

Unclassified

ENV/JM/MONO(2010)31

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

20-Jul-2010

English - Or. English

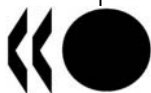
**ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY**

**Series on Testing and Assessment
No. 132**

**MULTI-LABORATORY VALIDATION REPORT OF THE H295R
STEROIDOGENESIS ASSAY TO IDENTIFY MODULATORS OF TESTOSTERONE AND
ESTRADIOL PRODUCTION**

JT03286843

Document complet disponible sur OLIS dans son format d'origine
Complete document available on OLIS in its original format



**ENV/JM/MONO(2010)31
Unclassified**

English - Or. English

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 132

***MULTI-LABORATORY VALIDATION REPORT OF THE H295R STEROIDOGENESIS ASSAY TO
IDENTIFY MODULATORS OF TESTOSTERONE AND ESTRADIOL PRODUCTION***

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among **FAO, ILO, UNEP, UNIDO, UNITAR, WHO and OECD**

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2010

Also published in the Series on Testing and Assessment:

No. 1, *Guidance Document for the Development of OECD Guidelines for Testing of Chemicals (1993; reformatted 1995, revised 2006 and 2009)*

No. 2, *Detailed Review Paper on Biodegradability Testing (1995)*

No. 3, *Guidance Document for Aquatic Effects Assessment (1995)*

No. 4, *Report of the OECD Workshop on Environmental Hazard/Risk Assessment (1995)*

No. 5, *Report of the SETAC/OECD Workshop on Avian Toxicity Testing (1996)*

No. 6, *Report of the Final Ring-test of the Daphnia magna Reproduction Test (1997)*

No. 7, *Guidance Document on Direct Phototransformation of Chemicals in Water (1997)*

No. 8, *Report of the OECD Workshop on Sharing Information about New Industrial Chemicals Assessment (1997)*

No. 9, *Guidance Document for the Conduct of Studies of Occupational Exposure to Pesticides during Agricultural Application (1997)*

No. 10, *Report of the OECD Workshop on Statistical Analysis of Aquatic Toxicity Data (1998)*

No. 11, *Detailed Review Paper on Aquatic Testing Methods for Pesticides and industrial Chemicals (1998)*

No. 12, *Detailed Review Document on Classification Systems for Germ Cell Mutagenicity in OECD Member Countries (1998)*

No. 13, *Detailed Review Document on Classification Systems for Sensitising Substances in OECD Member Countries 1998)*

No. 14, *Detailed Review Document on Classification Systems for Eye Irritation/Corrosion in OECD Member Countries (1998)*

No. 15, *Detailed Review Document on Classification Systems for Reproductive Toxicity in OECD Member Countries (1998)*

No. 16, *Detailed Review Document on Classification Systems for Skin Irritation/Corrosion in OECD Member Countries (1998)*

No. 17, *Environmental Exposure Assessment Strategies for Existing Industrial Chemicals in OECD Member Countries (1999)*

No. 18, *Report of the OECD Workshop on Improving the Use of Monitoring Data in the Exposure Assessment of Industrial Chemicals (2000)*

No. 19, *Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals used in Safety Evaluation (1999)*

No. 20, *Revised Draft Guidance Document for Neurotoxicity Testing (2004)*

No. 21, *Detailed Review Paper: Appraisal of Test Methods for Sex Hormone Disrupting Chemicals (2000)*

No. 22, *Guidance Document for the Performance of Out-door Monolith Lysimeter Studies (2000)*

No. 23, *Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (2000)*

No. 24, *Guidance Document on Acute Oral Toxicity Testing (2001)*

No. 25, *Detailed Review Document on Hazard Classification Systems for Specifics Target Organ Systemic Toxicity Repeated Exposure in OECD Member Countries (2001)*

No. 26, *Revised Analysis of Responses Received from Member Countries to the Questionnaire on Regulatory Acute Toxicity Data Needs (2001)*

No. 27, *Guidance Document on the Use of the Harmonised System for the Classification of Chemicals which are Hazardous for the Aquatic Environment (2001)*

No 28, *Guidance Document for the Conduct of Skin Absorption Studies (2004)*

No 29, *Guidance Document on Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media (2001)*

No 30, *Detailed Review Document on Hazard Classification Systems for Mixtures (2001)*

No 31, *Detailed Review Paper on Non-Genotoxic Carcinogens Detection: The Performance of In-Vitro Cell Transformation Assays (2007)*

No. 32, *Guidance Notes for Analysis and Evaluation of Repeat-Dose Toxicity Studies (2000)*

No. 33, *Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures (2001)*

No. 34, *Guidance Document on the Development, Validation and Regulatory Acceptance of New and Updated Internationally Acceptable Test Methods in Hazard Assessment (2005)*

No. 35, *Guidance notes for analysis and evaluation of chronic toxicity and carcinogenicity studies (2002)*

No. 36, *Report of the OECD/UNEP Workshop on the use of Multimedia Models for estimating overall Environmental Persistence and long range Transport in the context of PBTS/POPS Assessment (2002)*

No. 37, *Detailed Review Document on Classification Systems for Substances Which Pose an Aspiration Hazard (2002)*

No. 38, *Detailed Background Review of the Uterotrophic Assay Summary of the Available Literature in Support of the Project of the OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) to Standardise and Validate the Uterotrophic Assay (2003)*

No. 39, *Guidance Document on Acute Inhalation Toxicity Testing (2009)*

No. 40, *Detailed Review Document on Classification in OECD Member Countries of Substances and Mixtures Which Cause Respiratory Tract Irritation and Corrosion (2003)*

No. 41, *Detailed Review Document on Classification in OECD Member Countries of Substances and Mixtures which in Contact with Water Release Toxic Gases (2003)*

No. 42, *Guidance Document on Reporting Summary Information on Environmental, Occupational and Consumer Exposure (2003)*

No. 43, *Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment (2008)*

No. 44, *Description of Selected Key Generic Terms Used in Chemical Hazard/Risk Assessment (2003)*

No. 45, *Guidance Document on the Use of Multimedia Models for Estimating Overall Environmental Persistence and Long-range Transport (2004)*

No. 46, *Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances (2004)*

No. 47, *Detailed Review Paper on Fish Screening Assays for the Detection of Endocrine Active Substances (2004)*

No. 48, *New Chemical Assessment Comparisons and Implications for Work Sharing (2004)*

No. 49, *Report from the Expert Group on (Quantitative) Structure-Activity Relationships [(Q)SARs] on the Principles for the Validation of (Q)SARs (2004)*

No. 50, *Report of the OECD/IPCS Workshop on Toxicogenomics (2005)*

No. 51, *Approaches to Exposure Assessment in OECD Member Countries: Report from the Policy Dialogue on Exposure Assessment in June 2005* (2006)

No. 52, *Comparison of emission estimation methods used in Pollutant Release and Transfer Registers (PRTRs) and Emission Scenario Documents (ESDs): Case study of pulp and paper and textile sectors* (2006)

No. 53, *Guidance Document on Simulated Freshwater Lentic Field Tests (Outdoor Microcosms and Mesocosms)* (2006)

No. 54, *Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application* (2006)

No. 55, *Detailed Review Paper on Aquatic Arthropods in Life Cycle Toxicity Tests with an Emphasis on Developmental, Reproductive and Endocrine Disruptive Effects* (2006)

No. 56, *Guidance Document on the Breakdown of Organic Matter in Litter Bags* (2006)

No. 57, *Detailed Review Paper on Thyroid Hormone Disruption Assays* (2006)

No. 58, *Report on the Regulatory Uses and Applications in OECD Member Countries of (Quantitative) Structure-Activity Relationship [(Q)SAR] Models in the Assessment of New and Existing Chemicals* (2006)

No. 59, *Report of the Validation of the Updated Test Guideline 407: Repeat Dose 28-Day Oral Toxicity Study in Laboratory Rats* (2006)

No. 60, *Report of the Initial Work Towards the Validation of the 21-Day Fish Screening Assay for the Detection of Endocrine Active Substances (Phase IA)* (2006)

No. 61, *Report of the Validation of the 21-Day Fish Screening Assay for the Detection of Endocrine Active Substances (Phase IB)* (2006)

No. 62, *Final OECD Report of the Initial Work Towards the Validation of the Rat Hershberger Assay: Phase-1, Androgenic Response to Testosterone Propionate, and Anti-Androgenic Effects of Flutamide (2006)*

No. 63, *Guidance Document on the Definition of Residue (2006, revised 2009)*

No. 64, *Guidance Document on Overview of Residue Chemistry Studies (2006, revised 2009)*

No. 65, *OECD Report of the Initial Work Towards the Validation of the Rodent Uterotrophic Assay - Phase 1 (2006)*

No. 66, *OECD Report of the Validation of the Rodent Uterotrophic Bioassay: Phase 2. Testing of Potent and Weak Oestrogen Agonists by Multiple Laboratories (2006)*

No. 67, *Additional data supporting the Test Guideline on the Uterotrophic Bioassay in rodents (2007)*

No. 68, *Summary Report of the Uterotrophic Bioassay Peer Review Panel, including Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the follow up of this report (2006)*

No. 69, *Guidance Document on the Validation of (Quantitative) Structure-Activity Relationship [(Q)SAR] Models (2007)*

No. 70, *Report on the Preparation of GHS Implementation by the OECD Countries (2007)*

No. 71, *Guidance Document on the Uterotrophic Bioassay - Procedure to Test for Antioestrogenicity (2007)*

No. 72, *Guidance Document on Pesticide Residue Analytical Methods (2007)*

No. 73, *Report of the Validation of the Rat Hershberger Assay: Phase 3: Coded Testing of Androgen Agonists, Androgen Antagonists and Negative Reference Chemicals by Multiple Laboratories. Surgical Castrate Model Protocol (2007)*

No. 74, *Detailed Review Paper for Avian Two-generation Toxicity Testing (2007)*

No. 75, *Guidance Document on the Honey Bee (Apis Mellifera L.) Brood test Under Semi-field Conditions (2007)*

No. 76, *Final Report of the Validation of the Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances: Phase 1 - Optimisation of the Test Protocol (2007)*

No. 77, *Final Report of the Validation of the Amphibian Metamorphosis Assay: Phase 2 - Multi-chemical Interlaboratory Study (2007)*

No. 78, *Final Report of the Validation of the 21-day Fish Screening Assay for the Detection of Endocrine Active Substances. Phase 2: Testing Negative Substances (2007)*

No. 79, *Validation Report of the Full Life-cycle Test with the Harpacticoid Copepods Nitocra Spinipes and Amphiascus Tenuiremis and the Calanoid Copepod Acartia Tonsa - Phase 1 (2007)*

No. 80, *Guidance on Grouping of Chemicals (2007)*

No. 81, *Summary Report of the Validation Peer Review for the Updated Test Guideline 407, and Agreement of the Working Group of National Coordinators of the Test Guidelines Programme on the follow-up of this report (2007)*

No. 82, *Guidance Document on Amphibian Thyroid Histology (2007)*

No. 83, *Summary Report of the Peer Review Panel on the Stably Transfected Transcriptional Activation Assay for Detecting Estrogenic Activity of Chemicals, and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-up of this Report (2007)*

No. 84, *Report on the Workshop on the Application of the GHS Classification Criteria to HPV Chemicals, 5-6 July Bern Switzerland (2007)*

No. 85, *Report of the Validation Peer Review for the Hershberger Bioassay, and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-up of this Report (2007)*

No. 86, *Report of the OECD Validation of the Rodent Hershberger Bioassay: Phase 2: Testing of Androgen Agonists, Androgen Antagonists and a 5 α -Reductase Inhibitor in Dose Response Studies by Multiple Laboratories (2008)*

No. 87, *Report of the Ring Test and Statistical Analysis of Performance of the Guidance on Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media (Transformation/Dissolution Protocol) (2008)*

No.88 *Workshop on Integrated Approaches to Testing and Assessment (2008)*

No.89 *Retrospective Performance Assessment of the Test Guideline 426 on Developmental Neurotoxicity (2008)*

No.90 *Background Review Document on the Rodent Hershberger Bioassay (2008)*

No.91 *Report of the Validation of the Amphibian Metamorphosis Assay (Phase 3) (2008)*

No.92 *Report of the Validation Peer Review for the Amphibian Metamorphosis Assay and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-Up of this Report (2008)*

No.93 *Report of the Validation of an Enhancement of OECD TG 211: Daphnia Magna Reproduction Test (2008)*

No.94 *Report of the Validation Peer Review for the 21-Day Fish Endocrine Screening Assay and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-up of this Report (2008)*

No.95 *Detailed Review Paper on Fish Life-Cycle Tests (2008)*

No.96 *Guidance Document on Magnitude of Pesticide Residues in Processed Commodities (2008)*

No.97 *Detailed Review Paper on the use of Metabolising Systems for In Vitro Testing of Endocrine Disruptors (2008)*

No. 98 *Considerations Regarding Applicability of the Guidance on Transformation/Dissolution of Metals Compounds in Aqueous Media (Transformation/Dissolution Protocol) (2008)*

No. 99 *Comparison between OECD Test Guidelines and ISO Standards in the Areas of Ecotoxicology and Health Effects (2008)*

No.100 *Report of the Second Survey on Available Omics Tools (2009)*

No.101 *Report on the Workshop on Structural Alerts for the OECD (Q)SAR Application Toolbox (2009)*

No.102 *Guidance Document for using the OECD (Q)SAR Application Toolbox to Develop Chemical Categories According to the OECD Guidance on Grouping of Chemicals (2009)*

No.103 *Detailed Review Paper on Transgenic Rodent Mutation Assays (2009)*

No.104 *Performance Assessment: Comparison of 403 and CxT Protocols via Simulation and for Selected Real Data Sets (2009)*

No. 105 *Report on Biostatistical Performance Assessment of the draft TG 436 Acute Toxic Class Testing Method for Acute Inhalation Toxicity (2009)*

No.106 *Guidance Document for Histologic Evaluation of Endocrine and Reproductive Test in Rodents (2009)*

No.107 *Preservative treated wood to the environment for wood held in storage after treatment and for wooden commodities that are not cover and are not in contact with ground. (2009)*

No.108 *Report of the validation of the Hershberger Bioassay (weanling model) (2009)*

No. 109 *Literature review on the 21-Day Fish Assay and the Fish Short-Term Reproduction Assay (2009)*

No. 110 *Report of the validation peer review for the weanling Hershberger Bioassay and agreement of the working of national coordinators of the test guidelines programme on the follow-up of this report (2009)*

No. 111 *Report of the Expert Consultation to Evaluate an Estrogen Receptor Binding Affinity Model for Hazard Identification (2009)*

No. 112 *The 2007 OECD List of High Production Volume Chemicals (2009)*

No. 113 *Report of The Focus Session On Current And Forthcoming Approaches For Chemical Safety And Animal Welfare (2010)*

No. 114 *Performance Assessment of Different Cytotoxic and Cytostatic Measures for the In Vitro Micronucleus Test (MNVT): Summary of results in the collaborative trial (2010)*

No. 115 *Guidance Document on the Weanling Hershberger Bioassay in Rats: A Short-term Screening Assay for (Anti) Androgenic Properties (2009)*

No. 116 *Guidance Document on the Design and Conduct of Chronic Toxicity and Carcinogenicity Studies, Supporting TG 451, 452 and 453 (2010)*

No. 118 *Workshop Report on OECD Countries Activities Regarding Testing, Assessment and Management of Endocrine Disrupters Part I and Part II (2010)*

No. 119 *Classification and Labelling of chemicals according to the UN Globally Harmonized System: Outcome of the Analysis of Classification of Selected Chemicals listed in Annex III of the Rotterdam Convention (2010)*

No. 120 *Explanatory Background Document to the OECD Draft Test Guideline on in vitro Skin Irritation Testing (2010)*

No. 121 *Detailed review paper (DRP) on Molluscs life-cycle Toxicity Testing (2010)*

No. 122 *Guidance Document on the determination of the Toxicity of a Test Chemical to the Dung Beetle *Aphodius Constans* (2010)*

No. 123 *Guidance Document on the Diagnosis of Endocrine-related Histopathology in Fish Gonads (2010)*

No. 124 *Draft guidance for the Derivation of an Acute Reference Dose (2010)*

No. 125 *Guidance Document on Histopathology for Inhalation Toxicity Studies, Supporting TG 412 (Subacute Inhalation Toxicity: 28-Day) and TG 413 (Subchronic Inhalation Toxicity: 90-Day) (2010)*

No. 126 *Short Guidance on the Threshold approach for Acute Fish Toxicity (2010)*

No. 127 *Peer review report of the validation of the 21-day androgenised female stickleback screening assay (2010)*

No. 128 *Validation Report of the 21-day Androgenised Female Stickleback Screening Assay (2010)*

No. 129 *Guidance Document on using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests*

No.130 *Draft Guidance Document On Using Cytotoxicity Tests To Estimate Starting Doses For Acute Oral Systemic Toxicity Tests (2010)*

No.131 *Report of the Test Method Validation of Avian Acute Oral Toxicity Test (OECD test guideline 223) (2010)*

No.132 *Report of the Multi-Laboratory Validation of the H295R Steroidogenesis Assay to Identify Modulators (2010)*

© **OECD 2010**

Applications for permission to reproduce or translate all or part of this material should be made to: Head of Publications Service, RIGHTS@oecd.org, OECD, 2 rue André-Pascal, 75775 Paris Cedex 16, France

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 31 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides and Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/ehs/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNEP, UNIDO, UNITAR, WHO and OECD. The World Bank and UNDP are observers. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

This publication is available electronically, at no charge.

**For this and many other Environment,
Health and Safety publications, consult the OECD's
World Wide Web site (www.oecd.org/ehs/)**

or contact:

**OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France**

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

FOREWORD

This validation report of the “*H295R Cell-Based Assay for Steroidogenesis*” was developed by the Environmental Protection Agency of the United States in 2008. It was reviewed by the Validation Management Group for Non-Animal Testing in November 2009, and endorsed by the Working Group of National Coordinators of the Test Guidelines Programme at its 22nd Meeting on 23-25 March 2010. It should be noted that three detailed test method components of the draft Steroidogenesis Test Guideline, have been attached to this validation report. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 19 July 2010.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

TABLE OF CONTENTS

1	Abbreviations	26
2	Key Personnel	27
3	Introduction.....	29
3.1	Time Lines.....	32
3.2	H295R Cell Line	33
3.3	The H295R Steroidogenesis Assay	34
3.4	Pre-validation Studies Overview	35
4	Objectives of THE inter-laboratory validation study.....	39
5	Selection of Participating Laboratories.....	40
5.1	Participating Laboratories	41
6	Selection of Chemicals.....	43
6.1	Core chemicals	44
6.2	Supplementary chemicals.....	45
7	Test Procedure.....	48
7.1	QA criteria.....	48
7.1.1	Performance of H295R Cells under Standard Culture Conditions	48
7.1.2	Performance of H295R Cells during Exposure Experiments	48
7.2	Testing of chemicals.....	52
7.2.1	Core chemicals	52
7.2.2	Supplementary chemicals	52
7.3	Data interpretation.....	52
8	Data Processing and Statistics.....	54
8.1	Intra-laboratory statistics.....	55
8.2	Inter-laboratory statistics.....	55
9	Results.....	56
9.1	QA criteria.....	56
9.1.1	Performance of H295R Cells under Standard Culture Conditions	56
9.1.1.1	Basal hormone Production (Blanks).....	56
9.1.1.2	Initial QC-Plate Experiment Responses	58
9.1.2	Performance of H295R Cells during Exposure Experiments	61
9.2	Testing of core chemicals.....	65
9.2.1	Cell Viability	65
9.2.2	Relative changes after Exposure to Core Chemicals	67
9.2.2.1	Testosterone	67
9.2.2.2	Estradiol	70
9.2.3	Confounding Factors – Interference with Hormone Detection Assays	73
10	Data Evaluation	74

10.1	Fold-Change Evaluation.....	75
10.2	Application of Regression Type Models – Effective Concentrations (ECs).....	78
11	Application of the H295R Steroidogenesis Assay to a Selection of Supplementary Chemicals.....	80
12	In Vivo Predictivity of the H295R Steroidogenesis Assay.....	93
13	Adjustments Made to the Draft Guidelines Based on the Validation Study.....	96
13.1	Laboratory Proficiency Test.....	96
13.2	QC Plate Prochloraz Concentration Adjustment.....	97
13.3	Cell Viability Assessment	98
14	Conclusions and Summary	99
15	References.....	100
16	Appendices	103

TABLE OF TABLES

Table 3.1: Timeline for the H295R Steroidogenesis Assay validation studies to be submitted to US-EPA's EDSP and OECD for peer review.	32
Table 6.1: Number of chemicals tested for interaction with the production of testosterone (T) and estradiol (E2) at each laboratory as of the time this report was written.	43
Table 6.2: Core chemicals and their hypothesized mode of action selected for the H295R Steroidogenesis Assay validation studies.	44
Table 6.3: Supplemental chemicals and their hypothesized mode of action selected for the H295R Steroidogenesis Assay validation studies.	46
Table 7.1: Performance criteria to be met by each laboratory during the qualifying experiments...	48
Table 7.2: Acceptable ranges and/or variation (%) for H295R assay QC parameters.....	50
Table 7.3: Data qualifying parameters for the pre-categorization of results obtained with the H295R Steroidogenesis Assay prior to statistical assessment.....	53
Table 9.1: Comparison of basal production of testosterone (T) and estradiol (E2) as measured in the blanks of the QC-plates among laboratories.....	57
Table 9.2: Among laboratory coefficients of variation (CVs) for changes in concentrations of testosterone (T) and estradiol (E2).	60
Table 9.3: Within laboratory coefficients of variation (CVs) for changes in concentrations of testosterone (T) and estradiol (E2).	60
Table 9.4: Experiments and chemicals for which basal hormone concentration of testosterone (T) and estradiol (E2) was such that no decreases and/or increases after chemical exposure could be observed.	61
Table 9.5: Interference (pg hormone/mL @ the greatest concentration tested) of the 12 core chemicals with the immunoassays used for the determination of testosterone (T) and estradiol (E2).....	74
Table 10.1: Classification system for the effects of the 12 core chemicals on testosterone (T) and estradiol (E2) production by H295R cells	75
Table 10.2: Lowest observed effect concentrations (LOECs; measured by Dunnett's or Mann Whitney U test ^{mu}) and strength and direction of change (arrows; see Table 10.1 for explanation) for testosterone (T) and estradiol (E2) after exposure to the twelve core chemicals.	77
Table 10.3: EC50s(μ M) calculated for the changes in testosterone (T) and estradiol (E2) production after exposure to the twelve core chemicals using a probit model. --- chemical not analyzed.	79

Table 11.1: Lowest observed effect concentrations (LOECs [μ M]; measured by Dunnett’s test), strength and direction of change (arrows; see Table 10.1 for explanation) observed for the 16 test chemicals.92

Table 12.1: Comparison of data obtained with the H295R Steroidogenesis Assay (this study) with in vivo data.95

Table 13.1: Dosing schematic for the laboratory proficiency study.97

Table 13.2: Quality control plate layout for testing performance of unexposed H295R cells and cells exposed to known inhibitors (PRO = prochloraz) and stimulators (FOR = forskolin) of E2 and T production.98

Table 13.3: Performance criteria for the Quality Control Plate.....98

TABLE OF FIGURES

Figure 3.1: OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals.	31
Figure 3.2: Steroidogenic pathway in H295R cells.	33
Figure 3.3: H295R Steroidogenesis Assay to measure effects of chemicals on production of testosterone (T) and estradiol (E2).....	35
Figure 3.4: Changes in T (A) and E2 (B) production by H295R cells measured by five (5) independent laboratories (Lab 1 – Lab 5).	36
Figure 3.5: Changes in T (A) and E2 (B) production by H295R cells measured by five (5) independent laboratories (Lab 1 – Lab 5).....	37
Figure 3.6: Changes in T (A) and E2 (B) production by H295R cells measured by five (5) independent laboratories (Lab 1 – Lab 5).....	38
Figure 7.1: Plate layout for QC-plate to be analyzed together with each chemical exposure experiment.	49
Figure 7.2: Example of hormone detection system standard curve indicating acceptable range of hormone concentration of solvent control (SC) sample (not corrected for dilution in assay).	51
Figure 9.1: Comparison of basal production of testosterone and estradiol by H295R cells as measured by LC-MS and ELISA.....	58
Figure 9.2: Comparison of changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) in the QC-plate experiment conducted to evaluate the performance of the H295R prior to initiation of the exposure experiments.....	59
Figure 9.3: Comparison of changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) in a QC-plate experiment when measured by LC-MS and ELISA at the lead laboratory (Lab 1).....	60
Figure 9.4: Comparison of changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) in the QC-plates among laboratories (Lab).....	62
Figure 9.5: Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) in the QC-plates run parallel to the exposure experiments at each laboratory (Lab).....	63
Figure 9.6: Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) in the QC-plates run parallel to the exposure experiments at each laboratory (Lab).....	64
Figure 9.7: Comparison of cell viability among laboratories (Lab) after exposure to Aminoglutethimide, Atrazine, Benomyl, EDS, Forskolin and HCG.....	66
Figure 9.8: Comparison of cell viability among laboratories (Lab) after exposure to Letrozole, Molinate, Nonoxynol-9, Paraben, Prochloraz and Trilostane.....	67

Figure 9.9: Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to forskolin, trilostane, and atrazine. 68

Figure 9.10: Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to prochloraz, aminoglutethimide, and letrozole. 69

Figure 9.11: Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to HCG, EDS, molinat, benomyl and nonoxynol-9..... 70

Figure 9.12: Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to forskolin, atrazine, trilostane,molinate,and paraben..... 71

Figure 9.13: Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to letrozole, prochloraz and aminoglutethimide, letrozole. 72

Figure 9.14: Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to HCG, EDS, and benomyl. 73

Figure 9.15: Changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) after exposure to trilostane with and without adjustment of final hormone concentration for interference with hormone detection system. 74

Figure 11.1: Cell viability after exposure to piperonyl butoxide, prometon, DEHP, vinclozolin, finasteride, and Dimethoate. 82

Figure 11.2: Cell viability after exposure to dinitrophenol, glyphosate, genistein ,tricrescyl phosphate, and spironolactone..... 83

Figure 11.3: Cell viability after exposure to ketoconazole, bisphenol A, fenarimol, flutamide, danazol and mifepristone 84

Figure 11.4: Changes in the relative concentrations (SC=1) of testosterone (T) after exposure to spironolactone, finasteride and danazol, and of estradiol (E2) after exposure to genistein after (grey bars) and before (white bars) subtraction of concentration at which the chemicals interfered with the hormone detection assay..... 85

Figure 11.5: Changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to ketoconazole, spironolactone, bisphenol A, piperonyl butoxide, DEHP, and dinitrophenol (DNP). Error bars = 1x standard deviation..... 86

Figure 11.6: Changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to fenarimole, finasteride, genistein, dimethoate, flutamide, and danazol. Error bars = 1x standard deviation..... 87

Figure 11.7: Changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to prometon, tricrescyl phosphate, mifepristone, and glyphosate. Error bars = 1x standard deviation..... 88

Figure 11.8: Changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to ketoconazole, spironolactone, bisphenol A, piperonyl butoxide, DEHP, and dinitrophenol (DNP)89

Figure 11.9: Changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to fenarimole, finasteride, genistein, dimethoate, flutamide, and danazol90

Figure 11.10: Changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to prometon, tricrescyl phosphate, mifepristone, and glyphosate.91

1 ABBREVIATIONS

AOE	City University of Hong Kong
DIFVR	Danish Institute for Food and Veterinary Research
EC	Effective Concentration
GER	University of Heidelberg
IHCP	Institute for Health and Consumer Protection
KFDA	Korea Food and Drug Administration
LOEC	Lowest Observable Effect Concentration
MSU	Michigan State University
NOEC	No Observable Effect Concentration
OECD	Organization for Economic Co-operation and Development
VMG NA	Validation Management Group for Non-animal Testing for Endocrine Disruptors
REACH	Registration, Evaluation and Authorization of Chemicals
RTP	US Environmental Protection Agency
T	Testosterone
E2	17 β -estradiol

2 KEY PERSONNEL

Project Officer:

Gary E. Timm
Office of Science Coordination and Policy (7201M)
U.S. Environmental Protection Agency
Washington, DC 20460, USA
Tel: +1-202-564-8474
FAX: +1-202-564-8483
Email: Timm.Gary@epamail.epa.gov

Signature

Date

Principal Investigators:

Prof. John P. Giesy, Ph.D.
University of Saskatchewan
Department of Veterinary Biomedical Sciences
Toxicology Centre
44 Campus Drive
Saskatoon, SK S7N 5B3
Canada

TEL: +1 (306) 966-2096
FAX: +1 (306) 966-4796
Email: jgiesy@aol.com

Signature

Date

Dr. Markus Hecker, Ph.D.
University of Saskatchewan
Toxicology Centre
44 Campus Drive
Saskatoon, SK S7N 5B3
Canada

TEL: +1 (306) 966-5233
FAX: +1 (306) 966-4796
Email: mhecker@ENTRIX.com

Signature

Date

Study Manager/Coordinator:

Dr. Markus Hecker, Ph.D.

