001	24.7	35.7	34.2	0.8	1.0	0.9	0.9	0.1	—
0.01	25.6	34.8	37.8	0.9	1.0	1.0	1.0	0.1	—
0.1	26.5	36.4	34.9	0.9	1.0	0.9	1.0	0.1	—
1	25.3	33.7	35.1	0.8	0.9	0.9	0.9	0.1	—
10	27.0	33.8	37.9	0.9	0.9	1.0	1.0	0.1	—
100	34.6	44.3	45.1	1.2	1.2	1.2	1.2	0.0	Runs 1, 2, 3

a Data were obtained from page 28 of the study report. The lower limit of quantification (LLQ) was 25 pg/mL for testosterone and 10 pg/mL for estradiol.

FIGURE 1. Change in Testosterone Production Relative to 2,4-D Concentration.





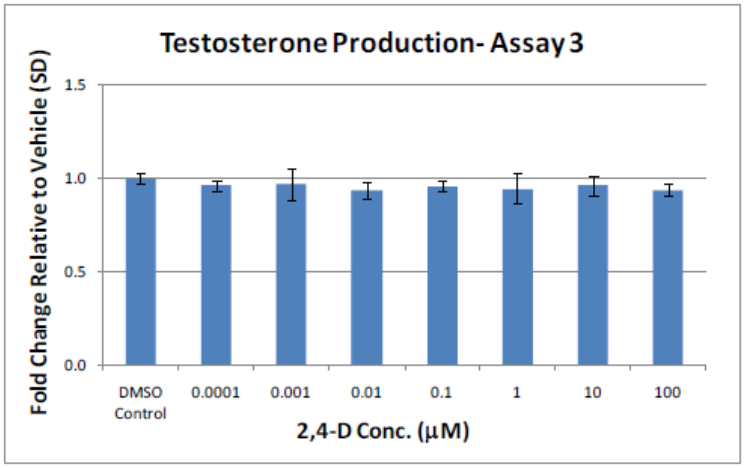
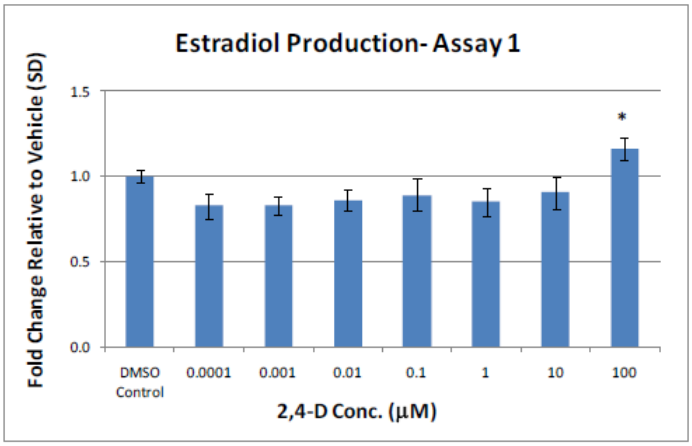
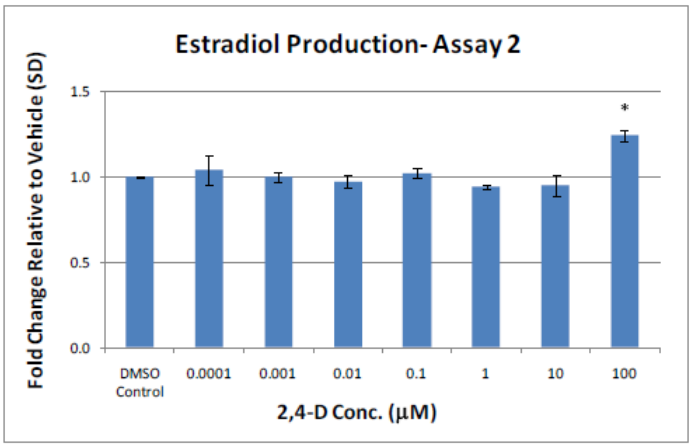
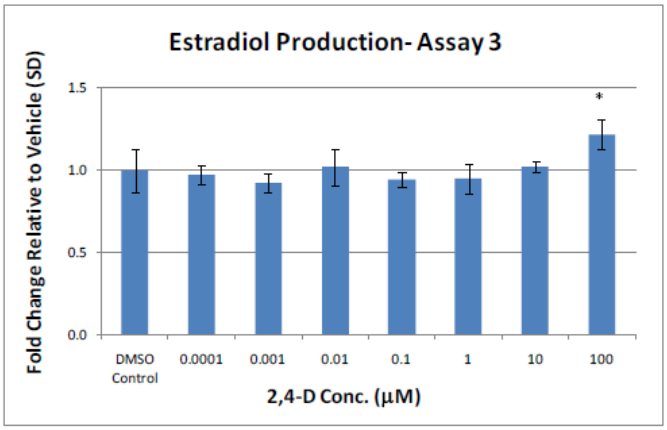


FIGURE 2. Change in Estradiol Production Relative to 2,4-D Concentration.





* Estradiol production significantly different from solvent control ($p<0.05$)



* Estradiol production significantly different from solvent control ($p<0.05$)

B. CYTOTOXICITY: 2,4-D was not cytotoxic to the H295R cells (Table 4). Viability in each run ranged from 92.5–133% relative to solvent controls.