ENTRIX. Inc.

Toxicology Centre

44 Campus Drive

Saskatoon, SK S7N 5B3

Canada

TEL: +1 (306) 966-5233

FAX: +1 (306) 966-4796

Email: mhecker@ENTRIX.com

Signature

Date

3 INTRODUCTION

Studies have indicated potential links between the exposure to natural and human-made substances in the environment and adverse effects on the endocrine and reproductive systems of mammals, birds, reptiles, amphibians, and fish (EPA, 1997; Kavlock et al. 1996). In response to concerns that these substances may alter the function of endocrine systems and result in adverse effects to human health, the U.S. Congress included a provision in the Food Quality Protection Act of 1996 adding section 408 to the Federal Food Drug and Cosmetic Act. This section of the FFDCA requires EPA to:

... develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect as the Administrator may designate [21 U.S.C. 346 (p)].

Subsequent to passage of the Act, EPA formed the Endocrine Disruptor screening and Testing Advisory Committee (EDSTAC), a committee of scientists and stakeholders that EPA charged to provide it with recommendations on how to implement its Endocrine Disruptor Screening Program (EDSP). EPA accepted the EDSTAC's recommendations for a two-tier screening program which included an assay for detecting chemicals that affect steroid hormone synthesis (EPA 1998).

The steroidogenic assay is intended to identify xenobiotics that have as their target site(s) the intracellular components that comprise the intracellular biochemical pathway beginning with the sequence of reactions occurring after the gonatotropin hormone receptors (FSHR and LHR), up through and including the production of the terminal sex steroid hormones, i.e. testosterone (males) and estradiol/estrone (females). The steroidogenic assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamus or pituitary gland. The most promising assay for use as a screen, which will meet the objectives as described above, will be a relatively fast, inexpensive, technically simple assay that identifies substances that alter sex steroid hormone production due to direct effects on the enzymes or other endogenous components of the steroidogenic pathway.

Based on studies conducted by ENTRIX for the EPA (Giesy et al. 2002; Hilscherova et al. 2004) and other reports as noted in section 3.2, the human H295R adreno-carcinoma cell line has been shown to possess all of the enzymes of the steroidogenic pathway and meet all of these criteria. It was therefore selected by EPA as the in vitro screen for chemicals that affect steroidogenesis. Development and standardization of the assay was carried in a multi-step process under EPA contracts with the ENTRIX Corporation.

In addition to the US-EPA activities, the Organization for Economic Cooperation and Development (OECD) initiated a high-priority activity in 1998 to revise existing, and to develop new, Test Guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals comprises five levels, each level corresponding to a different level of biological complexity (Figure 3.1). After initial assay development, US EPA presented a progress report on the development of the H295R assay to a committee of the OECD. The OECD adopted the H295R assay as a Level 2 in vitro assay to detect any substance that would disrupt estrogen and/or androgen gonadal steroid hormone production.

The U.S. invited OECD member countries to join the U.S. in its further standardization and validation. This invitation was accepted by Japan and Denmark. Laboratories in Germany, Hong Kong, and Korea also joined the validation effort. The final product of the validation effort will be an OECD Test Guideline for assessing the potential of chemicals to affect steroid hormone synthesis.

This document reports on the validation of a cell-based screening assay using the H295R cell line to identify chemicals that act to alter steroidogenic process downstream of the

gonadotropin hormone receptors in humans and wildlife as part of the EDSP and OECD validation programs.

Validation:

Validation is a scientific process designed to characterize the operational characteristics and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose. OECD Guidance Document 34 provides the principles of test validation and practical guidance for validation that are followed by OECD. These principles were set forth in the report from a workshop on validation in Solna (OECD 1996) and are consistent with the approaches used in Europe by the European Center for Validation of Alternative Methods (ECVAM 1995) and the U.S. Interagency Coordinating Committee on Validation of Alternative Methods (ICCVAM 1997).

OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

<p>Level 1 Sorting & prioritization based upon existing information</p>	<ul style="list-style-type: none"> •physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability •human & environmental exposure, e.g., production volume, release, use patterns hazard, e.g., available toxicological data
<p>Level 2 <i>In vitro</i> assays providing mechanistic data</p>	<ul style="list-style-type: none"> •ER, AR, TR receptor binding affinity •Transcriptional activation •Aromatase & Steroidogenesis <i>in vitro</i> •High Through Put Prescreens •Thyroid function •Fish hepatocyte VTG assay
<p>Level 3 <i>In vivo</i> assays providing data about single endocrine Mechanisms and effects</p>	<ul style="list-style-type: none"> •Aromatase & Steroidogenesis <i>in vitro</i> •Hershberger Assay (androgenic related) •Fish VTG assay (estrogenic related)
<p>Level 4 <i>In vivo</i> assays providing data about multiple endocrine mechanisms and effects</p>	<ul style="list-style-type: none"> •Enhanced OECD 407 (endpoints based on endocrine mechanisms) •Male and female pubertal assaysAdult •Fish gonadal histopathology assay
<p>Level 5 <i>In vivo</i> assays providing data on effectst from endocrine & other mechanisms</p>	<ul style="list-style-type: none"> •1-generation assay (TG415 enhanced) •2-generation assay (TG416 enhanced) •Reproductive screening (TG421 enhanced) •Partial and full life cycle assays in fish, birds, amphibians & invertebrates (develop. & reprod.)

Note: Document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force

Figure 3.1: OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

3.1 Time Lines

Because the H295R Steroidogenesis Assay is being validated both as part of US-EPA’s EDSP and the OECD Test Guidelines Program, the proposed validation efforts were conducted under two different timelines (Table 3.1). The overall duration of the validation studies was set at 19 months (April 2007 through October 2008). Due to time restraints regarding the EDSP implementation— battery selection for Tier 1 needed to be completed in early 2008 to meet an August 2008 target for beginning testing¹ — a peer review to meet the needs of the US EDSP was to be held after completion of studies on the 12 core chemicals (the Interim Report). The validation studies for OECD purposes were then to be completed by including the extended set of 16 additional chemicals with a final report covering all chemicals being submitted to OECD in 2009.

Table 3.1: Timeline for the H295R Steroidogenesis Assay validation studies to be submitted to US-EPA’s EDSP and OECD for peer review. Gray shaded bars: EDSP & OECD; Diagonal pattern bars: OECD; Square pattern bar: Report to OECD at VMG NA meeting

	2007												2008												
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	
Preparation of Assay (Cell Culture; QC Requirements, Cross Reactivity)				■	■	■	■						■	■	■										
Exposure Studies with Core Chemicals							■	■	■	■	■	■													
Exposure Studies with Supplementary Chemicals																	■	■	■	■					
Data Evaluation											■	■	■								■	■			
Report												■	■								■	■	■	■	■

¹ EPA’s 2008 Fiscal Year Appropriation specified that EPA should begin issuing Test Orders to pesticide registrants and manufacturers by August 2008.

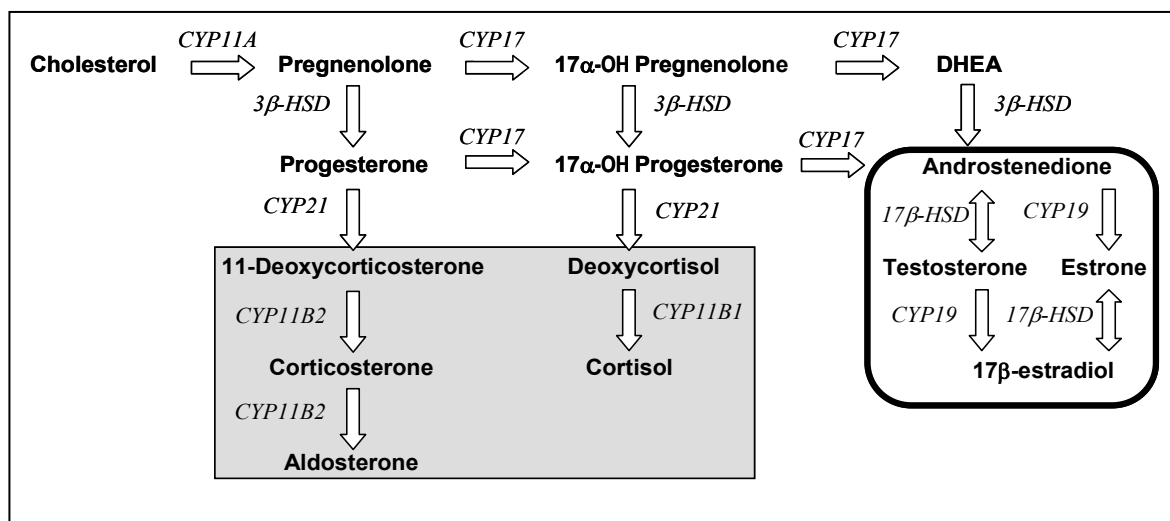


Figure 3.2: Steroidogenic pathway in H295R cells. Enzymes are in italics, hormones are bolded and arrows indicate the direction of synthesis. Gray background indicates corticosteroid pathways/products. Sex steroid pathways/products are circled. CYP = cytochrome P450; HSD = hydroxysteroid hydrogenase.

3.2 H295R Cell Line

The human H295R adreno-carcinoma cell line has been shown to be a useful *in vitro* model for steroidogenic pathways and processes (Hecker et al. 2006; Hilscherova et al. 2004; Sanderson et al. 2002). The H295R cell line expresses genes that encode for all the key enzymes for steroidogenesis (Gazdar et al. 1990; Rainey et al. 1993) (Figure 3.2). This is a unique property because *in vivo* expression of these genes is tissue and developmental stage-specific with typically no one tissue or one developmental stage expressing all of the genes involved in steroidogenesis. H295R cells have physiological characteristics of zonally undifferentiated human fetal adrenal cells (Gazdar et al. 1990). The cells represent a unique *in vitro* system in that they have the ability to produce the steroid hormones found in the adult adrenal cortex and the gonads, allowing testing for effects on both corticosteroid synthesis and the production of sex steroid hormones such as androgens and estrogens. There are several additional advantages to the use of the H295R cell line over other systems currently being evaluated as Tier I assays. One advantage the H295R *in vitro* assay has over tissue-based assays is that it permits the direct assessment of the potential impact of a chemical on cell viability/cytotoxicity. This is an important feature as it allows for the discrimination between effects that are due to cytotoxicity or due to the direct interaction of chemicals with steroidogenic pathways, which is not possible in tissue explants systems that consist of multiple cell types of varying sensitivities and functionalities.² In addition, the NCI-H295R cells are commercially available from the American Type Culture Collections (ATCC CRL-2128; ATCC, Manassas, VA, USA). Thus, these cells are available to everybody and no costly permissions are required as is the case for many other cell systems. The H295R has the advantage over other tests such as the minced testis assay that it allows for the detection of both increases and decreases in the production of both T and E2, and thus permits to assess chemicals with the potential to induce or inhibit this enzyme. Furthermore, H295R cells contain the complete suite of steroidogenic enzymes for the production of corticosteroids and sex steroids, and thus, enable the research of any target site within the steroidogenic pathway downstream of cholesterol in addition to those investigated in this study. Finally, the use of the immortalized H295R cells addresses the need for *in vitro* test systems as alternatives to tissue explant assays which require the use of live animals.

² The seemingly insurmountable problem of assessing cytotoxicity specific to Leydig cells lead EPA's advisory committee to recommend that EPA abandon further work on the sliced testes assay (EDMVAC, 2005).

3.3 The H295R Steroidogenesis Assay

Based on the promising results obtained during initial studies researching the potential of the H295R cells to detect effects of chemicals on steroidogenesis including the production of testosterone, estradiol, and progestins (Hecker et al. 2006), a standardized H295R Steroidogenesis Assay protocol was developed (*Figure 3.3*; APPENDIX I). In brief, the assay is performed under standard cell culture conditions in 24-well culture plates. After an acclimation period of 24 h, cells are exposed for 48 h to multiple concentrations of the test chemical in triplicate. In parallel, a plate with known inhibitors and inducers of hormone production is run as a quality control (QC). At the end of the exposure period, the medium is removed from each well and hormones are extracted using ethyl ether (note: some hormone detection assays may not need extraction; in these cases the medium can directly be used in the assay). Cell viability in each well is analyzed immediately after removal of medium. Concentrations of hormones in medium can be measured using a variety of methods including the use commercially available hormone detection kits and/or instrumental techniques (LC-MS), again making the assay accessible to most laboratories.

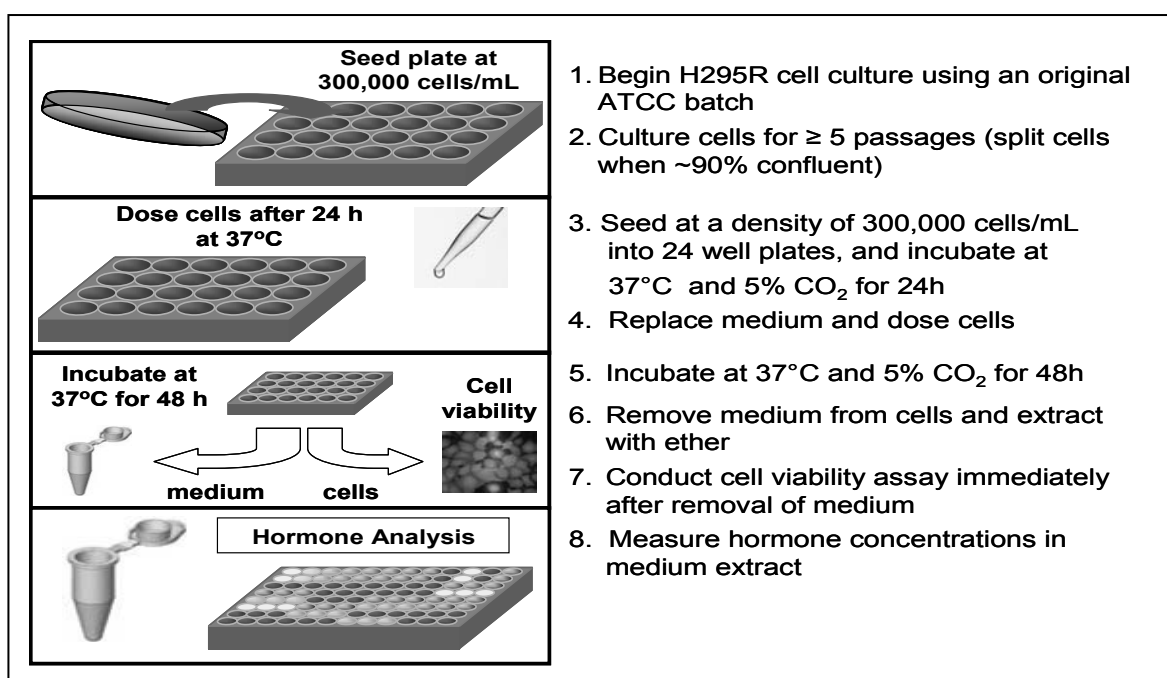


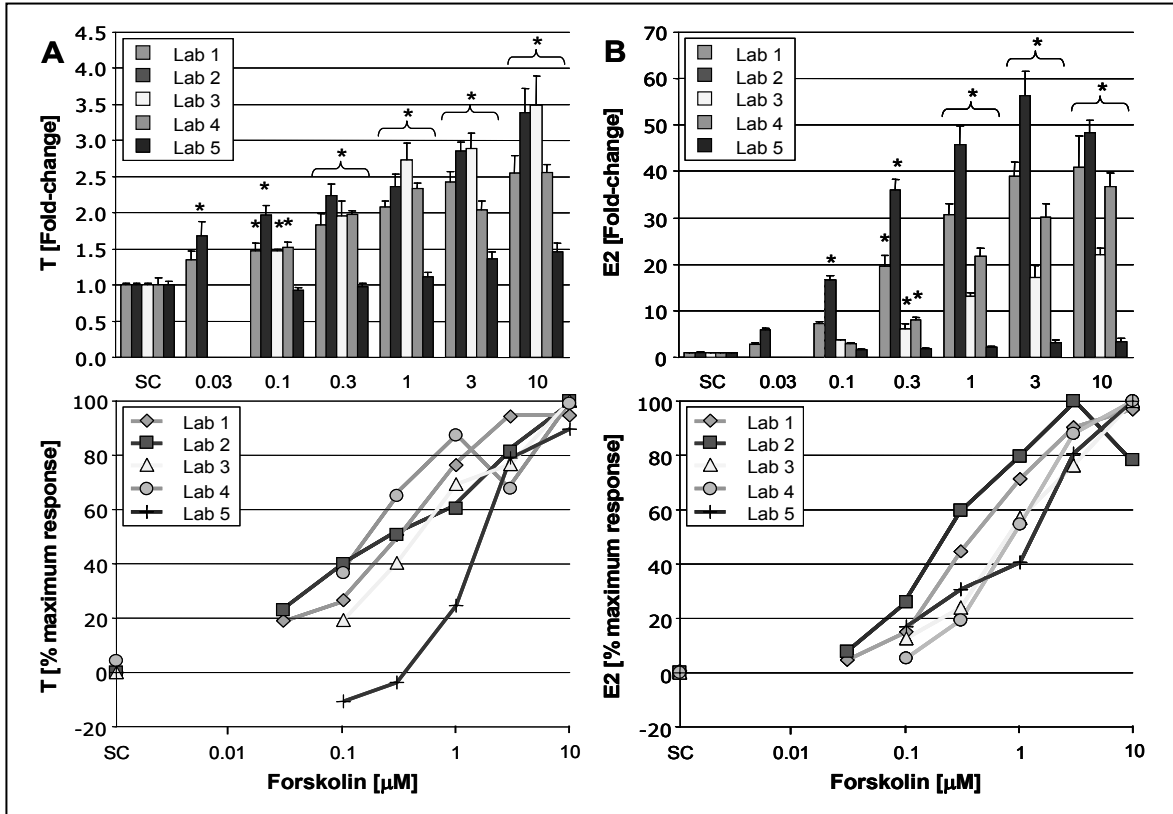
Figure 3.3: H295R Steroidogenesis Assay to measure effects of chemicals on production of testosterone (T) and estradiol (E2).

3.4 Pre-validation Studies Overview

An initial inter-laboratory pre-validation study was conducted to evaluate the H295R Steroidogenesis protocol described in Section 3.3 using a limited set of three model chemicals at five independent laboratories. These studies demonstrated that the test protocol was very promising as a reproducible, transferable, sensitive, economic, and precise method to test for chemical effects on the production of T and E2 (Hecker et al. 2007). The three model compounds used in this study included forskolin, prochloraz, and fadrozole that had known modes of interaction with steroidogenic pathway. Comparison of changes in hormone production by H295R cells treated with these chemicals revealed good reproducibility of the tested protocol among five independent laboratories (Figure 3.4-Figure 3.6). However, the results also indicated that H295R cells appear to maintain some variability concerning their hormone producing capacities that was related to cell passage, which is likely to be due to the undifferentiated characteristics of the cells (Gazdar et al. 1990). Interestingly, both the direction and extent of the changes in hormone production with cell passage were predictable and reproducible among different laboratories (Hecker et al. 2006b). Furthermore, it was demonstrated that, despite the differences in absolute production of hormones that occurred as a function of cell passage, the relative response of the H295R cells exposed to chemicals remained constant among the different laboratories (Hecker et al. 2007a; Figure 3.3). In addition, different data evaluation techniques that normalize the data to correct for differences due to cell passage showed promise in terms of accounting for these variations in hormone production. For instance, the expression of responses both as changes relative to the controls and as a percent of maximum efficacy represented promising approaches. The development of appropriate data evaluation approaches is part of the validation study described in this document.

Based on the findings of these pre-validation studies, the protocols to be used in this validation study were revised such that quality criteria for the exposure to known inducers and inhibitors of T and E2 production to serve as positive controls were defined and that the cell culture protocols were adapted to reflect optimum hormone production patterns of the cells (APPENDIX I).

Figure 3.4: Changes in T (A) and E2 (B) production by H295R cells measured by five (5) independent laboratories (Lab 1 – Lab 5). Data are expressed as relative changes compared to the SC (upper graphs) and percent of the



maximum hormone concentration measured across all concentrations (maximum induction = 100%; lower graphs) observed after exposure to forskolin for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

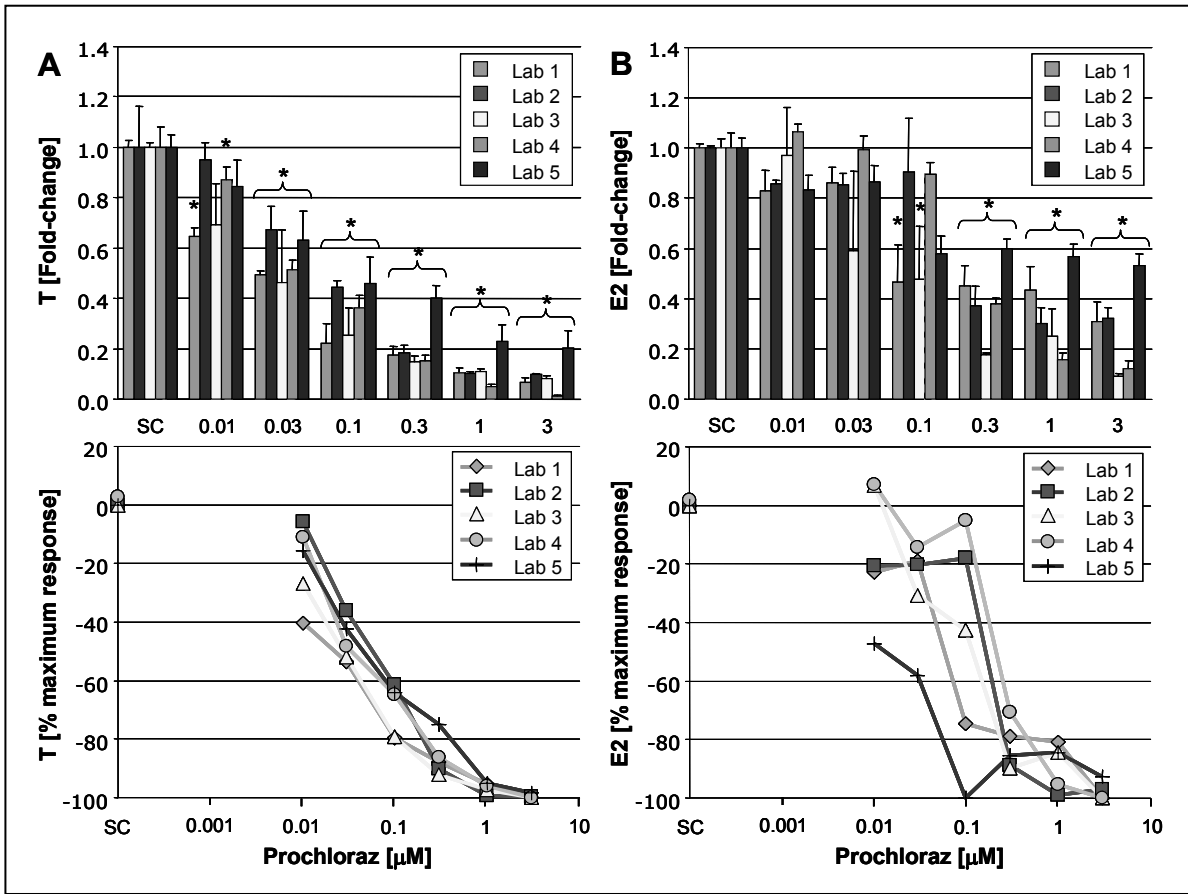


Figure 3.5: Changes in T (A) and E2 (B) production by H295R cells measured by five (5) independent laboratories (Lab 1 – Lab 5). Data are expressed as relative changes compared to the SC (upper graph) and percent of the least hormone concentration measured across all concentrations (maximum suppression = -100%; lower graph) observed after exposure to prochloraz for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

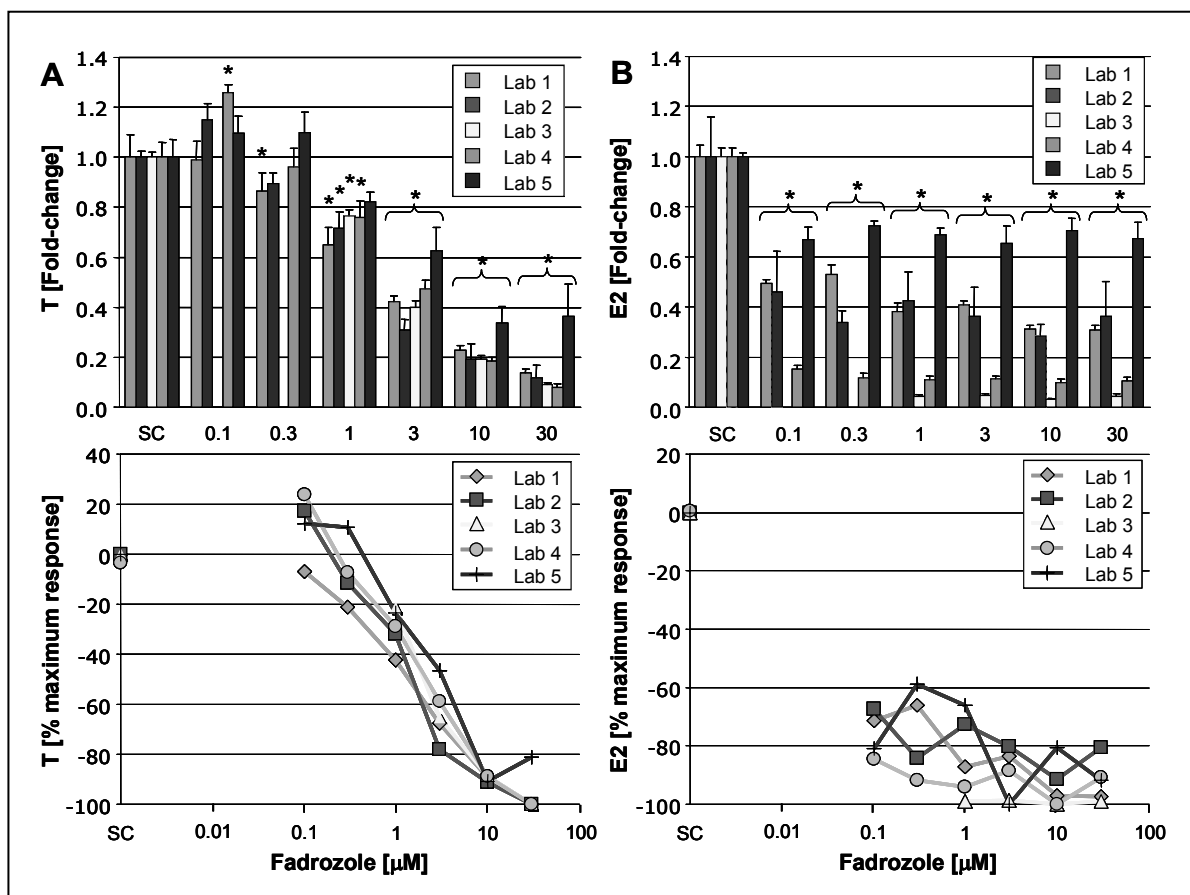


Figure 3.6: Changes in T (A) and E2 (B) production by H295R cells measured by five (5) independent laboratories (Lab 1 – Lab 5). Data are expressed as relative changes compared to the SC (upper graph) and percent of the least hormone concentration measured across all concentrations (maximum suppression = -100%; lower graph) observed after exposure to fadrozole for 48 h. Data represents the mean of three independent exposure experiments. Error bars = standard error of the mean (SEM). * = $p < 0.05$.

4 OBJECTIVES OF THE INTER-LABORATORY VALIDATION STUDY

The inter-laboratory comparison and validation study was conducted to evaluate the performance of the optimized H295R cell culture and exposure protocols (APPENDICES I & II) in different laboratories.

The specific objectives of this study are as follows:

1. Test the revised H295R Steroidogenesis Assay protocol at multiple independent laboratories;
2. Compare the specific changes in the production of T and E2 in response to 12 model compounds with different mode of interactions with steroidogenic pathways (weak, medium and strong inhibitors and inducers; negative chemicals);
3. Validate the quality control criteria to be met when conducting the H295R Steroidogenesis Assay;
4. Assess the transferability, reproducibility, sensitivity and applicability of the assay;
5. Identify and validate appropriate data evaluation procedures.
6. Apply the developed protocols and evaluation procedures to 16 supplemental chemicals with largely unknown types of interaction with the production of T and E2.

5 SELECTION OF PARTICIPATING LABORATORIES

A total of seven laboratories with different levels of experience in conducting the H295R Steroidogenesis Assay were selected to participate in the validation of this assay. Different proficiencies regarding the utilization of the assay are essential for an objective evaluation of the appropriateness of the test protocols and their transferability. As a consequence, the proficiency levels of the laboratories that are part of the validation group range from extensive to no previous experience. Extensive experience is represented by the lead laboratory (U of S/MSU), through which the assay was originally developed, and by three core laboratories (RTP, DIFVR, and CERI) that have been involved in the early pre-validation and optimization studies since 2005. A second group, consisting of two laboratories (GER and AOE), was chosen based on their participation in the most recent series of pre-validation studies that were conducted in 2006 and are representative of laboratories with limited experience with the assay. Finally, one laboratory (KFDA) was included that had never conducted the H295R Steroidogenesis Assay protocol. All laboratories that were part of the international validation team and their contact information are listed below (Chapter 5.1). Two of the originally identified seven laboratories only conducted partial analysis of only some of the chemicals and then decided not to participate further in the validation studies. As a consequence, this report presents complete data sets for the 12 core chemicals obtained by five laboratories (CERI, DIFVR, GER, RTP and UofS/MSU), and data sets from two laboratories for the 16 supplemental chemicals (see Chapters 1 and 7.2 for a detailed description of the specific experiments conducted by each laboratory).

5.1 Participating Laboratories

University of Saskatchewan (U of S; *Lead Laboratory*)

Toxicology Centre

Contact:

Dr. Markus Hecker, Ph.D.
44 Campus Drive
Saskatoon, SK S7N 5B3, Canada
TEL: +1 (306) 966-5233
FAX: +1 (306) 966-4796
Email: mhecker@ENTRIX.com

US Environmental Protection Agency (RTP)

Endocrinology Laboratory

Research Triangle Park

Contact:

Dr. Ralph Cooper, Ph.D.
Endocrinology Branch, MD-72
TRD, NHEERL, US EPA, USA
TEL: +1-919-541-4084
FAX: +1-919-541-5138
Email: cooper.Ralph@epamail.epa.gov

Chemicals Evaluation and Research Institute (CERI)

Chemicals Assessment Center

Contact:

Dr. Yumi Akahori, Ph.D.
1600, Shimo-Takano, Sugito-machi,
Kitakatsuhika-gun, Saitama, Japan
TEL: +81-3-5804-6136
FAX: +81-3-5804-6149
Email: akahori-yumi@ceri.jp

**Department of Toxicology and Risk Assessment (DIFVR)
National Food Institute
Technical University of Denmark**

Contact:

Christine Nellemann, Ph.D.
Mørkhøj Bygade 19
DK-2860 Søborg
Denmark
TEL: +45-72-34-7549
Email: CNE@food.dtu.dk

**University of Heidelberg (GER)
Heidelberger Institut für Zoologie**

Contact:

Dr. Henner Hollert, Ph.D.
Im Neuenheimer Feld 230
69120 Heidelberg, Germany
TEL: ++ 49 6221 54-5650
FAX: ++ 49 6221 54-6162
Email: Henner.Hollert@urz.uni-heidelberg.de

**City University of Hong Kong (AOE)
Department of Biology and Chemistry**

Contact:

Dr. Margaret Murphy, Ph.D.
83 Tat Chee Avenue
Hong Kong, SAR, China
TEL: +852-2788-7368
Email: mbmurphy@cityu.edu.hk

**National Institute of Toxicological Research (KFDA)
Korea Food and Drug Administration (KFDA)**

Contact:

Soon Young HAN, Ph.D.
Endocrine Toxicology Team
194 Tongil-ro, Eunpyung-gu
Seoul 122-704, Korea
TEL: +82-2-380-1877~9
FAX: +82-2-380-1879
Email: soonyoungh@kfda.go.kr

6 SELECTION OF CHEMICALS

To evaluate the validity of the H295R Steroidogenesis Assay as a screen for potential effects of suspected endocrine disrupting chemicals on the production of T and E2, a total of 28 chemicals were identified. These chemicals were selected based on their known or suspected endocrine activity, or a lack thereof, and include various inhibitors and inducers of different strengths as well as positive and negative controls. Prior to initiation of the validation studies with the international laboratory team, all chemicals were to be pre-analyzed by the lead laboratory (Lab1) using the H295 Steroidogenesis Assay. As noted above two laboratories dropped out of the validation program; thus, the 12 core chemicals were tested by the remaining five laboratories. The additional 16 chemicals were only tested by the lead laboratory and three other groups (Labs 2, 3 and 4).

Table 6.1: Number of chemicals tested for interaction with the production of testosterone (T) and estradiol (E2) at each laboratory as of the time this report was written. The number refer to the code number assigned to each chemical at the initiation of studies, and do not reflect the order in which compounds are listed in tables 6.1 and 6.2. This was done because all chemicals were to be tested blind to avoid any potential bias. Note: at the time this report was written not all laboratories had tested all chemicals yet.

Lab #	Core chemicals [code #]		Supplementary chemicals [code #]	
	T	E2	T	E2
1	1-12	1-12	13-29	13-29
2	1-12	1-12	13, 15, 17, 19, 21, 23	13, 15, 17, 19, 21, 23
3	1-12	1-12	14, 18, 20, 22, 24	14, 18, 20, 22, 24
4	1-12	1-12	25, 26, 27, 28, 29	25, 26, 27, 28, 29
5	---	---	---	---
6	1-12	1-12	---	---
7	---	---	---	---

To reduce the work load for individual laboratories, each group tested a total of 17 to 18 chemicals. These were comprised of the so called “core group” of 12 compounds that are tested in parallel by all laboratories. In addition to these 12 compounds, three laboratories conducted assays of a different set of five or six of the 16 supplementary chemicals in the assay. That is, the 16 chemicals were divided into three sub-groups of five to six chemicals and each chemical sub-group was to be tested by one laboratory (total number of laboratories = 3, so that with two laboratories [lead and one participating lab] testing five to six different compounds all 16 remaining compounds were analyzed).

Where possible the test set of chemicals was harmonized with those used in other steroidogenesis assays currently under development or in validation (e.g. the REACH program³).

³ REACH is a new European Community Regulation on chemicals and their safe use (EC 1907/2006). It deals with the Registration, Evaluation, Authorisation and Restriction of Chemical substances.

6.1 Core chemicals

Out of the 28 chemicals, a core set of 12 compounds was established that were analyzed by all laboratories participating in the validation studies (Table 6.2). Selection of these core chemicals was made in accordance with accepted guidelines for the validation of screening type test systems (OECD 2005, Hartung 2004). All compounds included in this core set were previously reported to exhibit a specific type of direct or indirect interaction with the production of T and/or E2 as measured either by means of the H295R Steroidogenesis Assay or by other steroidogenic tests including tissue explant assays and/or *in vivo* studies. In addition, this core chemical test set included a number of negative chemicals that were not expected to elicit any effect on the measured endpoints here at non-cytotoxic concentrations. The inclusion of such negative chemicals is of importance because it allows evaluation of the specificity of a test system with regard to the endpoints of interest. The suite of positive chemicals utilized in this core chemical set was chosen to reflect different types and strengths of interactions with the production of the hormones analyzed here.

Table 6.2: Core chemicals and their hypothesized mode of action selected for the H295R Steroidogenesis Assay validation studies. Due to the nature of the validation studies, conduct of experiments using coded chemicals, here we do not distinguish between core and supplementary chemicals. Chemicals are sorted in alphabetical order.

Code#	Name	CAS#	Mode of action	Product class	Effect type
2	Aminoglutethimide	125-84-8	Inhibits CYP19 aromatase and other cytochrome P450 enzymes	Pharmaceutical (phased out)	Medium to weak inhibitor of T and E2 production.
1	Atrazine	1912-24-9	Aromatase inducer in vitro	Herbicide	Weak inducer of E2 production.
11	Benomyl	17804-35-2	Aromatase inducer in vitro	Fungicide	Weak inhibitor and weak inducer or negative of T and E2 production, respectively. Has been shown to induce aromatase activity in human ovarian tumor cells (KGN).
8	Ethane dimethane sulfonate (EDS)	4672-49-5	Cytotoxic		No effect expected at non-cytotoxic concentrations.
3	Forskolin	66575-29-9	Cyclic-AMP second messenger system	Pharmaceutical	Strong inducer of T and E2 production.
10	Human chorionic gonadotropin (hcG)	9002-61-3	Binds to GtH receptor	Peptide hormone	No effect on T and E2 production in H295R cells.
5	Letrozole	112809-51-5	Specifically inhibits catalytic aromatase activity.	Pharmaceutical	Strong inhibitor of E2 production. Weak inhibitor of T production.
7	Molinate	2212-67-1	Anti-cholinesterase/ neurotoxicant. Note: In vitro, molinate is a poor inhibitor of esterase activity, whereas molinate sulfoxide, a major metabolite of molinate in rats, and molinate sulfone were shown to be potent inhibitors of esterase activity, suggesting that metabolic activation of molinate is required in vivo.	Pesticide	Weak inducer of E2 and negative/weak inhibitor of T production.

9	Nonoxynol-9	26027-38-3	Unknown	Excipients, Pharmaceutical aid [surfactant], Pharmaceutical aid [wetting and or solubilizing agent], Spermicide	Unknown.
6	Paraben (Butyl paraben)	94-26-8	ER binder	Preservative in food, cosmetics, toiletries, pharmaceutical.	Weak inducer of E2, and weak inhibitor of T production.
4	Prochloraz	67747-09-5	General inhibitor of microsomal cytochrome P450 mixed function oxidases.	Fungicide	Strong inhibitor of T and E2 production.
12	Trilostane	13647-35-3	3B-HSD competitive inhibitor	Pharmaceutical, used in treatment of Cushings disease	Strong inducer of T and E2 production.

6.2 Supplementary chemicals

In addition to the 12 core chemicals described in the previous section, 16 compounds were selected for additional testing (Table 6.3). To reduce the burden on each laboratory, the 16 chemicals were divided into 3-subsets of five to six chemicals each, and each subset will be tested by two laboratories (lead lab and one participating laboratory).

Selection of these additional 16 chemicals was made based on the range of putative effects, as well as general toxic properties and technical feasibility (e.g. availability of the compound, ownership rights, etc.). The types of effects were categorized as strong, medium, and weak inducers and inhibitors of production of testosterone, estradiol, or both hormones as well as negative compounds. All decisions were discussed and made in agreement with the US-EPA and the OECD advisory group.

Table 6.3: Supplemental chemicals and their hypothesized mode of action selected for the H295R Steroidogenesis Assay validation studies. Due to the nature of the validation studies, conduct of experiments using coded chemicals, here we do not distinguish between core and supplementary chemicals. Chemicals are sorted in alphabetical order.

Code#	Name	CAS#	Mode of action	Product class	Effect type
24	2,4-Dinitrophenol	51-28-5	Cell toxicant: phosphorylation uncoupler	Industrial chemical	No known endocrine function other than cell toxicity and altered bioenergetics.
14	Bisphenol A	80-05-7	Cyclic-AMP second messenger system; reported ER binder	Monomer in polycarbonate plastics	Unknown. Some evidence that alters Progesterone in vitro, but mechanism may or may not be c-AMP second messenger system. For all steroidogenesis assay, will need to be specific for endpoint of assay. Tested positive for ER binding in vitro and in uterotrophic assay.
23	Danazol	17230-88-5	3HSD; P450c17 (17 hydroxylase/C17-20 lyase); 17KSR	Agricultural Chemical, Antineoplastic agents, Contraceptives, postcoital, synthetic, Drug / Therapeutic Agent	Unknown
19	Di (2-ethylhexyl) phthalate (DEHP)	117-81-7	Inhibits FSH-stimulated cAMP accumulation. Effects have been demonstrated at the level of P450c17 and aromatase. Note: Compound that has been hypothesized to be active is the metabolite MEHP, not DEHP.	Polyvinyl additive	Metabolite monoethylhexyl phthalate (MEHP) has been shown to suppress aromatase and estradiol production in female rat primary granulosa cells. Parent compound is not considered active.
28	Dimethoate	60-51-5	Inhibits steroidogenesis by disrupting transcription of StAR	Organophosphorus insecticide	Has been shown to decrease progesterone synthesis in vitro; does not affect aromatase activity in vitro
18	Fenarimol	60168-88-9	Aromatase inhibition	Fungicide	Shown to inhibit aromatase (CYP19) in vitro, evidence from in vivo studies not as unequivocal
22	Finasteride	98319-26-7	5- α reductase inhibitor	Pharmaceutical, therapeutic agent for prostate cancer, hirsutism, and alopecia	Unknown
21	Flutamide	13311-84-7	P450c17 (17 hydroxylase/C17-20 lyase)	Pharmaceutical	Unknown
20	Genistein	446-72-0	Anti-oxidant, topoisomerase inhibitor/ tyrosine kinase inhibitor	Pharmaceutical	Weak inducer of E2 and weak inhibitor of T production. Weak estrogen receptor agonist
29	Glyphosate (Roundup)	1071-83-6		Herbicide	Unknown. Has not shown to conclusively affect reproduction in laboratory in vivo studies.

15	Ketoconazole	65277-42-1	Inhibiting the microsomal cytochrome P450 mixed function oxidases. This drug inhibits 17 alpha-hydroxylase, C17-20 lyase, and the cholesterol-side-chain cleavage enzyme	Fungicide	Strong inhibitor of T production; Medium inhibitor of E2 production; Induces progesterone production.
13	Piperonyl butoxide	51-03-6	Cytochrome P450 inhibitor	Pesticide synergist	Unknown. This compound is used to inhibit several P450s involved in metabolism but not necessarily steroidogenesis.
17	Prometon	1610-18-0	Photosynthetic inhibitor	Wide-spectrum herbicide	Weak inducer of E2 production; Negative for T.
26	RU-486/mifepristone	84371-65-3	Negative for ER very weakly positive for AR at high conc., blocking the progesterone receptor, incr. levels of EST.	Pharmaceutical	Unknown
25	Spirolactone	52-01-7	Antiandrogen action through inhibition of 17 α hydroxylase; Glucocorticoid & PXR-ligand	Pharmaceutical	Unknown

7 TEST PROCEDURE

7.1 QA criteria

Laboratories were required to demonstrate competence in performing all of the procedures that are part of the H295R Steroidogenesis Assay prior to testing chemicals. The quality controls that were part of the actual conduct of the assay to allow for the evaluation of the assay performance during each experiment also served as the benchmarks for determining laboratory competence prior to the initiation of chemical testing. Finally, at the lead laboratory the ELISA method based analysis of T and E2 concentrations in a selection of samples (QC-plate) was confirmed using LC-MS to identify any potential issues related to the use of antibody-based hormone detection systems.

7.1.1 Performance of H295R Cells under Standard Culture Conditions

The qualifying experiment required growing cells for 5 passages, seeding and exposing them as described for the QC plates in the H295R exposure protocol, and measuring E2 and T in the cell media using pre-validated hormone detection methods. The threshold concentrations for basal production of testosterone (T) and estradiol (E2) relative to the minimum detectable level (MDL) of the assay method employed at each laboratory is given for each test chemical (Table 7.1). Since production of E2 in passage 5 cells may not be sufficient to detect decreases greater than 1.5-times the response after exposure to an inhibitor (Hecker et al. 2006b), during the qualifying experiments it was only expected that the laboratory showed conformance with the performance criteria for E2 induction after exposure to the stimulator forskolin (Table 7.1).

Table 7.1: Performance criteria to be met by each laboratory during the qualifying experiments. Criteria were selected based on the previous pre-validation studies investigating the effects of forskolin and prochloraz on the production of T and E2 by H295R cells in five independent laboratories (Hecker et al. 2007b). Numbers represent average (replicate wells of a given concentration) change in hormone production relative to the solvent control (SC = 1) in a given plate.

	<i>Testosterone</i>	<i>Estradiol</i>
<i>Basal Production</i>	$\geq 2.5\text{-times MDL}$	$\geq 2.5\text{-times MDL}$
<i>Induction (10uM forskolin)</i>	$\geq 1.5\text{-times SC}$	$\geq 5\text{-times SC}$
<i>Inhibition (3uM prochloraz)</i>	$\leq 0.5\text{-times SC}$	n/a

7.1.2 Performance of H295R Cells during Exposure Experiments

A necessary step in the validation of an assay is to define the quality control (QC) criteria. A QC plate was designed to include wells for control (i.e., blank), non-treated wells, solvent control wells, wells with a known inducer (forskolin) and wells with a known inhibitor (prochloraz). The first step in the QC validation studies was to measure the alterations in hormone production in cells exposed to forskolin and prochloraz in the QC plates. In addition, selected wells are dedicated to the evaluation of maximum cytotoxicity and the viability assay is performed on the entire plate. (Figure 7.1) The target performance criteria to be achieved for the QC plate are described below and were selected based on the data obtained for the exposure to these chemicals at five laboratories during the pre-validation studies (Hecker et al. 2007b):

1. Blank: basal hormone production of T and E2 had to be at least 2.5-times the MDL of the hormone detection assay used.
2. Solvent control: basal hormone production of T and E2 had to be at 2.5-times the MDL of the hormone detection assay used.
3. Inducer: *Forskolin* (10 μM): ≥ 7 -times induction of E2 production, and ≥ 2 -times induction of T production after 48 h.
4. Inhibitor: *Prochloraz* (3 μM): $\geq 50\%$ reduction of E2 and T production compared to the SCs after 48 h.

	1	2	3	4	5	6
A	Blank ^a	Blank ^a	Blank ^a	Blank ^a + MeOH ^b	Blank ^a + MeOH ^b	Blank ^a + MeOH ^b
B	DMSO 1uL	DMSO 1uL	DMSO 1uL	DMSO 1uL + MeOH ^b	DMSO 1uL + MeOH ^b	DMSO 1uL + MeOH ^b
C	FOR 1uM	FOR 1uM	FOR 1uM	PRO 0.3uM ^c	PRO 0.3 M ^c	PRO 0.3uM ^c
D	FOR 10uM	FOR 10uM	FOR 10uM	PRO 3uM ^c	PRO 3uM ^c	PRO 3uM ^c

^a Blank wells receive medium only.

^b Methanol (MeOH) was added **after** the exposure is terminated and the medium is removed from these wells.

^cNote: These concentrations were changed in the final guideline based on the findings of this validation effort

Figure 7.1: Plate layout for QC-plate to be analyzed together with each chemical exposure experiment. PRO = prochloraz; FOR = forskolin; MeOH = methanol.

Other quality criteria include an evaluation of the variation between replicate wells, replicate experiments, linearity, and sensitivity of hormone detection systems, variability between replicate hormone measures of the same sample. One criterion for the acceptance of data generated during an experiment was the validity of the SC in terms of its position within the standard curve range. Allowable location of the SC was within the 75% range below the upper part (maximum optical density [OD] or similar response measured by hormone detection system) and 75% above the lower part (minimum OD or similar response measured by hormone detection system) of the linear range of standard curve for inducers, and inhibitors, respectively (Figure 7.2). Dilutions of medium (extracts) in the hormone detection assay were to be selected accordingly.

Table 7.2: Acceptable ranges and/or variation (%) for H295R assay QC parameters.

	Comparison Between	T	E2
<i>Basal hormone production in blanks and SCs</i>	<i>Fold-greater than MDL</i>	$\geq 5\text{-fold}$	$\geq 2.5\text{-fold}$
<i>Exposure Experiments - Within Plate CV for SCs (Replicate Wells)</i>	<i>Absolute Concentrations</i>	$\leq 30\%$	$\leq 30\%$
<i>Exposure Experiments - Between Plate CV for SCs (Replicate Experiments)</i>	<i>Fold-Change</i>	$\leq 30\%$	$\leq 30\%$
<i>Hormone Detection System – Sensitivity</i>	<i>Detectable fold-decrease relative to SC</i>	$\geq 5\text{-fold}$	$\geq 2\text{-fold}$
<i>Hormone Detection System – Replicate Measure CV for SCs</i>	<i>Absolute Concentrations</i>	$\leq 25\%$	$\leq 25\%$
<i>Medium Extraction – Recovery of Internal ^3H Standard (If Applicable)</i>	<i>CPM</i>	$\geq 65\% \text{ Nominal}^a$	

^a Note: Hormone concentration should be normalized for % recovery in each well.

The QC plate was used in all assays, and the ability to meet these criteria was intended to be used as the qualifier to accept data generated during an experiment. In those instances where the data did not meet these criteria, the experiment typically was to be repeated. A second level of QC was required for the acceptance of the test chemical data generated during the validation studies. These QC criteria were to be defined on the basis of the variation observed during the validation experiments, and were to include but were not limited to measures of effective concentrations (EC5, EC10 and EC50) and fold-changes relative to the SCs (see section 10).