TABLE 4. Mean (±SD) MTT Cell Viability Results after Treatment with Forskolin, Prochloraz, or 2,4-D for 48 Hours. [a]

Compound	Concentration (µM)	Cell Viability	
		Mean	SD
Media	NA	96.6	1.59
Media + Methanol	NA	29.4	2.35
DMSO + Methanol	NA	30.9	3.18
Forskolin	1	121.7	9.91
Forskolin	10	114.1	5.25
Prochloraz	0.1	110.1	9.50
Prochloraz	1	99.9	7.40
2,4-D	0.0001	116.6	8.05
2,4-D	0.001	110.9	5.37
2,4-D	0.01	111.7	8.35
2,4-D	0.1	104.9	4.92
2,4-D	1	106.4	5.89
2,4-D	10	110.7	5.16
2,4-D	100	106.9	5.12

a Values were calculated by the reviewers from data obtained from page 41 of the study report.

C. QC PLATE: The hormone concentrations after exposure to the reference chemicals, SC, and blank samples, as well as the fold difference change relative to SC (individual and mean ± SD) for the three assay runs are presented in Table 5. The minimum basal hormone production levels (500 pg/mL for testosterone, 40 pg/mL for estradiol) were met in both blank and SC wells for testosterone (Table 6). The required concentration for estradiol was not met in any run for blank or SC, with values ranging from 26.7–38.3 pg/mL. Forskolin at 10 µM induced testosterone an average of 2.4-fold and estradiol 20-fold over the SC. Prochloraz at 1 µM inhibited concentrations of testosterone by 0.3-fold and estradiol by 0.6-fold compared to SC. Guideline requirements for basal hormone induction and inhibition were met for testosterone and estradiol, except that all three runs of 1 µM prochloraz only reduced estradiol to 0.6-fold that of the SC rather than 0.5-fold.

The variability (%CV) between the runs (calculated by the reviewer) based on the absolute hormone concentrations in the SC were 4.6% for testosterone and 14.6% for estradiol, and within the guideline criteria of ≤30% for the assays. The %CVs within each run for the QC plates were 2.5-6.6% for testosterone and 3.8-10.1% for estradiol, which were within the guideline criteria (≤30%).

TABLE 5. Mean (±SD) Hormone Concentrations Following Treatment with Forskolin or Prochloraz for 48 Hours.[a]

Concentration (µM)	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Mean	± SD
	Testosterone (pg/mL)			Fold Difference (Relative to DMSO)				
Blank	1041	936	1040	—	—	—	—	—
DMSO	778	719	781	—	—	—	—	—
1 µM Forskolin	1377	1217	1310	1.8	1..7	1.7	1.7	0.0
10 µM Forskolin	2007	1757	1733	2.6	2.4	2.2	2.4	0.2
0.1 µM Prochloraz	515	514	534	0.7	0.7	0.7	0.7	0.0
1 µM Prochloraz	250	233	218	0.3	0.3	0.3	0.3	0.0
	Estradiol (pg/mL)			Fold Difference (Relative to DMSO)				
Blank	26.3	34.8	37.2	—	—	—	—	—
DMSO	28.5	34.2	38.3	—	—	—	—	—
1 µM Forskolin	348	366	492	12.2	10.7	12.8	11.9	1.1
10 µM Forskolin	610	626	780	21.4	18.3	20.4	20.0	1.6
0.1 µM Prochloraz	26.4	29.7	28.6	0.9	0.9	0.5	0.8	0.3
1 µM Prochloraz	17.6	19.5	21.9	0.6	0.6	0.6	0.6	0.0

a Data were obtained from page 29 of the study report. The lower limit of quantification (LLQ) was 25 pg/mL for testosterone and 10 pg/mL for estradiol.

III. DISCUSSION AND CONCLUSIONS

A. <u>INVESTIGATOR'S CONCLUSIONS</u>: Based on the combined hormone responses in each of three independent H295R steroidogenesis assays, it was determined that 2,4-D treatment resulted in a statistically significant increase in estradiol production at the assay limit-concentration of 100 µM (10^{-4} M), while no effects on estradiol production were seen at lower concentrations. There were no effects on testosterone production at any concentration of 2,4-D. The slight (1.2-fold or less) increase in estradiol production did not meet the 1.5-fold cut-off criteria established in the validation program for this assay, and hence was not interpreted to be biologically relevant.

B. <u>AGENCY COMMENTS</u>: Guideline acceptability recommendations and requirements were generally met, including lack of cytotoxicity, adequate production of testosterone and estradiol, acceptable reproducibility (low %CV), and appropriate induction and inhibition with positive controls. The exceptions included: the required concentration for estradiol production (40 pg/mL) was not met in any run for blank or solvent control, with values ranging from 26.7–38.3 pg/mL; and all three runs of 1 µM prochloraz only reduced estradiol to 0.6-fold that of the solvent control rather than 0.5-fold.

2,4-D had no effect on testosterone production at concentrations up to 10^{-4} M, and no effect on estradiol production at concentration up to 10^{-5} M. However, at the highest concentration tested (10^{-4} M), 2,4-D increased ($p \leq 0.05$) estradiol levels by 20% in all three runs relative to the DMSO-treated cells.

Based on hormone responses in each of the independent runs, 2,4-D treatment resulted in a statistically significant and reproducible alteration in estradiol production.

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:

- Stability of 2,4-D in the test system was not reported.
- The report erroneously specified an LLQ of 25 mg/mL. The assay must be able to quantify 500 pg/mL according to the Guideline, and this assay was able to do so.
- There were two minor departures from the Guideline: (i) the required concentration for estradiol was not met in any run for blank or SC, ranging from 26.7 to 38.3 pg/mL rather than 40 pg/mL; and (ii) all three runs of 1 µM prochloraz only reduced estradiol to 0.6-fold that of the SC rather than 0.5-fold.