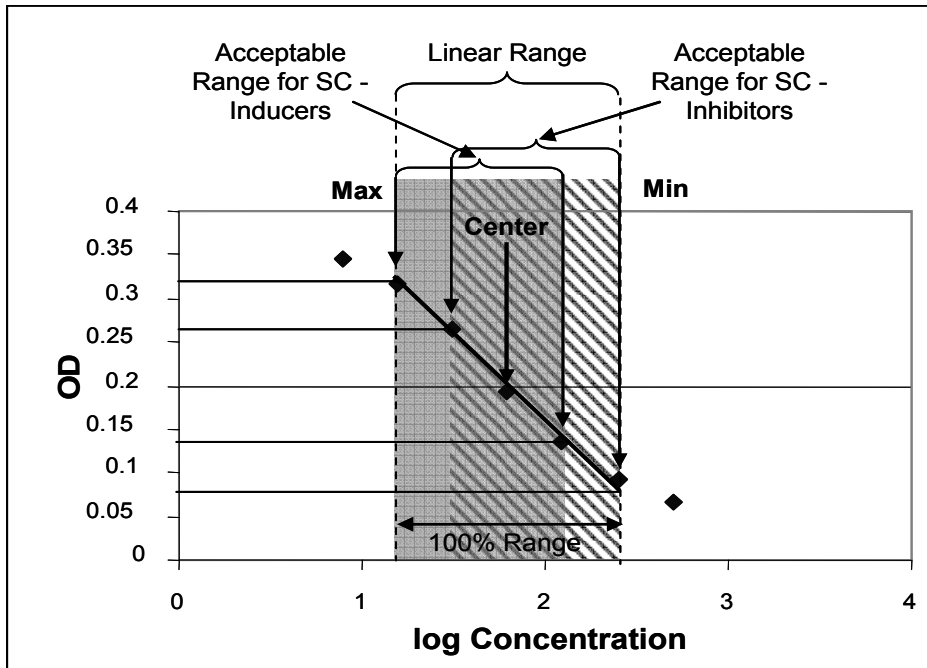


Figure 7.2: Example of hormone detection system standard curve indicating acceptable range of hormone concentration of solvent control (SC) sample (not corrected for dilution in assay). Max = upper limit of linear range; Min = lower limit of linear range. Grey shaded area = 75% range below the maximum OD of the linear part of the standard curve. Diagonally striped area = 75% range above the minimum OD of the linear part of the standard curve.

7.2 Testing of chemicals

Validation of the H295R Steroidogenesis Assay was conducted in two main phases, each focusing on a certain suite of chemicals. In the first phase, the core set of 12 chemicals were to be analyzed by all laboratories. In the second phase, the remaining 16 compounds were to be tested in three sub-sets of five to six chemicals, each of which were analyzed by two laboratories (lead laboratory and one participating group).

Prior to initiation of the actual exposure experiments each chemical had to be tested for potential interference with the hormone detection system utilized. This was of particular relevance for antibody based assays such as ELISAs and RIAs because it has been previously shown that some chemicals can interfere to a certain extent with these tests (Shapiro and Page 1976; Puddefoot et al. 2002; Villeneuve, personal communication; Hecker, personal communication). The results from a simple test can not be used to correct for possible interference with the hormone detection system, nor can it be used to eliminate the compound of interest from being evaluated in the H295R assay, it will provide critical information regarding the validity of the data generated.

7.2.1 Core chemicals

After satisfactorily demonstrating that the laboratory could meet the performance criteria listed in Table 7.1, the laboratory was allowed to proceed with evaluation of the 12 core chemicals. In this phase each chemical was to be tested in two-three independent experiments as a measure of intra-laboratory variation. Testing of core chemicals represents the key aspect of these studies and the results are critical in confirming the basic utility of the assay. As pointed out in Section 6, the majority of the core chemicals have been well characterized in terms of their interaction with the production of T and E2 or other endocrine processes. It was decided in conjunction with the US-EPA that completion of this stage of the validation studies and the result of the supplementary chemicals in the lead lab will suffice for the submission of the assay for peer-review and validation through the EDSP and would allow EPA to include the H295R assay in the Tier 1 battery for the first group of chemicals for which screening were to begin in August 2008.

7.2.2 Supplementary chemicals

For validation purposes through OECD, an additional 16 chemicals were tested using the H295R Steroidogenesis Assay following the procedures given above. These studies were initiated after phase I (testing of core chemicals) had been completed. The aim of this second phase of supplementary chemical testing was to evaluate the broader application of the assay to chemicals of greater structural diversity, including less well characterized compounds and chemicals of unknown or uncertain modes of action.

7.3 Data interpretation

One purpose of this validation study was to come to a decision regarding the interpretation and evaluation of data obtained with the H295R Steroidogenesis Assay. It was the aim to identify and harmonize appropriate statistical procedures to be used with the assay. Defining data evaluation criteria is essential in the validation and later implementation of the assay and will be a key component of the standardized study protocols that will come out of these efforts, and that are to be submitted for review through OECD member countries. To achieve these goals the data collected during the experiments involving the 12 core chemicals have been subjected to a number of different data evaluation procedures, and from which the most appropriate approach was to be selected for the final study protocols.

In previous discussions with both the US-EPA and OECD, it was decided that results obtained with

the H295R Steroidogenesis Assay would be evaluated using a semi-quantitative approach that will group data as negative, weak, medium or strong inducers or inhibitors of steroidogenesis. Decisions regarding the evaluation approach utilized in this validation study were decided together with the laboratories involved in the OECD validation of the assay as well as with the OECD advisory group. Prior to being subject to the analysis procedures discussed below, data were to be screened using specific qualifiers. A summary of these parameters is provided (*Table 7.3*), and a detailed discussion and selection of criteria and approaches based on the data generated during this validation effort is given in section 10.

Table 7.3: Data qualifying parameters for the pre-categorization of results obtained with the H295R Steroidogenesis Assay prior to statistical assessment.

Parameter	Criterion
Interference with hormone detection assay	When marked interference of the chemical of interest with the hormone detection system utilized occurs ($\geq 30\%$ of hormone concentration measured at the same concentration at which interference occurred), this data is to be omitted. In the case of weak to moderate interference ($< 30\%$ of hormone concentration measured at the same concentration at which interference occurred), results may be corrected for the % interference.
Solubility	The results at concentrations for which cloudiness or a precipitate is observed should not be included.
Cell viability	Only non-cytotoxic concentration ($> 80\%$ cell viability) are to be included

If data passed this initial screening using the above provided criteria (*Table 7.3*), it was to be subjected to basic parametric or non-parametric (as appropriate) statistical evaluation (e.g. ANOVA; pair wise comparisons). For statistically significant differences (≤ 0.05) to be further considered for data evaluation, data were to follow a concentration response type profile at non-cytotoxic concentrations, or concentrations that did not interfere with the hormone detection assay. This response could be bi-phasic such as an increase at lower and a decrease at higher concentrations, but changes randomly observed at only a few concentrations within the concentration range were to be excluded.

In addition, where permitted by the data, concentration-response curves were to be defined that would allow both the determination of effective concentrations (ECs) of a chemical to alter a hormone concentration and its relative potency. It was assumed that the study protocols would only permit the description of concentration-response curves for medium and strong inhibitors/inducers. This was because the concentration-range for chemicals to be tested in the validation studies covered a wide range of concentrations due to the relatively wide spacing of concentrations; however, this results in lower resolution. Weak inducers that only show effects at the highest concentrations, therefore, would be required to be re-analyzed using a different dosing regime to allow for the description of full concentration-response curves. The OECD validation management group decided that such retesting was not necessary given the primary purpose of this assay: a screen to identify potential inducers and/or inhibitors of T and E2 production. The proposed approach allowed the grouping of chemicals based on their potency to alter the production of T and E2, and provided additional toxicological information for medium and strong effectors.

A key component of the data evaluation procedure was the integration of cell viability data from each assay to identify acceptable concentration-ranges that can be used in evaluating the potency of a test chemical. Care must be taken when strong cytotoxicity is observed for a chemical. In this study all concentrations that exhibited cytotoxicity greater 20% were omitted for further evaluation.

8 DATA PROCESSING AND STATISTICS

All data are expressed as mean +/- standard deviation (SD). To evaluate the relative increase/decrease in chemically altered hormone production, results were normalized to the mean solvent control (SC) value for each assay (i.e., each 24-well plate of cells used to test a given chemical), and results are expressed as changes relative to the SC in each exposure plate (Equation 1). Furthermore, the average response in each well was divided by the relative cell viability measured in the same well to normalize for possible differences due to variations in the number of live cells (note: as stated in 7.3; Data interpretation, all data obtained for wells with > 20% cytotoxicity was omitted).

Fold-change = Hormone concentration per well / average hormone concentration of SCs on the same plate
(1)

Prior to conducting statistical analyses, the assumption of normality and variance homogeneity was evaluated. Normality was evaluated using standard probability plots or any other appropriate statistical method (e.g. Shapiro-Wilk's test). If the data were normally distributed or approximate normal distribution, differences between chemical treatments and solvent controls (SCs) were analyzed using a one way ANOVA design followed by Dunnett's test. If data were not normally distributed, the Kruskal Wallis test followed by the Mann Whitney U test was used. Effective concentrations (EC₅₀s) were calculated using a probit model. Differences were considered significant at $p \leq 0.05$.

8.1 Intra-laboratory statistics

The participating laboratories provided all data to the lead laboratory for statistical analysis. Data was reported both in form of mean responses \pm 1 SD for each well measured and as raw data. Mean response data were entered by each laboratory into the data sheet templates provided for this purpose by the lead laboratory. Raw data was submitted to the lead laboratory in a format of choice by the participating laboratories, and included the following information:

1. Standard and calibration curves for all analytical assays conducted
2. Each replicate measure in form of the original data provided by the instrument utilized for a specific analysis (e.g. as OD, fluorescence units, CPM, etc.)
3. Hormone extraction recovery data (if applicable)

Each laboratory was responsible for conducting assay-related statistical analysis (e.g. variation between replicate measures of the same well) in compliance with the QC criteria required for this assay. In those cases where significant deviations from target criteria were observed (e.g. increased variation between replicate measures of the same sample, significant differences between standard curves of the same assay), the deviations were to be reported to the lead laboratory. The lead laboratory then decided in conjunction with the participating laboratory and the OECD advisory group regarding the acceptability of these data.

8.2 Inter-laboratory statistics

Conduct of inter-laboratory data evaluation and statistical analyses was the responsibility of the lead laboratory. Data was subjected to the statistical procedures described above (Chapter 9.0). Statistical analysis procedures and results were submitted to the US-EPA and the OECD advisory group for independent review. All data and analysis results are provided in form of summarized data and raw data including all analysis procedures and steps the data was subjected to (APPENDICES III – IX).

9 RESULTS

Note: In the subsequent sections only data from five of the seven laboratories that originally agreed to participate in the validation studies is presented. This is because during the conduct of the studies two laboratories (Lab 5 and 7) decided to not further participate in these studies for different reasons. At the time when these decisions were made only partial data was available, which was not included in this report.

9.1 QA criteria

9.1.1 Performance of H295R Cells under Standard Culture Conditions

9.1.1.1 Basal hormone Production (Blanks)

Threshold concentrations for basal production of testosterone (T) and estradiol (E2) varied significantly among laboratories (Table 9.1). The greatest and the least T concentrations were observed at Labs 1 and 6 with 7729 and 1409 pg/mL, respectively. For E2, concentrations ranged between 311 and 13 pg/mL at Labs 6 and 3, respectively. With the exception of E2 at Labs 1 and 6, the coefficients of variation (CVs) at the different laboratories were between 18 and 32% for T, and between 27 and 36% for E2. Among laboratory CVs were 77 and 70% for T and E2, respectively. All laboratories met the performance criterion for the basal hormone production, which was defined as a 2.5-fold greater concentration than the MDL for each hormone (

Table 7.2).

Table 9.1: Comparison of basal production of testosterone (T) and estradiol (E2) as measured in the blanks of the QC-plates among laboratories. SD = Standard deviation; CV = Coefficient of variation (%); Max = Maximum hormone concentration observed during all experiments; Min = Minimum hormone concentration observed during all experiments; Lab = Laboratory.

	Lab 1		Lab 2		Lab 3		Lab 4		Lab 6	
	T	E2	T	E2	T	E2	T	E2	T	E2
Mean	5512	96	1894	50	4560	20	1877	54	1943	155
SD	1764	49	488	14	818	6	390	19	365	109
CV	32	51	26	27	18	31	21	36	19	70
Max	7729	143	2411	61	5451	24	2204	77	2202	311
Min	3287	25	1441	35	4278	13	1445	31	1409	63

The comparison of basal hormone production when measured by LC-MS vs ELISA at the lead laboratory revealed comparable concentrations for E2 (*Figure 3.1*). In contrast, T production appeared to be overestimated by approximately 50% when measured by ELISA. As noted below, this is likely due to the cross-reaction of the T antibody with androstenedione (3.7%), which is produced at much greater concentrations (Hecker, personal communication) between 8,000 and 30,000 pg/mL.

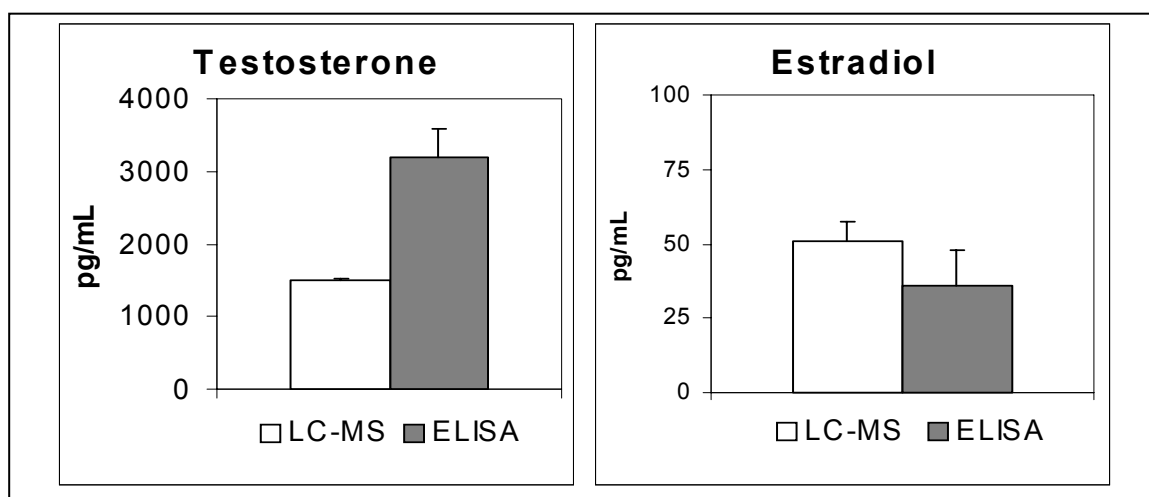


Figure 9.1: Comparison of basal production of testosterone and estradiol by H295R cells as measured by LC-MS and ELISA. Error bars = 1x standard deviation. Bars represent means of two (LC-MS) or three replicate wells (ELISA).

9.1.1.2 Initial QC-Plate Experiment Responses

Changes in hormone production relative to the solvent controls as determined in the QC-plate experiment conducted to evaluate the performance of the H295R prior to initiation of the exposure experiments showed a comparable trend among laboratories with the exception of E2 production after exposure to 0.3 μ M prochloraz at Labs 3 and 4 (Figure 9.2). However, these laboratories achieved similar responses compared with all other groups during later QC-plate experiments (Figures 9.5 and 9.6), and thus, all subsequent data from these laboratories was accepted if not stated otherwise. There was some variability among laboratories for the production of both hormones (

Table 9.2), and maximum CVs were 79% for the forskolin exposures, and 100% for the T data in the prochloraz exposures. Within laboratory variation was substantially less with CVs not exceeding 34% for either the SCs or the forskolin treatment groups (Table 9.3). The greater CVs observed after exposure to prochloraz are due to the fact that hormone concentrations were close to the method detection limits of the utilized hormone quantification assays. Regardless of the observed inter-laboratory variation, all laboratories could demonstrate conformance with the data performance criteria outlined in chapter 7.1.2 with the exception of T measured by Labs 2 & 3 after exposure to forskolin. Here only 1.5- and 1.6-fold instead of the desired 2-fold increases were observed. Normally these data would be disqualified; however, considering that the trend for T after exposure of cells to forskolin was similar to that reported by the other groups, and because in the subsequent experiments greater than 2-fold changes were observed at these labs (see chapter 9.1.2), it was decided to accept data from these laboratories. Nevertheless, care should be taken when evaluating T data of laboratories for potential inducers when less than 2-fold changes are observed in the respective QC-plate of an experiment, and it is recommended that in such situations the number of replicate experiments should be increased such that the effect can be described with great certainty (a minimum of three out of four experiments should confirm the effect).

In the previous chapter (Chapter 9.1.1.1) it was shown for the lead laboratory that there seem to be an overestimation of T concentrations measured by the utilized ELISA when compared to LC-MS. It was hypothesized that this difference was likely due to a low percentage of cross-reactivity of the antibody of the ELISA system used to measure T with androstenedione. To assess whether this interference could possibly affect the relative change in hormone production as a function of chemical exposure one additional QC-plate experiment was conducted comparing the concentrations of hormones when measured

by ELISA with those determined by means of LC-MS. This analysis revealed that while there was a slight overestimation and underestimation of T and E2, respectively, by the ELISAs when compared to the LC-MS results, both methods were very comparable (Figure 9.3). Considering the hypothesized interference of the T antibody with androstenedione as true, the only concern of the overestimating tendency of the T ELISA derived data would be if a test chemical interferes with 17 β -HSD, This would be expected to cause a shift in the ratio of androstenedione and T and the interference with 17 β -HSD would not be detected in the measurement of T. However, given that no comparable overestimation for E2 was observed, potential effects due to changes in this enzyme would be captured through the measurement of E2, which would be expected to decrease under the scenario described above.

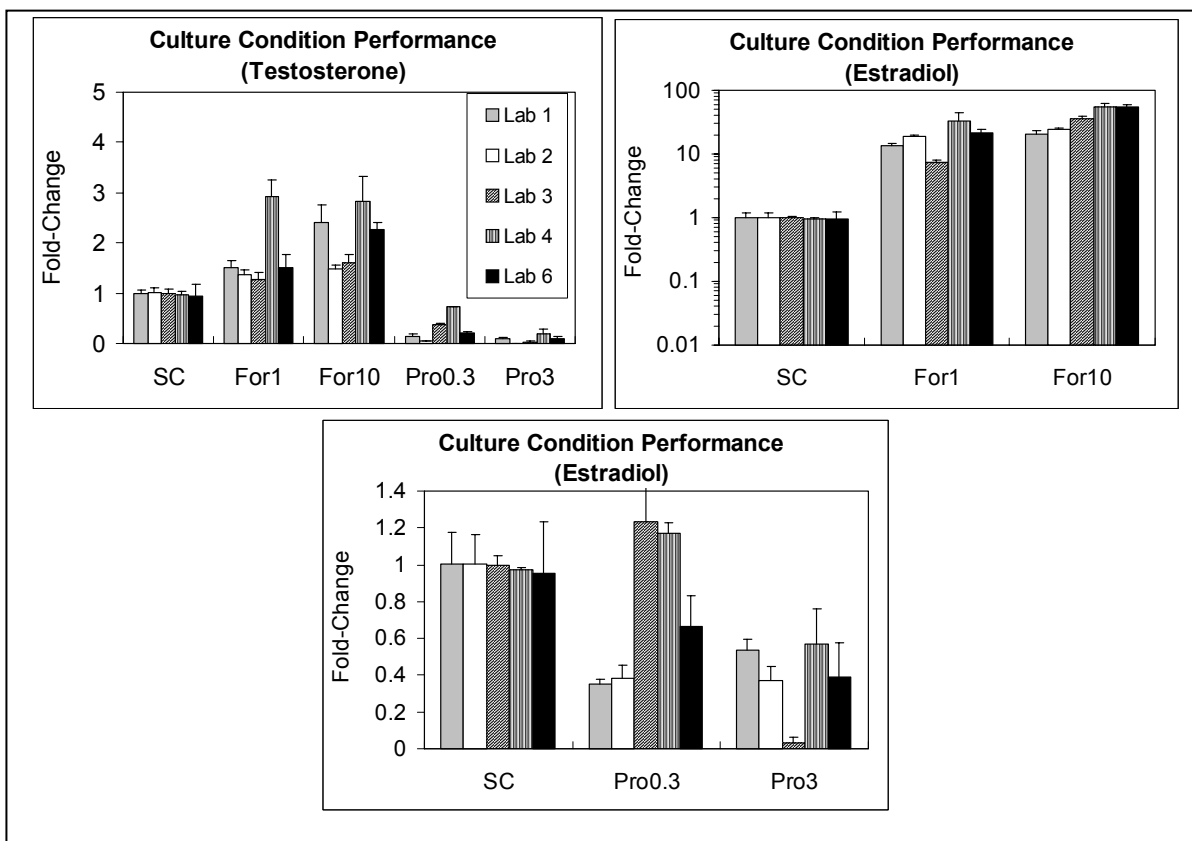


Figure 9.2: Comparison of changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) in the QC-plate experiment conducted to evaluate the performance of the H295R prior to initiation of the exposure experiments. For 1 = 1 μ M Forskolin; For 10 = 10 μ M Forskolin; Pro 0.3 = 0.3 μ M Prochloraz; Pro 3 = 3 μ M Prochloraz. Error bars = 1x standard deviation. Bars represent means of three replicate well (Lab 4 only 2 replicate wells).

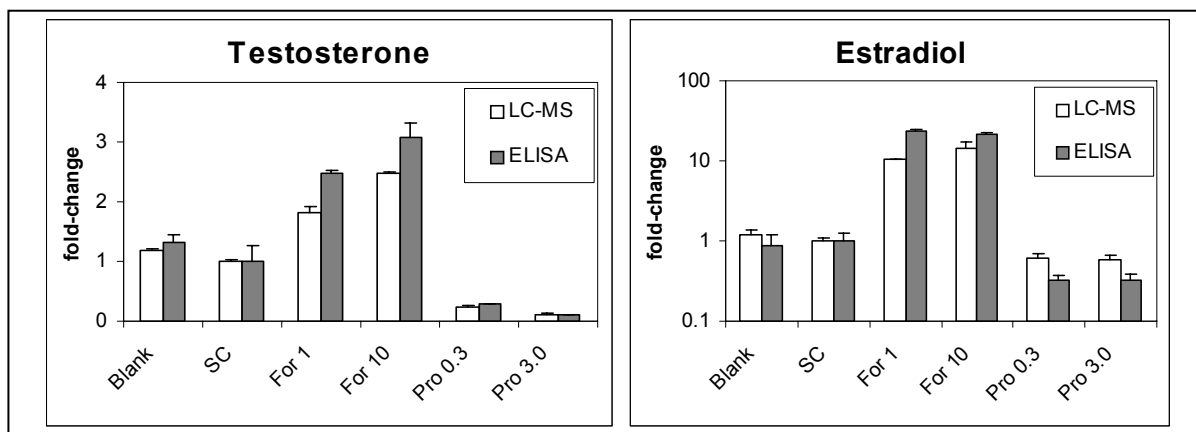


Figure 9.3: Comparison of changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) in a QC-plate experiment when measured by LC-MS and ELISA at the lead laboratory (Lab 1). For 1 = 1 μ M Forskolin; For 10 = 10 μ M Forskolin; Pro 0.3 = 0.3 μ M Prochloraz; Pro 3 = 3 μ M Prochloraz. Error bars = 1x standard deviation. Bars represent means of two (LC-MS) and three (ELISA) replicate wells.

Table 9.2: Among laboratory coefficients of variation (CVs) for changes in concentrations of testosterone (T) and estradiol (E2). Calculations were based on data expressed relative to the solvent controls (SC=1) from the QC-plate experiment conducted to evaluate the performance of the H295R prior to initiation of the exposure experiments. For 1 = 1 μ M Forskolin; For 10 = 10 μ M Forskolin; Pro 0.3 = 0.3 μ M Prochloraz; Pro 3 = 3 μ M Prochloraz.

	Among Lab CV	
	T	E2
For 1	45	79
For 10	34	67
Pro 0.3	100	69
Pro 3	97	61

Table 9.3: Within laboratory coefficients of variation (CVs) for changes in concentrations of testosterone (T) and estradiol (E2). Calculations were based on absolute data from the QC-plate experiment conducted to evaluate the performance of the H295R prior to initiation of the exposure experiments. For 1 = 1 μ M Forskolin; For 10 = 10 μ M Forskolin; Pro 0.3 = 0.3 μ M Prochloraz; Pro 3 = 3 μ M Prochloraz.

Testosterone					
	Lab1	Lab2	Lab3	Lab4	Lab6
SC	6	10	10	8	24
For 1	9	7	12	11	18
For 10	14	3	10	17	6
Pro 0.3	29	30	8	0.5	11
Pro 3	41	64	50	52	41

Estradiol					
	Lab1	Lab2	Lab3	Lab4	Lab6
SC	18	16	5	1	30
For 1	7	6	8	34	13
For 10	12	5	6	11	9
Pro 0.3	8	18	42	5	25
Pro 3	11	21	89	33	47

9.1.2 Performance of H295R Cells during Exposure Experiments

As in the standard culture performance experiments, with a few exceptions, all laboratories met the key quality performance parameters such as fold induction or percent inhibition of hormone production after exposure to forskolin and prochloraz, respectively and observable fold-change (Figures 9.4, 9.5 & 9.6). However, in rare cases basal hormone production was so low that no decreases in E2, or T (one laboratory only) production could be measured (Table 9.4). Another issue that was observed in rare occasions was an increase in variation among replicate wells such that the data could not be used for further evaluations. This, however, occurred only at one laboratory during a single experiment (Lab 4; Chemicals 5-8; Experiment 1), where the average CV of the SCs was 48%, which is almost 20% greater than the QC criterion of 30% for this parameter. None of the results obtained during these experiments were used for the data evaluation as described in Chapter 10.2 (Table 9.4). However, it should be noted that these events were rare and did not impact the overall validity and usability of data produced during these studies. Overall, only 2 or 7% of all experiments for T and E2 were excluded due to these issues. The reason for the slightly greater percentage of non-usable data for E2 is probably due to the fact that basal E2 production was relatively low compared to T (Hecker et al. 2006b).

Relative changes in the production of T and E2 after exposure to forskolin and prochloraz in the QC plates was comparable both within and among laboratories (Figures 9.4, 9.5 & 9.6), indicating that the H295R Steroidogenesis Assay functioned properly at all laboratories. Coefficients of variation for relative changes measured after exposure to forskolin and prochloraz were between 12 and 13% and between 44 and 77%, respectively, for T, and between 62 and 73% and between 31 and 55%, respectively, for E2.

Table 9.4: Experiments and chemicals for which basal hormone concentration of testosterone (T) and estradiol (E2) was such that no decreases and/or increases after chemical exposure could be observed. Number indicate number of laboratories in which these issues occurred. Exp = Number of Repeat Experiment in which incidence occurred

Chemical	Testosterone			Estradiol		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
Atrazine		1				
Aminoglutethimide		1	1		1	2
Benomyl				2		1
EDS						
Forskolin						
HCG				2	1	
Letrozole		1				
Molinate				1		
Nonoxynol-9						
Paraben				1		
Prochloraz					1	1
Trilostane						

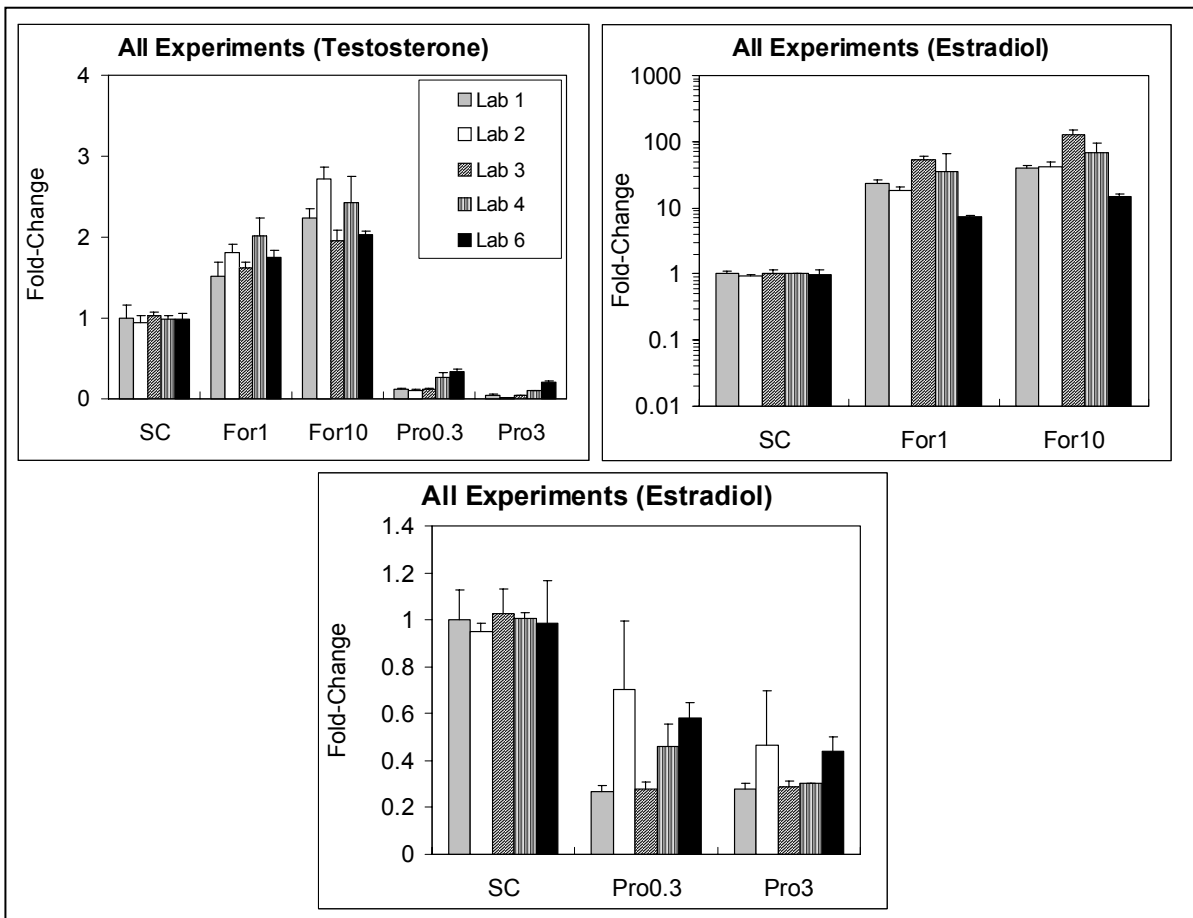


Figure 9.4: Comparison of changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) in the QC-plates among laboratories (Lab). For 1 = 1 μ M Forskolin; For 10 = 10 μ M Forskolin; Pro 0.3 = 0.3 μ M Prochloraz; Pro 3 = 3 μ M Prochloraz. Error bars = 1x standard deviation. Bars represent means of three replicate well (Lab 4 only 2 replicate wells).

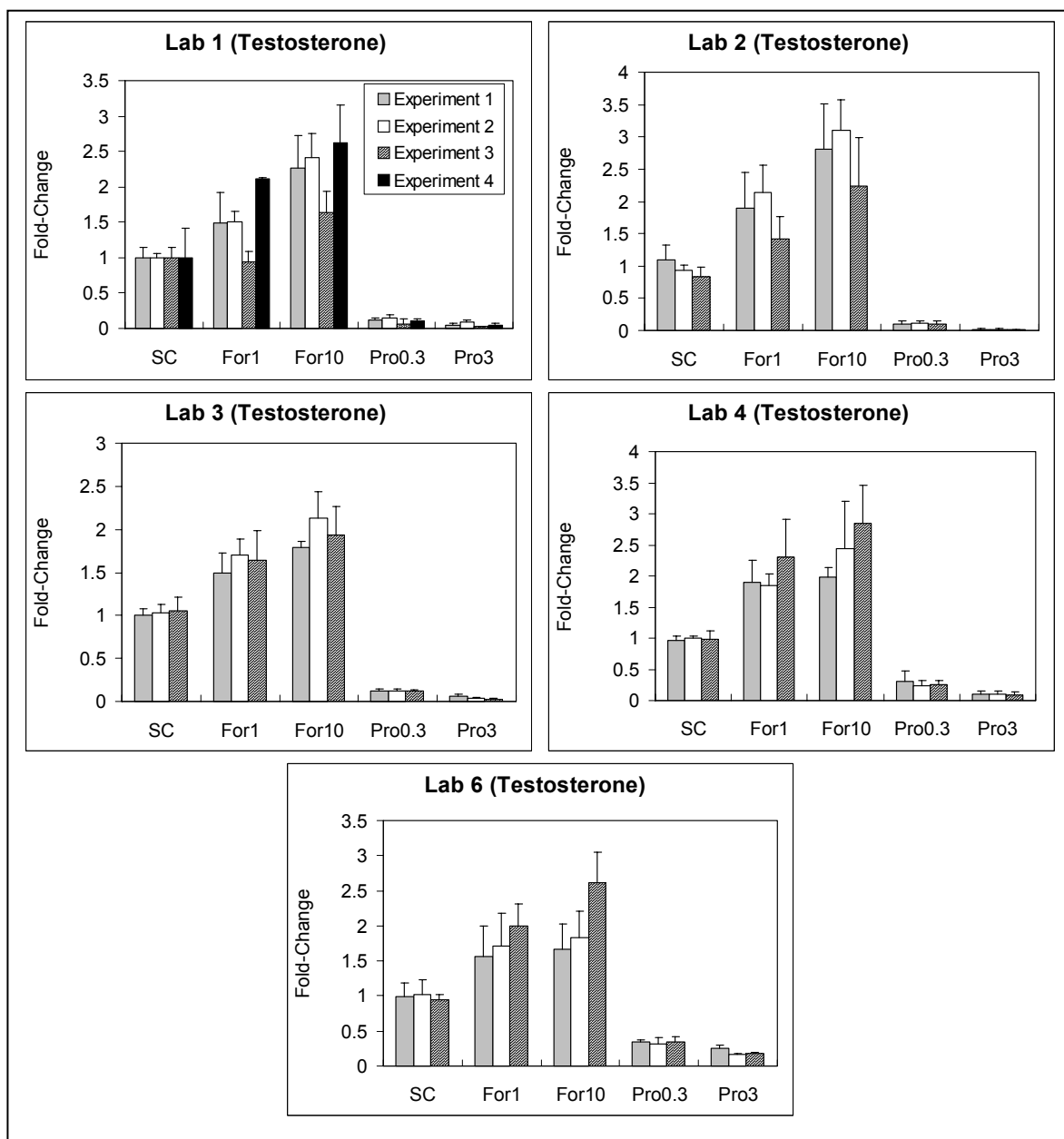


Figure 9.5: Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) in the QC-plats run parallel to the exposure experiments at each laboratory (Lab). Each group represents a batch of chemicals for which a parallel QC-plate was run. For 1 = 1 μ M Forskolin; For 10 = 10 μ M Forskolin; Pro 0.3 = 0.3 μ M Prochloraz; Pro 3 = 3 μ M Prochloraz. Error bars = 1x standard deviation. Bars represent means of three replicate well (Lab 4 and 7 only 2 replicate wells).

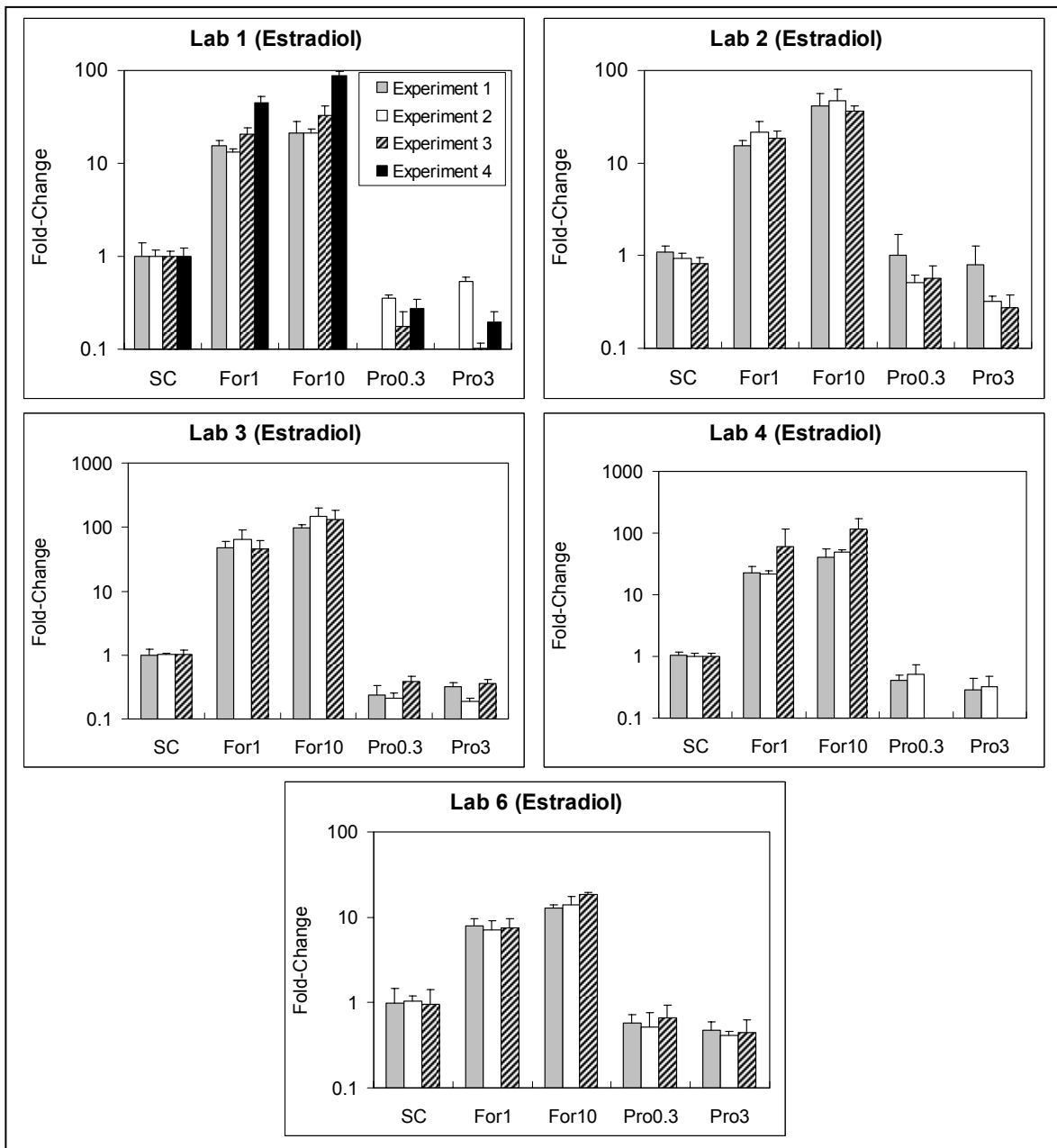


Figure 9.6: Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) in the QC-plates run parallel to the exposure experiments at each laboratory (Lab). Each group represents a batch of chemicals for which a parallel QC-plate was run. For 1 = 1 μ M Forskolin; For 10 = 10 μ M Forskolin; Pro 0.3 = 0.3 μ M Prochloraz; Pro 3 = 3 μ M Prochloraz. Error bars = 1x standard deviation. Bars represent means of three replicate well (Lab 4 and 7 only 2 replicate wells).

9.2 Testing of core chemicals

Five laboratories (Labs 1, 2, 3, 4, 6) provided a complete data set for both T and E2 concentrations in medium for all 12 core chemicals tested. As discussed in Section 9.1.3, 2% of the T experiment and 7% of the E2 experiment were excluded due to issues related to basal hormone production (Table 9.4). Four of the five remaining laboratories (1, 2, 3 and 4) had participated in the pre-validation studies, and therefore, were considered experienced with regard to the assay. However, the last laboratory (Lab 6) had no experience with the H295R Steroidogenesis Assay protocols used in this study, and thus, provided information regarding the true transferability of the assay protocol. With very few exceptions (see subsequent sections) the performance of this laboratory was comparable to that of the other groups. Therefore, the data set presented here from the four laboratories can be assumed to be representative of the performance of the H295R Steroidogenesis Assay.

9.2.1 Cell Viability

Out of the 12 chemicals tested, four compounds were found to be cytotoxic (Figures 9.7 & 9.8). Paraben, and prochloraz were cytotoxic at only the greatest concentration tested (100 μM). Benomyl was found to be cytotoxic at the two greatest concentrations at Lab 1 and at the greatest concentration in Labs 2 and 3. No cytotoxicity was observed for benomyl at Lab 6. For nonoxyenol-9, effects on cell viability were observed at 10 μM for 2 out of 5 laboratories that evaluated this compound. As a result, the cytotoxic concentrations for these chemicals were excluded from further data analysis. An increase in cell viability greater than that observed in the solvent controls was observed in 4 out of 5 laboratories in the forskolin experiments (Fig. 9.7). Maximum inductions in cell viability relative to the controls observed for this chemical were 126 and 136% at the greatest concentration at Labs 1 and 2, respectively, and 137% at Lab 6 at 10 μM . In addition to the forskolin exposures, a greater than 20% increase in cell viability was observed for Lab 2 experiments with nonoxynol-9 (10 μM), trilostane (100 μM) and prochloraz (10 and 100 μM). It is assumed that there might have been a technical problem with the cell viability assay in Lab 2 because the hormone concentration data obtained for these concentrations behaved in a manner that was similar to those reported by the other laboratories (see Chapter 9.2.2). To verify this hypothesis, the cell viability experiments for prochloraz, benomyl, and trilostane were re-run by this laboratory. The data obtained during this second set of experiments was in accordance with those obtained by the other labs (Figure 9.8). Lab 6 observed an increase in cell viability with molinate for concentrations greater or equal to 1 μM , and and for trilostane at all exposure concentrations, a trend that was not observed by the other labs. It is unclear what the reason for this increase in cell viability was. Finally, for some chemicals there was a great variation in some of the cell viability data obtained by Lab 6, which was likely to be due to technical issues. Thus, the consensus cytotoxicity data from other laboratories were used in making decisions concentration to exclude data points from the analysis of Lab 6 test results.

Given the variations (slight inductions or reductions in the number of viable cells), it was decided to normalize all data from wells with cell viabilities greater 80% for cell viability by dividing the hormone response by the relative viability (SC=1) in each well. All data from wells with cell viability of equal to or less than 80% was not considered for further evaluation due to potential interference through cytotoxicity.

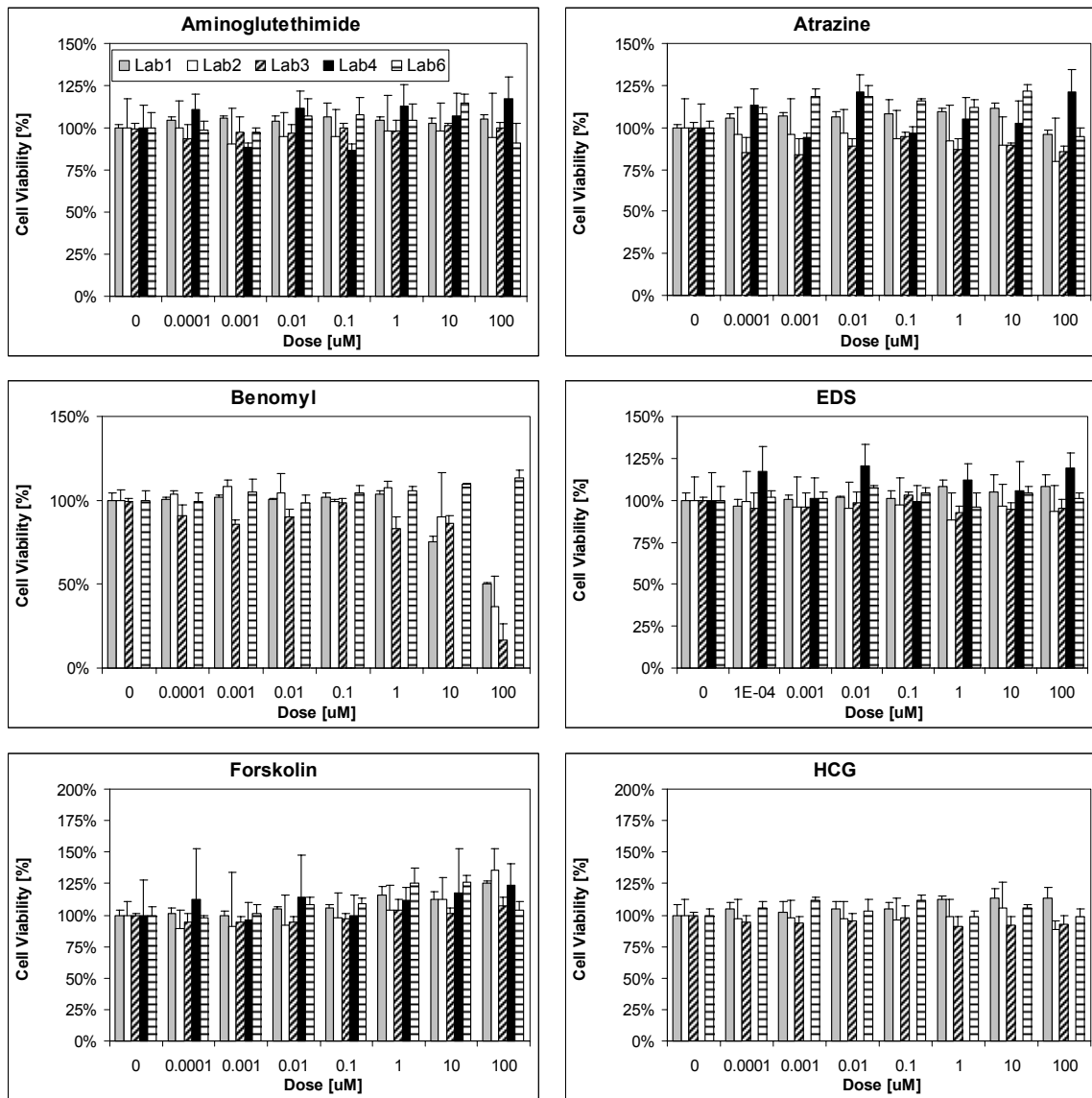


Figure 9.7: Comparison of cell viability among laboratories (Lab) after exposure to Aminogluthetimide, Atrazine, BenomyI, EDS, Forskolin and HCG. Cell viability is expressed relative to the solvent controls (SC = 100%) in each plate. Error bars = 1 x SD. Lab4: No data available for HCG and benomyI. Lab6: No data available for aminogluthetimide, atrazine, forskolin and HCG.

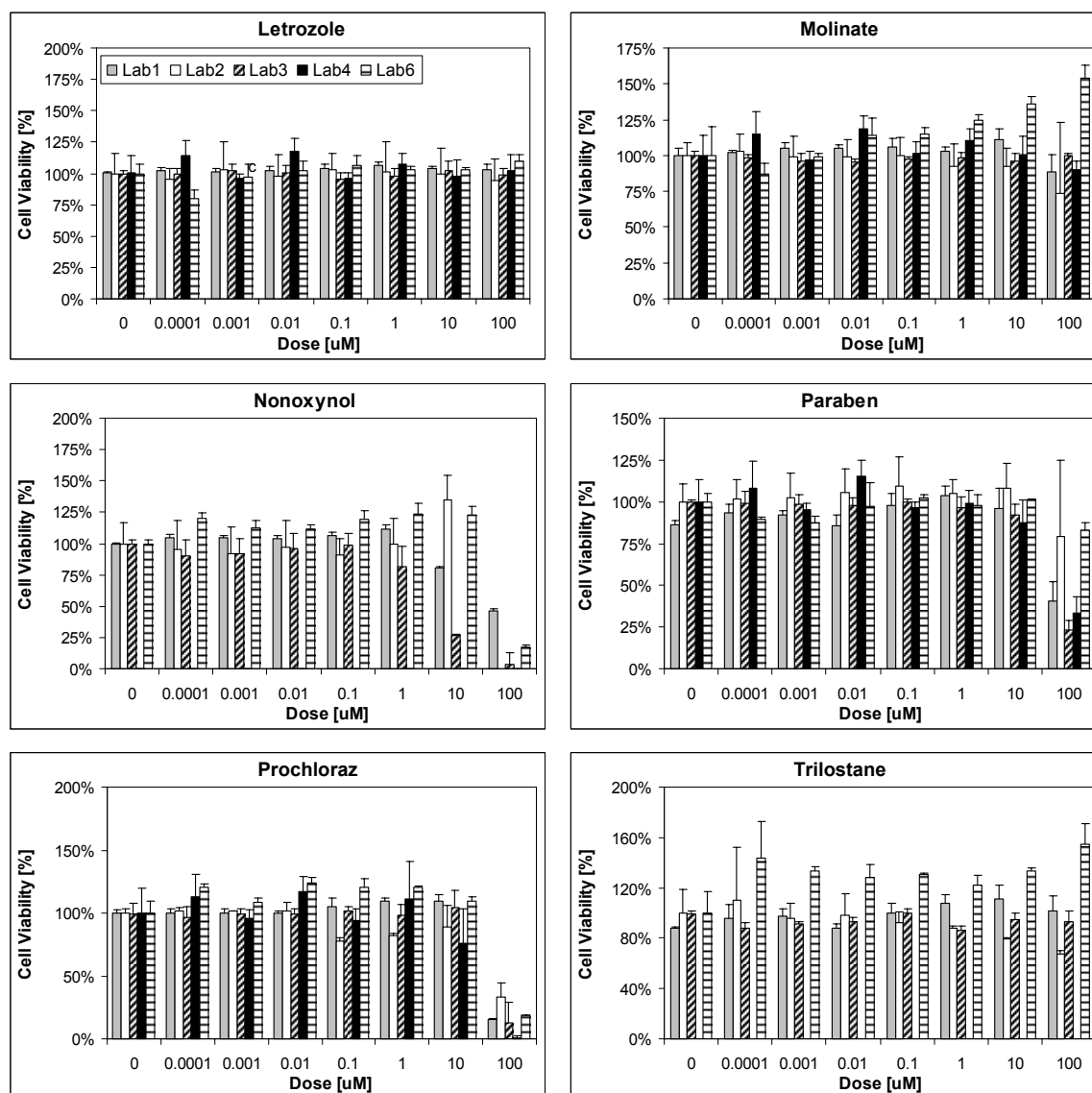


Figure 9.8: Comparison of cell viability among laboratories (Lab) after exposure to Letrozole, Molinate, Nonoxynol-9, Paraben, Prochloraz and Trilostane. Cell viability is expressed relative to the solvent controls (SC = 100%) in each plate. Error bars = 1 x SD. Lab4: No data available for nonoxynol-9 and trilostane. Lab6: No data available for nonoxynol-9, prochloraz and trilostane.

9.2.2 Relative changes after Exposure to Core Chemicals

9.2.2.1 Testosterone

With few exceptions, the effects on T and E2 production that were observed after exposure to the 12 model chemicals were comparable among laboratories and could be grouped in three different types of effects: inducers (Figure 9.9), inhibitors (Figure 9.10) and negative reference chemicals (Figure 9.11). Among the inducers, exposure to trilostane resulted in greatest fold changes (>10-fold induction) in T concentration when compared to solvent controls. The least fold-changes were observed for the atrazine exposures where induction of T production all were less than 1.5-fold with the exception of Lab 2, at which maximum induction was 2.4-fold. No effect on T production was observed after exposure to atrazine at Lab 6. Exposure to prochloraz resulted in a greater than 15-fold reduction of T production at the greatest concentration tested (100 µM) at all

laboratories with the exception of Lab 4 where an up to 4.5-fold reduction was observed. Exposure to the other inhibitors resulted in less than 4-fold changes in T production.

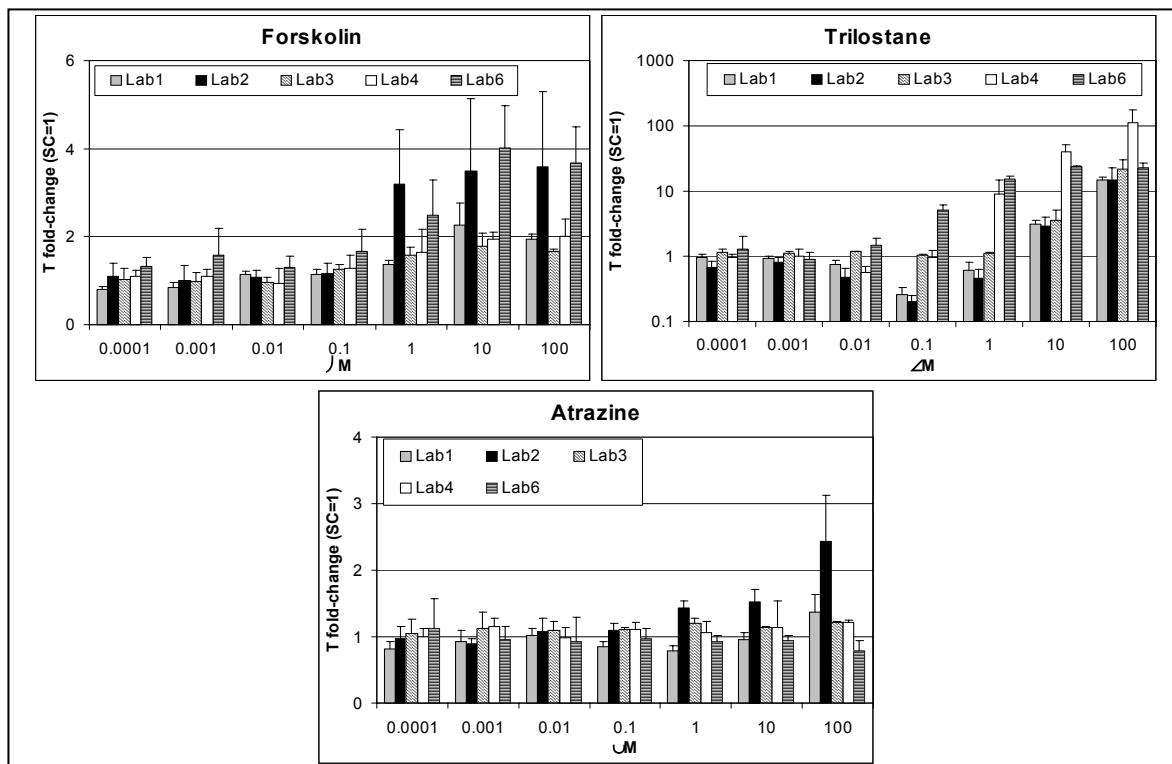


Figure 9.9: Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to forskolin, trilostane, and atrazine. Error bars = 1x standard deviation. Bars represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Lab 3– only two replicate experiments were conducted).

When chemicals exhibited no statistically significant response following a concentration response type profile at non-cytotoxic concentrations, or concentrations that did not interfere with the hormone detection assay they were categorized as negatives (Fig. 9.11). Some of these negative chemicals could have been categorized as inhibitors in individual cases (Molinate: Lab 4; Benomyl: Lab1). However, even in situations where inhibition was observed at an individual laboratory, changes were always less than 2-fold, and typically did not follow a concentration-dependent trend. In the case of nonoxynol-9, a decrease in T concentrations at non-cytotoxic concentrations at two of five laboratories for which data was available was observed. Inhibitions were 29 and 47% relative to the SCs for Labs 1 (1 μM) and 2 (10 μM), respectively. However, it should be noted that at Lab 2 10μM nonoxynol-9 caused an average increase in cell viability of 38%, and thus, the observed reduction in T production may be an artifact due to the correction for cell viability, especially as no such increase was observed at any of the other groups. The greatest letrozole concentration resulted in a significant decrease in T production at all laboratories with the exception of Lab 6 (data not shown). Therefore, the letrozole experiment was re-run by this group using a new batch of letrozole. This re-run produced data that was comparable to that observed by the other groups, indicating that the chemical stock of Letrozole sent to Lab 6 was not correctly made (Figure 9.10).

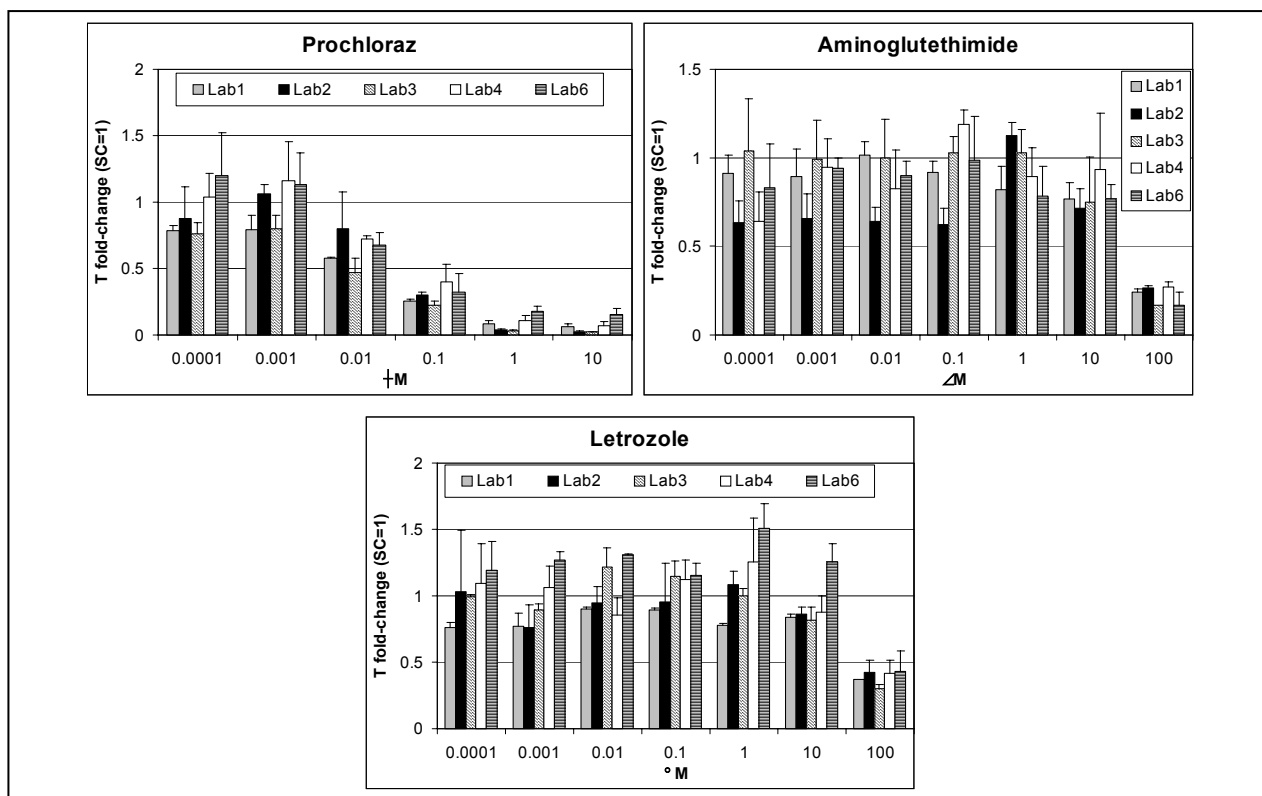


Figure 9.10: Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls ($SC=1$) after exposure to prochloraz, aminoglutethimide, and letrozole. Error bars = 1x standard deviation. Data points represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Labs 3 – only two replicate experiments were conducted).

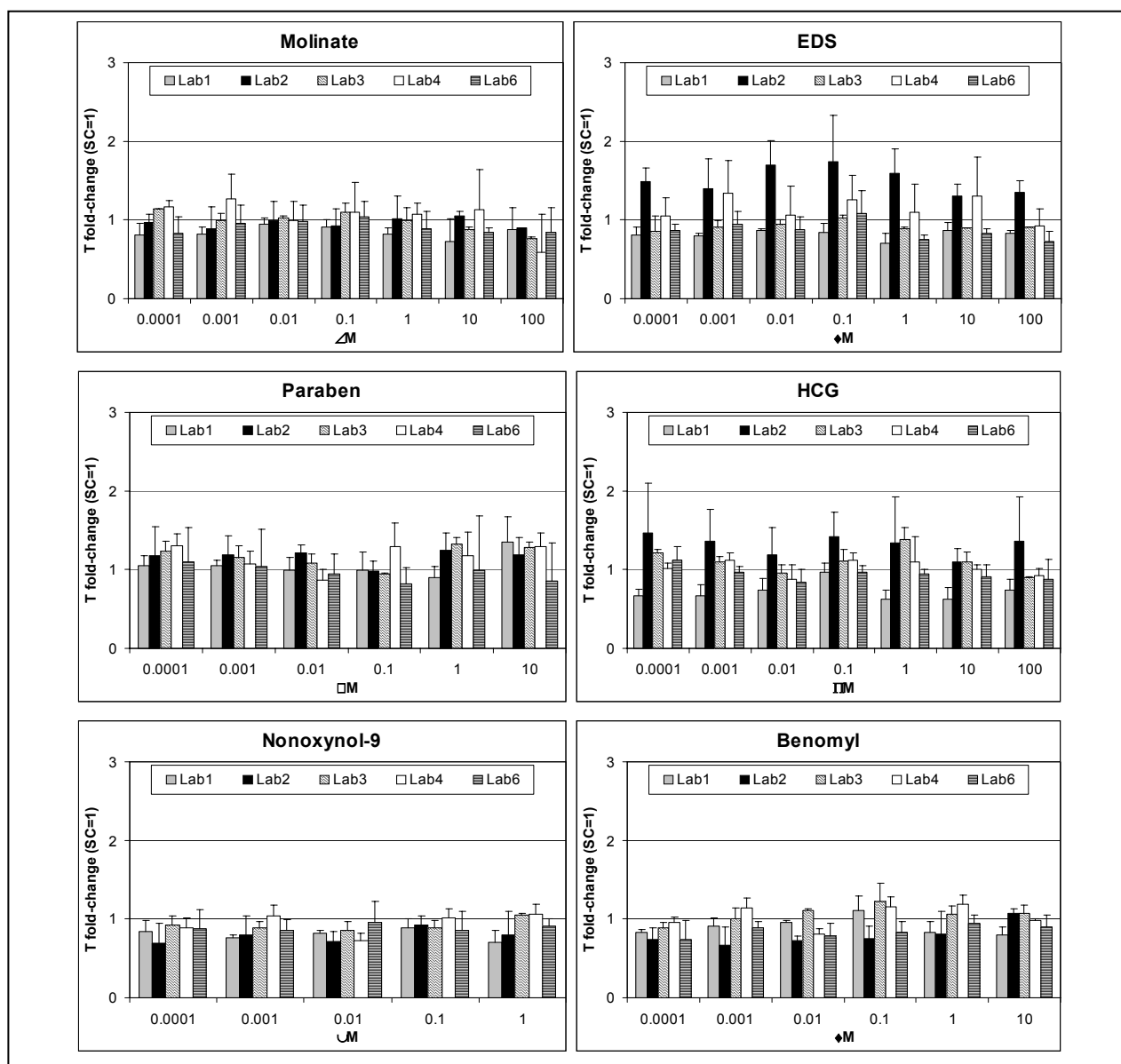


Figure 9.11: Comparison of changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to HCG, EDS, molinat, benomyl and nonoxynol-9. Error bars = 1x standard deviation. Data points represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Labs 3 – only two replicate experiments were conducted).

9.2.2.2 Estradiol

Significant differences in the response of E2 production was observed for H295R cells exposed to 12 core chemicals (Figures 9.12 – 9.14). The direction of effects for each chemical was comparable among laboratories with the exception of the Lab 2 trilostane data (see Chapter 9.2.3 for discussion). Overall, the types of effect were slightly different than those observed for T with the majority of the chemicals acting as inducers of E2 production (Figure 9.12). Three chemicals inhibited E2 concentrations (letrozole, prochloraz and aminoglutethimide; Figure 9.13) while HCG, EDS, benomyl and nonoxynol-9 (Figure 9.14) did not elicit any clear (> 1.5-fold) effects at non-cytotoxic concentrations.

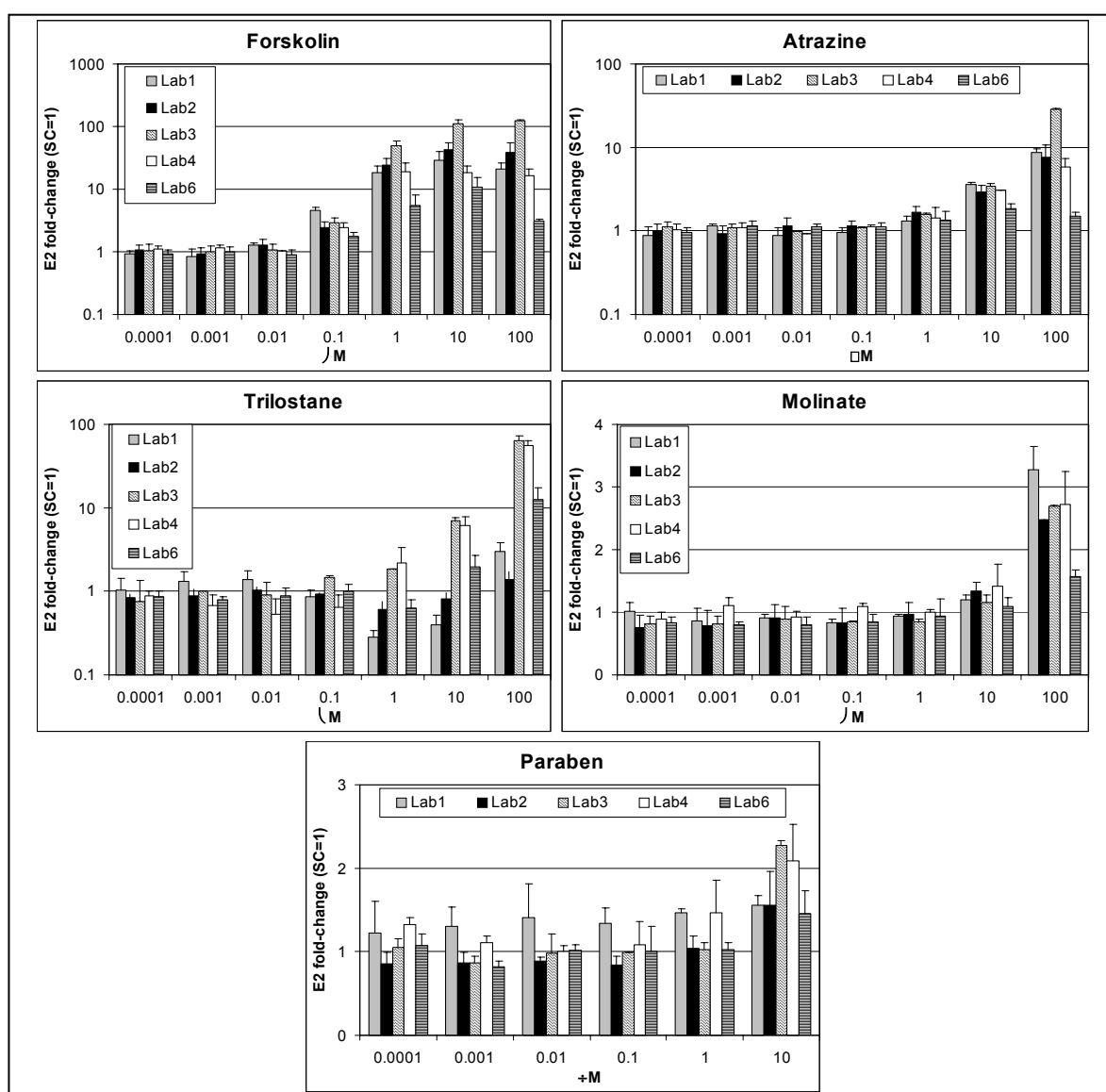


Figure 9.12: Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to forskolin, atrazine, trilostane, molinate, and paraben. Error bars = 1x standard deviation. Bars represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Lab 3 – only two replicate experiments were conducted).

The strength of the response to the exposure with chemicals that increased production of E2 ranged between 20-fold or greater (Forskolin) to <3-fold (Paraben). The concentration at which effects occurred was not related to the magnitude of the response. While forskolin resulted in increases in E2 production at concentrations greater or equal to 0.1 μM exposure to other inducers typically did not reveal effects at concentrations less than 1 μM . Exposure to letrozole and prochloraz resulted in marked reductions of E2 at concentrations greater 0.001 and 0.1 μM , respectively, with the exception of letrozole at Lab 6, for which significant reductions occurred at concentrations greater than 0.01 μM . Exposure to aminoglutethimide, in contrast, only caused a clear reduction in E2 concentrations at the greatest concentration tested.

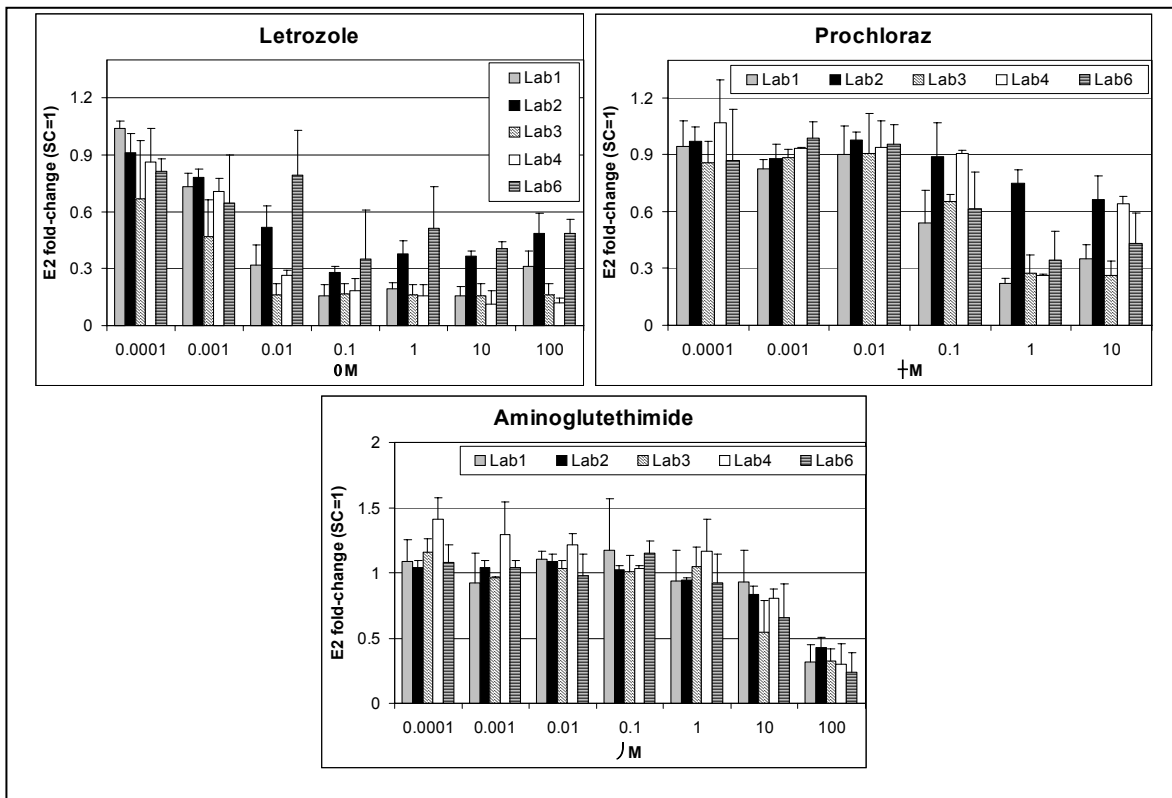


Figure 9.13: Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to letrozole, prochloraz and aminoglutethimide, letrozole. Error bars = 1x standard deviation. Bars represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Lab 3 – only two replicate experiments were conducted).

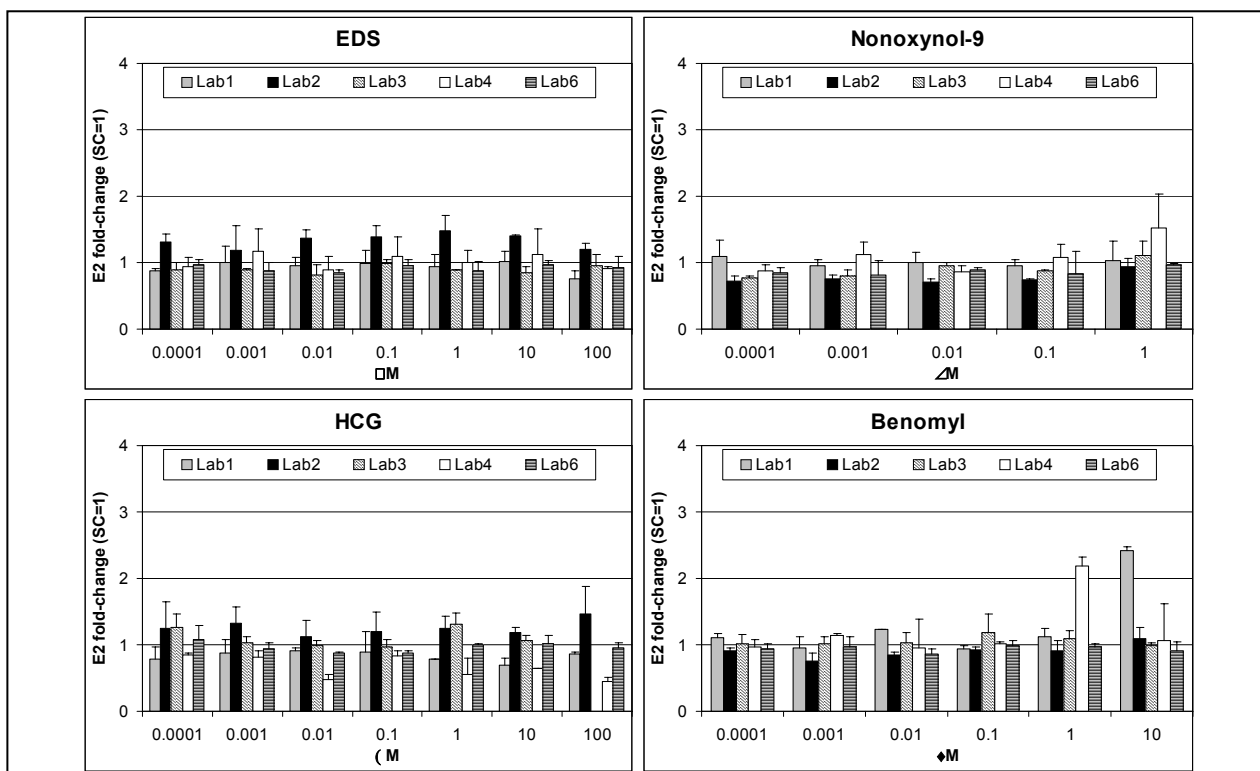


Figure 9.14: Comparison of changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to HCG, EDS, and benemyl. Error bars = 1x standard deviation. Bars represent means of three replicate experiments (exceptions: Lab1 – only one replicate experiment was conducted; Lab 3 – only two replicate experiments were conducted).

9.2.3 Confounding Factors – Interference with Hormone Detection Assays

The analysis of cross-reactivity of the greatest concentrations of each chemical with the antibodies of the immunoassays utilized at most of the laboratories revealed marked interaction with a few test compounds (Table 9.5). Interestingly, all laboratories with the exception of Lab 2 showed a very great interaction of the estradiol immunoassay with trilostane. Similarly, cross-reactivity of this chemical was also reported for the T antibodies, albeit less pronounced. Since only the greatest chemical concentration was analyzed for cross-reactivity with the antibodies no adjustment of the concentration-response curves could be performed. However, an initial attempt to correct for the interaction with the antibodies at this greatest concentration (greatest highest three concentrations for Lab 1) indicated that while the induction of E2 after exposure to trilostane is likely to be solely due to this cross-reactivity, the induction of T could not be explained by this factor (Figure 9.12). Similar interaction of trilostane with hormone detection systems have been also observed by other authors (Shapiro and Page 1976; Puddefoot et al. 2002; Villeneuve, personal communication). Furthermore, nonoxynol-9, paraben, and prochloraz also interacted with the E2 immunoassays. However, since the cross-reactivity for prochloraz, paraben, and nonoxynol-9 was either low at the greatest concentrations tested or these concentrations were excluded due to marked cytotoxicity, this factor had no effect on the interpretation of the results. However, further analyses are required to address possible uncertainties resulting from the interference of a test chemical with the hormone detection system utilized.

Table 9.5: Interference (pg hormone/mL @ the greatest concentration tested) of the 12 core chemicals with the immunoassays used for the determination of testosterone (T) and estradiol (E2). The numbers indicate the concentration (pg/mL) of T or E2 measured by the respective hormone immunoassay in supplemented stock medium with 100µM of the chemical of interest added. Note: This medium has not been in contact with the cells.

	Interference with Hormone Detection Assay									
	Testosterone (pg/ml @ 100µM)					Estradiol (pg/ml @ 100µM)				
	Lab 1*	Lab 2*	Lab 3*	Lab 4*	Lab 6*	Lab 1*	Lab 2*	Lab 3+	Lab 4*	Lab 6
Aminoglutethimide					76				24	17
Atrazine				59						24
Benomyl									29	15
EDS					100				22	20
Forskolin					106					19
HCG					103				30	25
Letrozole	33		237		64				12	
Molinate									11	
Nonoxynol	137				90	394	11			35
Paraben	n/d					n/d	15	24	20	16
Prochloraz	94		169	57				20	11	
Trilostane	3719	6554	44303	58806	49939	802	9	1112	1075	1515

* only chemicals that were @ >50pg/mL interference were considered
 + only chemicals that were @ >10pg/mL interference were considered

Bold numbers Result has or is likely to have impact on final result
Shaded bold numbers Not relevant due to parallel cytotoxicity that resulted in omission of data point

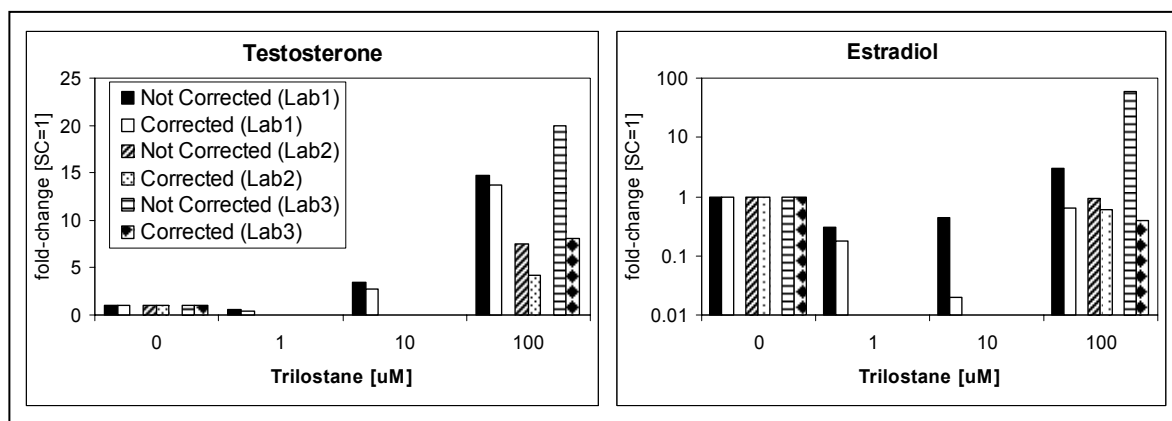


Figure 9.15: Changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) after exposure to trilostane with and without adjustment of final hormone concentration for interference with hormone detection system.

10 DATA EVALUATION

In the previous sections, despite some minor remaining uncertainties, it was demonstrated that the H295R Steroidogenesis Assay is a sensitive, reproducible, transferable, and specific test for the determination of chemical effects on the production of T and E2. One of the key questions that needed to be addressed during the development and validation of an *in vitro* assay for screening purposes is the

format in which the data are presented and the type of analyses that will provide meaningful interpretation of these data and classification of chemicals of concern. In this section we discuss several different approaches for the presentation and evaluation of the data obtained during this phase of the validation studies then select an approach for the assessment of data obtained with the H295R Steroidogenesis Assay. In a concluding section, we then have subjected an extended data set that included 16 additional chemicals, produced by four of the seven laboratories, to these data evaluation procedures to verify this approach with a number of compounds with unknown modes of action.

Based on results obtained during the pre-validation studies (Hecker et al. 2006b & 2007b) it was decided that data should be expressed as fold-change relative to the solvent control due to variation in basal hormone production that was a function of cell passage and/or freeze/thaw cycle (for a detailed discussion please refer to the above listed reports). For this validation study, several different data evaluation approaches were utilized including the definition of induction and inhibition thresholds, and calculation of effective concentrations (EC50) where possible.

10.1 Fold-Change Evaluation

Data expressed as fold-change relative to the SC was subjected to either an ANOVA followed by the Dunnett's test, or to a Kruskal Wallis analysis followed by a Mann Whitney U test when data was normally or not-normally distributed, respectively, to identify significant changes from the SC. Furthermore, for purposes of comparing laboratory results in the validation studies, changes were classified into four categories to allow for separation of chemicals by the magnitude of effect (Table 10.1).

Table 10.1: Classification system for the effects of the 12 core chemicals on testosterone (T) and estradiol (E2) production by H295R cells. Note: Only statistically significant effects are to be considered.

Inducer	Change	Inhibitor	Change
↑	<2-fold	↓	<0.5-fold
↑↑	2- to <4-fold	↓↓	0.5- to <0.25-fold
↑↑↑	4- to <20-fold	↓↓↓	0.25- to <0.1-fold
↑↑↑↑	≥20-fold	↓↓↓↓	≥0.1-fold

A comparison of lowest observed effect concentrations (LOECs), which was defined as the least concentration at which a statistically significant change occurs, revealed comparable concentration-ranges among laboratories with only a few exceptions (Table 10.2). In most cases these exceptions were represented by the absence of responses. These included the lack of a response for T production after exposure to atrazine, letrozole, and molinate when measured at Labs 4 & 5, 5, and 3, respectively, and E2 production for the paraben experiment conducted at Lab 6. The only situation in which the minority of laboratories showed an effect was for the weak induction of T after exposure to the greatest non-cytotoxic concentration of paraben at Labs 1 & 3. Most of the differences were observed for weak to moderate inducers or inhibitors.

The magnitude of change in T and E2 production for experiments conducted with the core chemicals was similar among laboratories. In 100% and 93% of all experiments, the results did not deviate by more than one category among laboratories for T and E2, respectively.

It should also be noted that the data obtained from the trilostane experiments should be assessed with care due to the uncertainties resulting from the marked cross-reactivity that this chemical exhibited with most of the hormone detection assays utilized by the laboratories.

In summary, fold change as exemplified in the categorical classification system (Table 10.1) in combination with the LOEC data was capable of clearly identifying inducers and inhibitors of different strengths for both T and E2 production in the H295R steroidogenesis assay. Although the assay can detect

moderate and strong effects on hormone production with great certainty, one limitation is that it is not always possible to distinguish between weak effectors and negative chemicals. In the subsequent sections we attempted to further characterize the results obtained during these validation studies to improve the evaluation approach by utilizing regression models, such as probit models, to determine effective concentrations and potency curves.

Table 10.2: Lowest observed effect concentrations (LOECs; measured by Dunnett’s or Mann Whitney U test ^{mu}) and strength and direction of change (arrows; see Table 10.1 for explanation) for testosterone (T) and estradiol (E2) after exposure to the twelve core chemicals. Ranges refer to maximum values measured in repeat-experiments. nd – not detectable; --- chemical not analyzed. Gray shaded cells – uncertainty due to cross-reactivity.

	Fold-Change (Testosterone)									
	Lab 1 ^a		Lab 2		Lab 3		Lab 4		Lab 6	
	LOEC	Max Change	LOEC	Max Change	LOEC	Max Change	LOEC	Max Change	LOEC	Max Change
Aminoglutethimide	100	↓↓↓	100	↓↓	10	↓↓↓	100	↓↓	100	↓↓↓
Atrazine	100	↑	1	↑	100	↑	nd		nd	
Benomyl	nd		nd		Nd		nd ^{mu}		nd	
EDS	nd		nd		nd		nd		nd	
Forskolin	10	↑↑	1	↑↑	1	↑	1	↑↑	1	↑↑
HCG	nd		nd		Nd		nd		nd	
Letrozole	100	↓↓	100	↓↓	100 ^a	↓↓	100	↓↓	100	↓↓
Molinate	nd		nd		100	↓	nd		nd	
Nonoxynol-9	10	↓	10	↓	nd ^c		10	↓	10	↓↓
Paraben	10	↑	nd		1	↑	nd		nd	
Prochloraz	0.0001	↓↓↓	0.1	↓↓↓	0.0001	↓↓↓	0.001	↓↓↓	0.01	↓↓↓
Trilostane	0.1 ^{mu}	↑↑↑	0.01 ^{mu}	↑↑↑	1 ^{MU}	↑↑↑	1 ^{mu}	↑↑↑	0.01 ^{mu}	↑↑↑

	Fold-Change (Estradiol)									
	Lab 1 ^a		Lab 2		Lab 3		Lab 4		Lab 6	
	LOEC	Max Change	LOEC	Max Change	LOEC	Max Change	LOEC	Max Change	LOEC	Max Change
Aminoglutethimide	100	↓↓	10 ^{mu}	↓↓	10	↓↓	100 ^b	↓↓	100	↓↓
Atrazine	10	↑↑↑	0.001	↑↑↑	1 ^{mu}	↑↑↑	10 ^{mu}	↑↑↑	0.1	↑
Benomyl	nd		nd		nd ^a		nd ^a		nd	
EDS	nd		nd		Nd		nd		nd	
Forskolin	0.01 ^{mu}	↑↑↑	0.1 ^{mu}	↑↑↑	0.1 ^{mu}	↑↑↑	0.1 ^{mu}	↑↑↑	0.01 ^{mu}	↑↑
HCG	nd		nd	↑	nd ^a		nd ^a		nd	
Letrozole	0.001	↓↓	0.001	↓↓	0.0001 ^{mu}	↓↓↓	0.01	↓↓↓	0.01	↓↓
Molinate	100	↑↑ ^{mu}	100	↑	100 ^{mu}	↑↑	100 ^{mu}	↑↑	100	↑↑
Nonoxynol-9	nd		nd		Nd		nd		nd	
Paraben	nd ^{mu}	↑↑↑	10	↑	10 ^{mu}	↑↑	nd	↑↑	nd	
Prochloraz	0.1	↓↓	1	↓	0.1	↓↓	1	↓↓	0.1	↓↓
Trilostane	1 ^{mu}	↑↑↑	100	↑	0.1 ^{mu}	↑↑↑	1 ^{mu}	↑↑↑	1 ^{mu}	↑↑↑

^a only one experiment was conducted or considered for data evaluation; ^b Not statistically significant; p = 0.051; ^c Cytotoxicity observed at concentration at which effects occurred at other laboratories = 10µM

10.2 Application of Regression Type Models – Effective Concentrations (ECs)

An alternative approach to further characterize the results obtained during the validation studies was to use regression models to calculate EC50s with probit-transformed data. The EC50 is defined as the chemical concentration that provokes a response halfway between the solvent control baseline and the maximum response for the chemical.

Where possible, effective concentrations were calculated for all chemicals that showed a significant increase or decrease relative to the SCs (see Section 10.1). For the 12 core chemicals analyzed in this study, only two chemicals had sufficient T data for conducting a probit analysis while for E2, only three chemicals had sufficient data for this type of analysis. For chemicals that had sufficient data, the analyses revealed comparable EC50s for most of these chemicals at the different laboratories. Inconsistencies in the determination whether a chemical could be categorized as an effector (either an inhibitor or an inducer) vs a negative occurred in 11 and 4% of all cases for T and E2, respectively, and the maximum difference between EC50s among all laboratories for a given chemical was less than one order of magnitude.

One major issue with using a regression model to calculate EC50 was the lack of sufficient data to fully describe concentration-response profiles for many of the chemicals tested. While the data from most of the strong effectors was sufficient to derive a full concentration-response curve necessary for conducting a probit regression analysis, medium and weak inducers/inhibitors of hormone production did not produce data that described maximum induction or inhibition values such that a regression model could be used. Therefore, in these cases Table 10.3 reports that the EC50 is a value greater than the no effect concentration (NOEC). Furthermore, for some chemicals calculation of effective concentrations was complicated by the occurrence of decreases in cell viability at concentrations near the upper testing range used in this study, and by the relatively large spacing (order of magnitude) of concentrations. Given these uncertainties and limitations, it can be concluded that the utilization of this regression approach, using the current H295R Steroidogenesis Assay protocol is of limited value in categorizing chemicals.

However, with the exception of chemicals that limit the concentration range due to cytotoxicity, the H295R assay protocol can be refined to allow for the determination of a more detailed and complete concentration-response curves that would allow the calculation of effective concentrations (ECx). Thus, the current H295R assay protocol could be used as an initial screen to identify chemicals of potential concern and then a second experiment could be conducted with chemical that tested positive, but with a dosing-regime more closely grouped around the concentration for which a response was observed.

Table 10.3: EC50s(μM) calculated for the changes in testosterone (T) and estradiol (E2) production after exposure to the twelve core chemicals using a probit model. --- chemical not analyzed. Greater than values indicate insufficient data to derive a complete concentration response curve, as a result the data is present as > NOEC. Gray shaded cells – uncertainty due to cross-reactivity.

	Testosterone				
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6
Aminoglutethimide	>10	>10	>10	>10	>10
Atrazine	>10	>10	>10		
Benomyl					
EDS					
Forskolin	>1	1.3	0.30	0.65	0.40
HCG					
Letrozole	>10	>10	>10	>10	>10
Molinate			>10		
Nonoxynol-9	>1	>1		>1	>1
Paraben	>10		>1		
Prochloraz	0.0099	0.057	0.0075	0.028	>0.01
Trilostane	>10	>10	>10	>10	0.58
	Estradiol				
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 6
Aminoglutethimide	>10	>10	>10	>10	>10
Atrazine	>10	>10	>10	>10	>1
Benomyl					
EDS					
Forskolin	0.65	1.9	1.8	1.6	1.3
HCG					
Letrozole	0.0022	0.0037	0.00052	0.0025	0.1
Molinate	>10	>10	>10	>10	>10
Nonoxynol-9				>0.1	
Paraben			>10		>1
Prochloraz	0.068	0.27	0.040	>0.1	>0.038
Trilostane	>10	>10	>10	47	>10

11 APPLICATION OF THE H295R STEROIDOGENESIS ASSAY TO A SELECTION OF SUPPLEMENTARY CHEMICALS

The approaches and protocols discussed in the previous sections were used to evaluate the potential of the 16 supplementary chemicals to interfere with hormone production. Of the 16 chemicals all were analyzed by the lead laboratory, while three additional laboratories (Lab 2, 3 and 4) each analyzed 5 to 6 chemicals as described in section 7.2.2 (Testing of Chemicals, Supplementary Chemicals) so that data sets are available from two different laboratories for each of the test compounds. It should also be noted that Lab 4 encountered issues with the conditions of their cells during the first replicate experiment as indicated by visual observations and parallel cell viability measurements (data not provided). Therefore, only data obtained during the second and third replicate experiments are provided for Lab 4.

As was observed in tests with the 12 core chemicals, data obtained from wells with less or equal to 80% cell viability were excluded from further analysis. While over 47% of all chemicals showed reductions in cell viability of greater or equal to 20%, no compound exhibited cytotoxicity at concentrations less than 100 μ M (Figures 11.1, 11.2 and 11.3). Also, in most cases, effects (decreases) in cell viability were comparable among laboratories with the exception of spironolactone when measured at Lab 4 and fenarimol at Lab 3. For fenarimol the trend in cell viability change at Labs 1 and 3 was the same (decrease) whereas Lab 1 observed a greater than 90% decrease in viability while cytotoxicity at Lab 3 only reached 23% at the greatest concentration. It is unclear why there was no cytotoxicity observed for spironolactone at the greatest concentration at Lab 4 or why there was such a large difference in cell viability found between Labs 1 and 3.

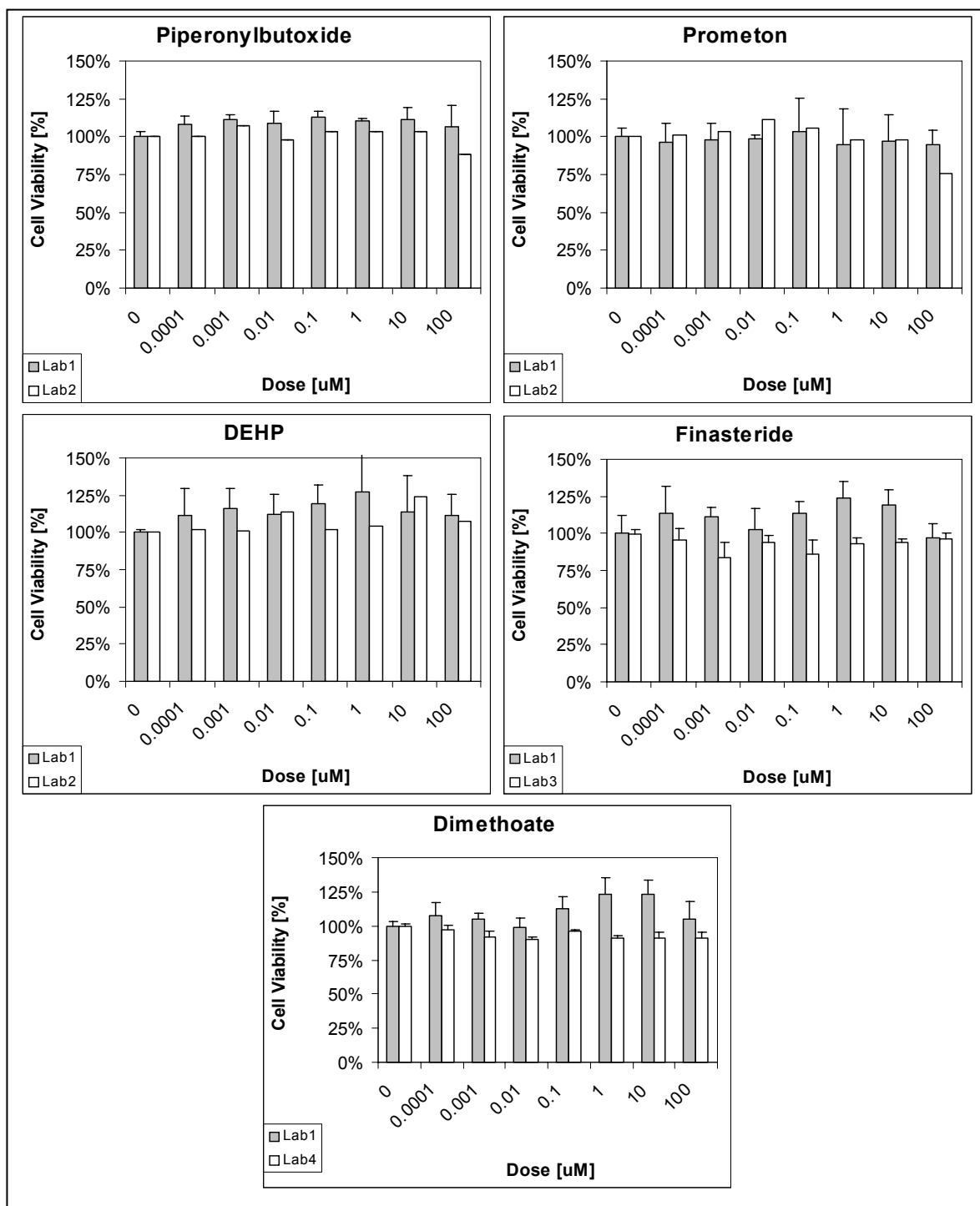
No data for the interference with the hormone detection assays were available from Labs 2 and 4. However, since Lab 4 used the same hormone ELISAs as Lab 1, it was assumed that potential interferences of the test chemicals with the hormone assays would be the same. Thus, the Lab 1 data were used to further evaluate the data generated at this laboratory. Significant interactions of the chemicals with the hormone detection assays that occurred at non-cytotoxic concentrations were only observed for T after exposure to spironolactone, finasteride and danazol at Lab 1, and for E2 after exposure to genistein at Labs 1 and 3. However, when the data for spironolactone, finasteride and danazol not corrected for this interference were compared to the data corrected for interference, no significant impact on the overall trend/response were observed (Figure 11.4). Similarly, while genistein interference of the E2 ELISA antibodies reduced the magnitude of the response by approximately 30%, it did not change the overall trend of the response (Figure 11.4).

As was observed for the 12 core chemicals, the H295R Steroidogenesis Assay distinguished between inducers and inhibitors of differing strength/potency for both T and E2 (Figures 11.5 – 11.10). Five and four of the 16 compounds (31 and 25%, respectively) tested negative in the assay for T and E2, respectively. DEHP, dimethoate, flutamide, glyphosate and prometon did not elicit significant concentration dependent responses for T, while glyphosate, dinitrophenol, piperonyl butoxide and spironolactone did not reveal any concentration-dependent effects on E2 production. One exception was dinitrophenol, which was identified as a significant inhibitor of T at all concentrations tested. However, no concentration-response trend was recognizable and the magnitude of the effect was weak. Therefore, it is possible that this response represents an artifact. Compared to the 12 core chemicals, there was greater variation among the responses observed at different laboratories. Approximately 25 and 38% of the chemicals showed a significant response for T and E2, respectively, at only one of the two laboratories where they were tested (Table 11.1). It is unclear what the basis for these differences are but it should be noted that in 7 out of the 10 cases where such incongruencies were observed, they were associated with one group (Lab 4). Six of these 7 compounds were identified as inducers by the Lab 1. Also, at the same laboratory some of the cell viability data revealed no effects where significant decreases were observed at the other laboratory (tricrescyl phosphate and spironolactone) indicating that there may have been some issues related to dosing. Finally, basal E2 production by Lab 4 was approximately a factor 3 to four greater than that by Lab 1 (~200 pg/mL vs. ~50 pg/mL), indicating that cells were at a suboptimal (late) passage when used for the experiment. This further supports the need for stringent conditions regarding the age of

the cells, and which should not be used beyond passage 10. When Lab 4 is excluded, there were 1 and 2 chemicals for T and E2, respectively, where the data obtained at different laboratories did not match.

Regardless of these remaining uncertainties, the H295R Steroidogenesis Assay protocol successfully identified chemicals with unknown modes of interaction with sex steroid synthesis as inducers and inhibitors of T and E2 production. Some of the chemicals identified as inhibitors of T (genistein, fenarimol, bisphenol A) showed a biphasic response where statistical significant increases in hormone production were observed up to concentrations of 0.1 to 1 μ M. However, with the exception of the genistein exposure experiments none of these changes exceeded 1.5-fold. This phenomenon did not affect the final categorization of a chemical. It is hypothesized that these minor changes are likely to be a compensatory mechanism, reflecting the integrative nature of the H295R Steroidogenesis Assay rendering a more realistic assay with regard to the identification of potential in vivo inducers/inhibitors of T and E2 production.

The specificity of the assay could be demonstrated by the relatively great number of chemicals that tested negative for the interference with the production of either T or E2 or both hormones.



Fig

re 11.1: Cell viability after exposure to piperonyl butoxide, prometon, DEHP, vinclozolin, finasteride, and Dimethoate. Cell viability is expressed relative to the solvent controls (SC = 100%) in each plate. Error bars = 1 x SD. Note: Lab2 did re-measure cell viability due to technical issues that were encountered during the initial experiments. Therefore, only one experimental data set is available from this lab, resulting in the lack of error bars for the cell viability results of this group.

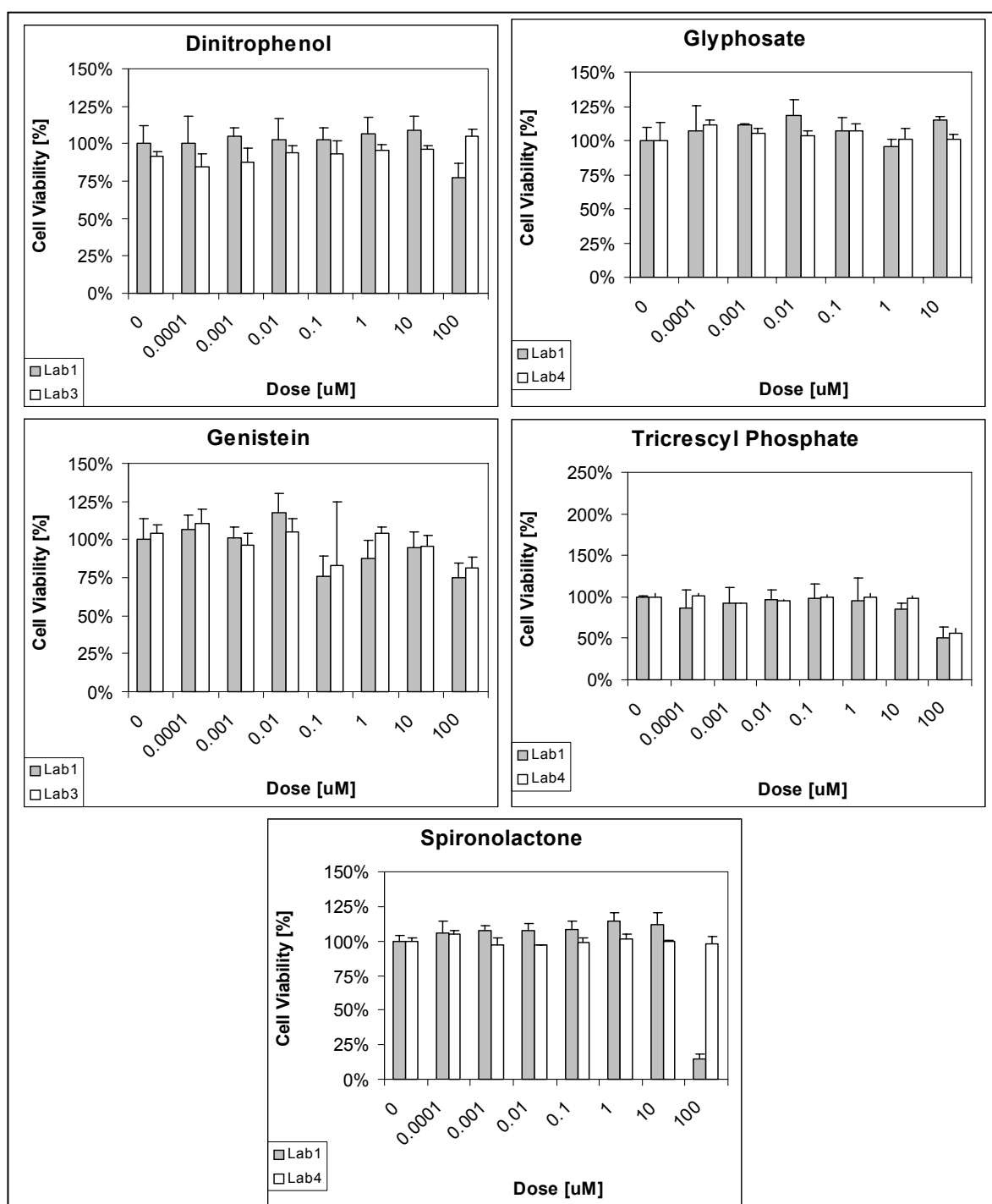


Figure 11.2: Cell viability after exposure to dinitrophenol, glyphosate, genistein, tricrescyl phosphate, and spiroinolactone. Cell viability is expressed relative to the solvent controls (SC = 100%) in each plate. Error bars = 1 x SD. Note: Lab2 did re-measure cell viability due to technical issues that were encountered during the initial experiments. Therefore, only one experimental data set is available from this lab, resulting in the lack of error bars for the cell viability results of this group.

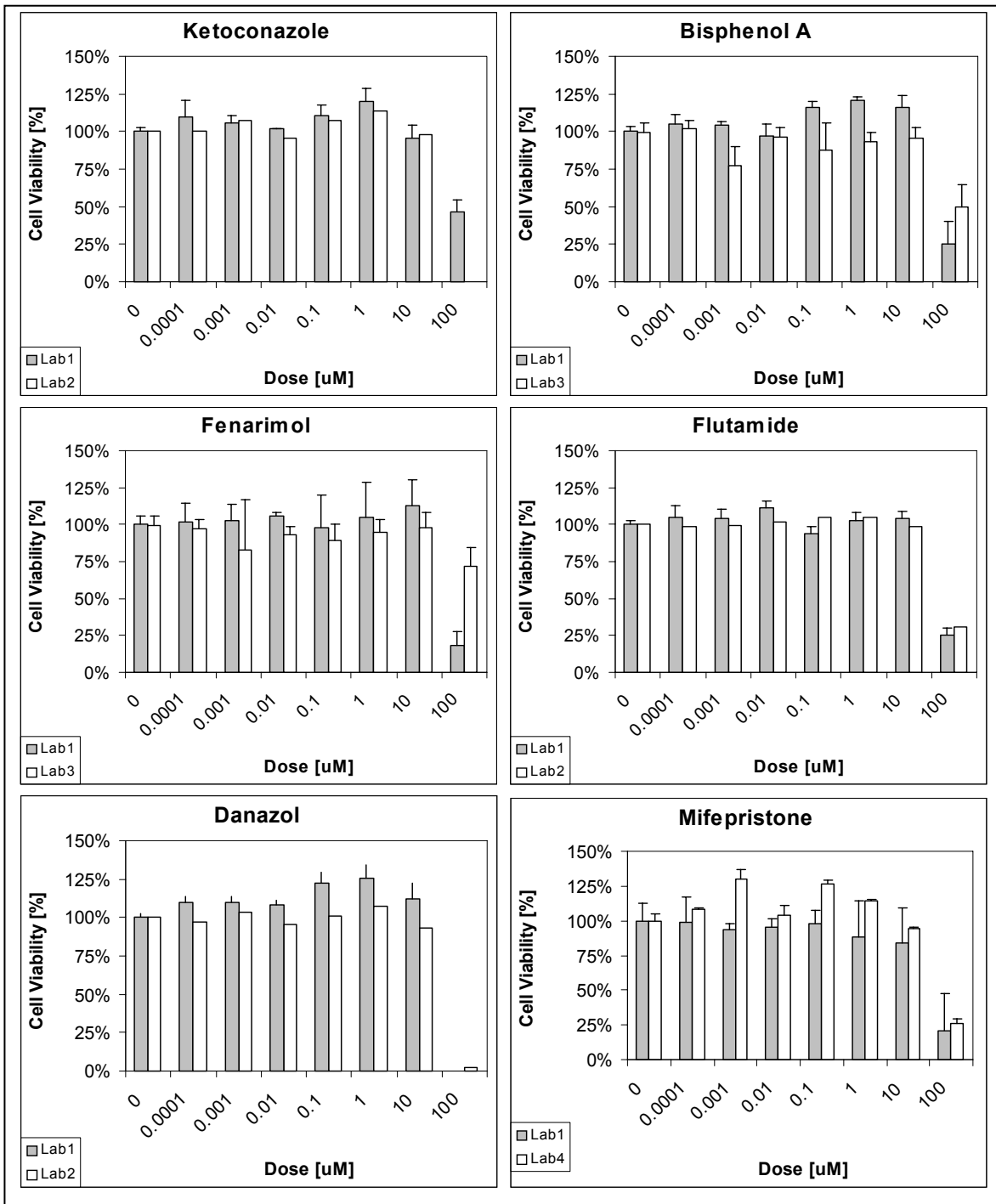


Figure 11.3: Cell viability after exposure to ketoconazole, bisphenol A, fenarimol, flutamide, danazol and mifepristone. Cell viability is expressed relative to the solvent controls (SC = 100%) in each plate. Error bars = 1 x SD. Note: Lab2 did re-measure cell viability due to technical issues that were encountered during the initial experiments. Therefore, only one experimental data set is available from this lab, resulting in the lack of error bars for the cell viability results of this group.

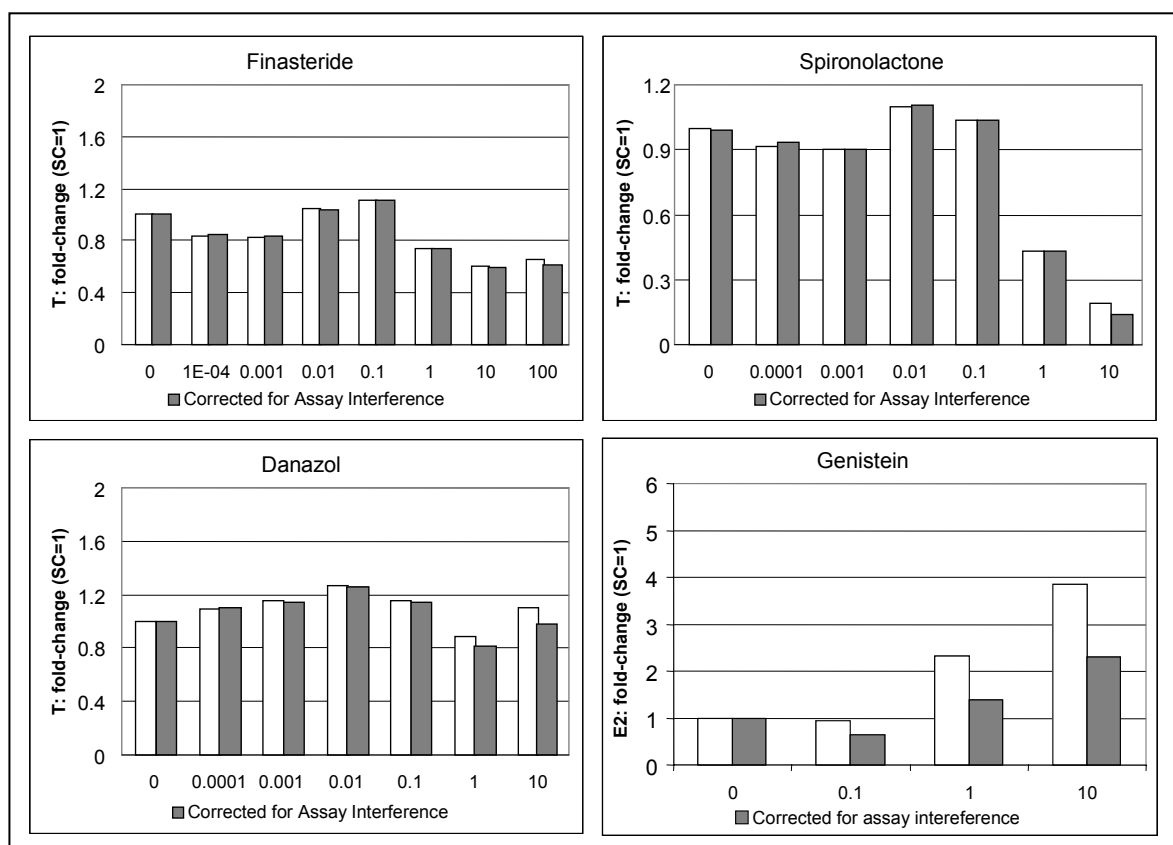


Figure 11.4: Changes in the relative concentrations (SC=1) of testosterone (T) after exposure to spironolactone, finasteride and danazol, and of estradiol (E2) after exposure to genistein after (grey bars) and before (white bars) subtraction of concentration at which the chemicals interfered with the hormone detection assay.

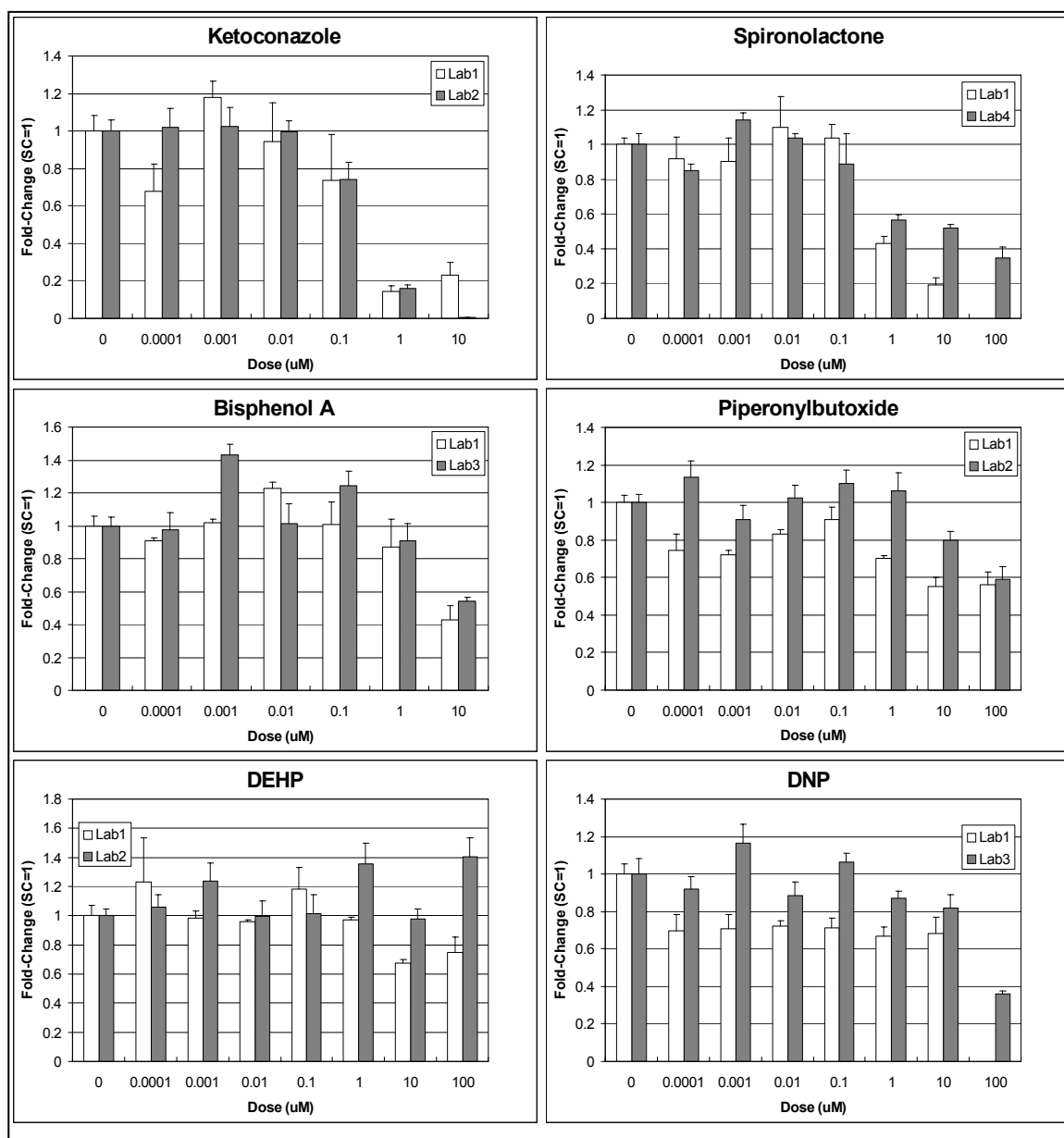


Figure 11.5: Changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to ketoconazole, spironolactone, bisphenol A, piperonyl butoxide, DEHP, and dinitrophenol (DNP). Error bars = 1x standard deviation.

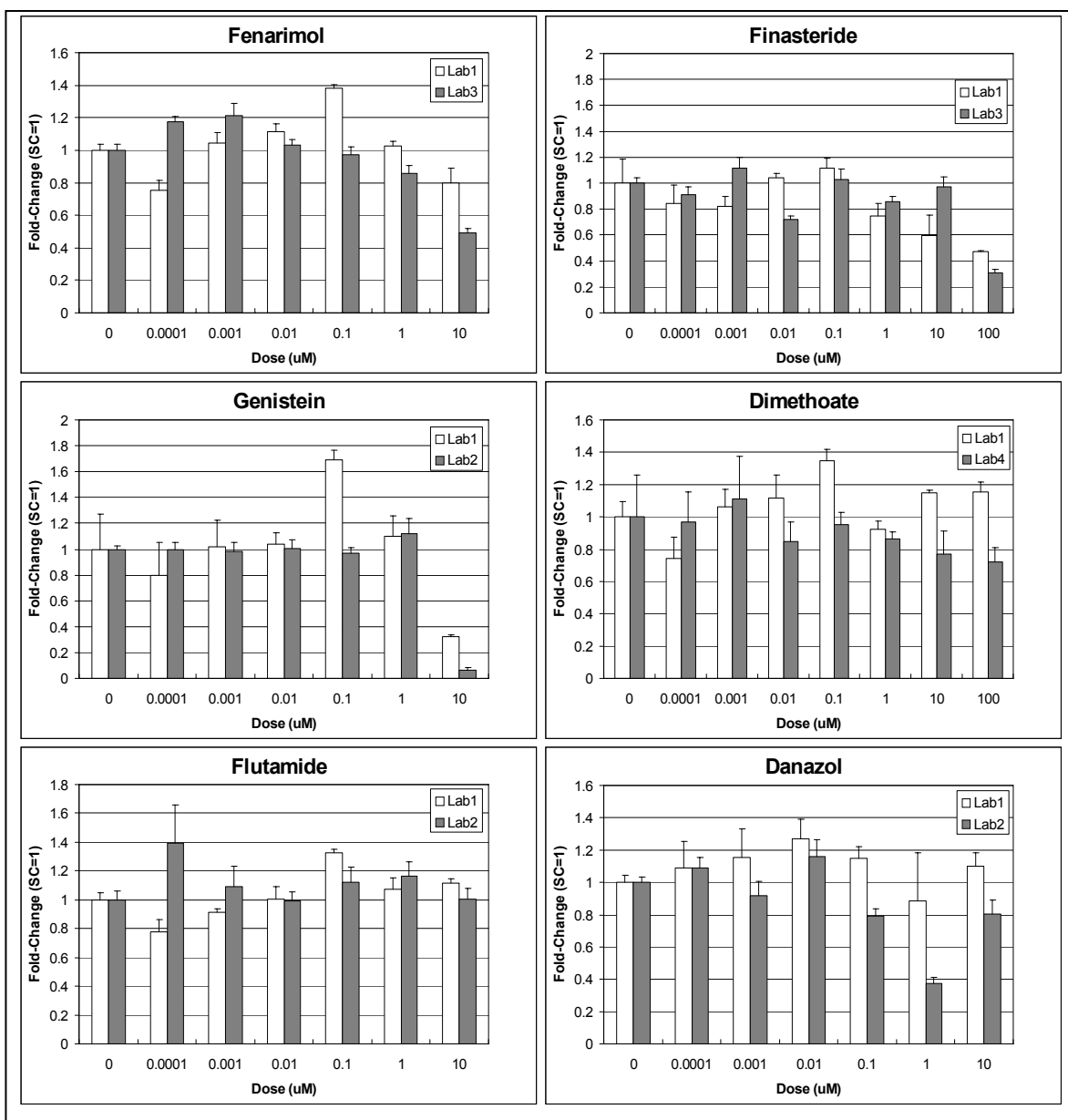


Figure 11.6: Changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to fenarimole, finasteride, genistein, dimethoate, flutamide, and danazol. Error bars = 1x standard deviation.

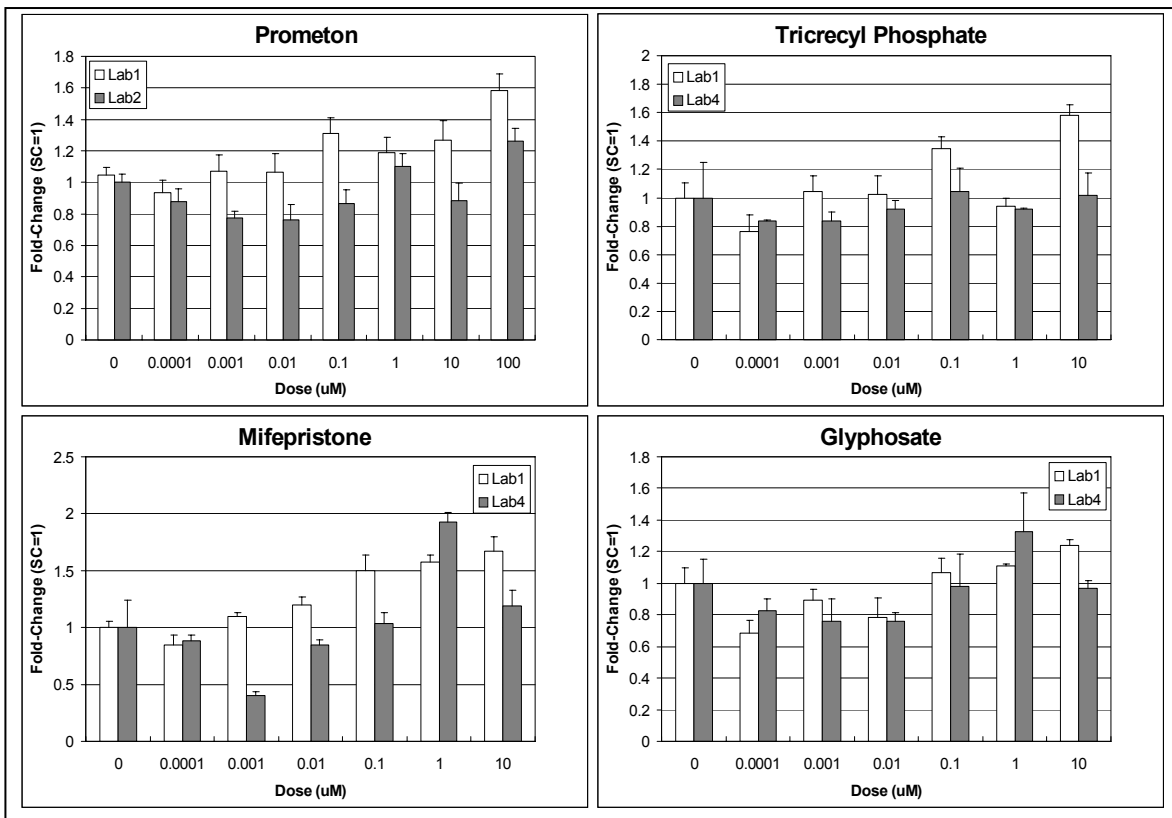


Figure 11.7: Changes in the concentrations of testosterone (T) relative to the solvent controls (SC=1) after exposure to prometon, tricresyl phosphate, mifepristone, and glyphosate. Error bars = 1x standard deviation.

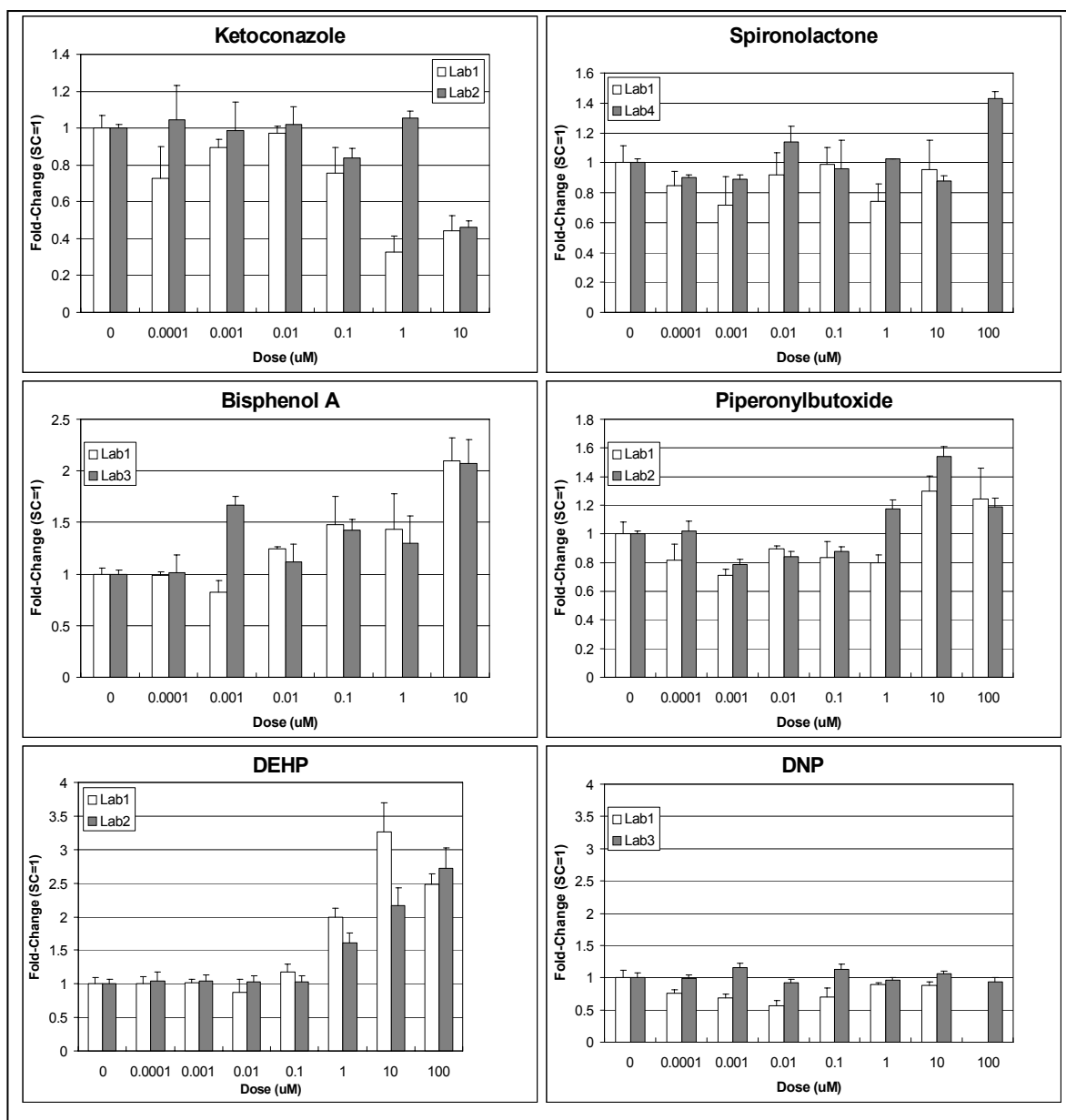


Figure 11.8: Changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to ketoconazole, spironolactone, bisphenol A, piperonyl butoxide, DEHP, and dinitrophenol (DNP). Error bars = 1x standard deviation.

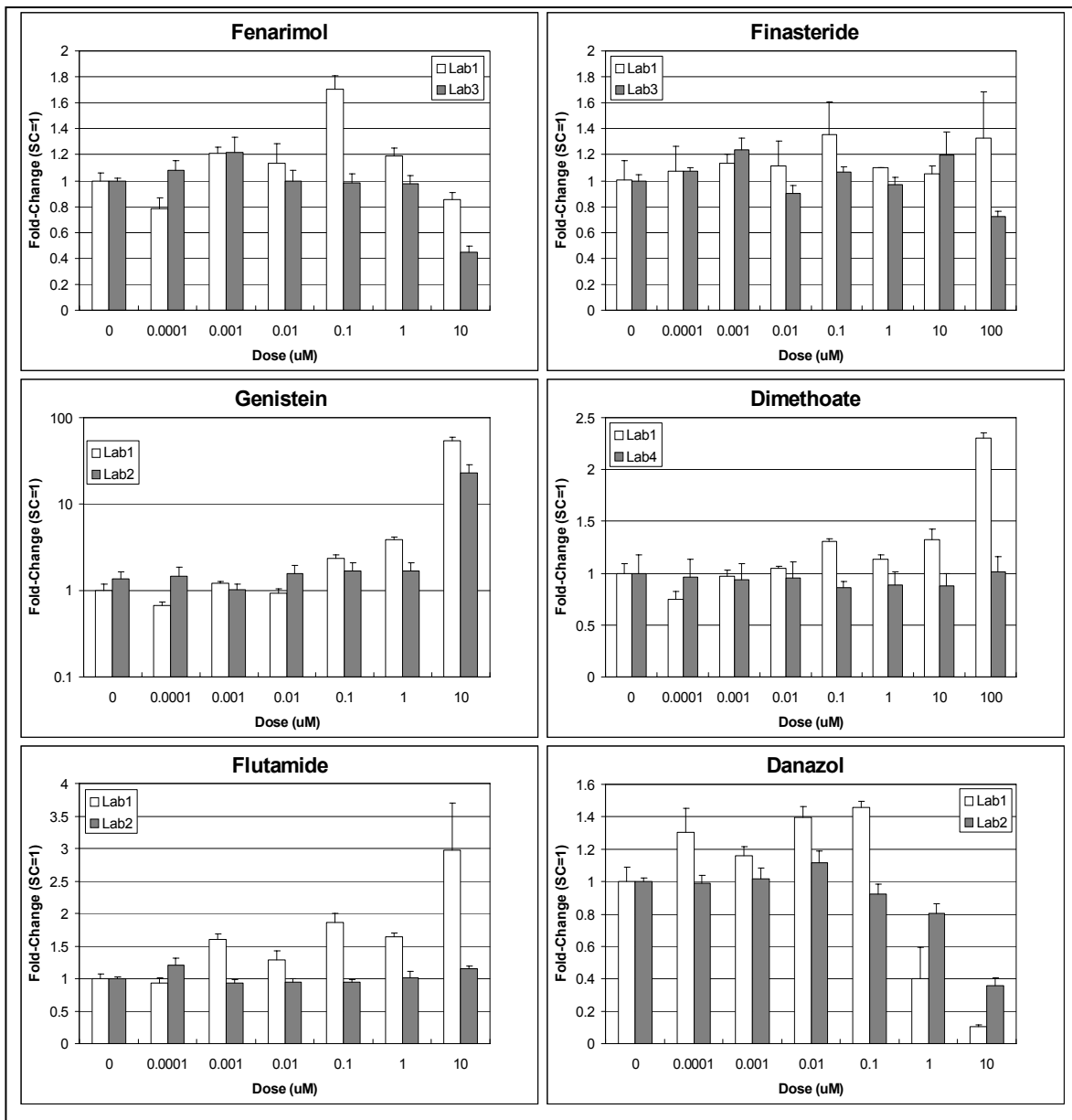


Figure 11.9: Changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to fenarimole, finasteride, genistein, dimethoate, flutamide, and danazol. Error bars = 1x standard deviation.

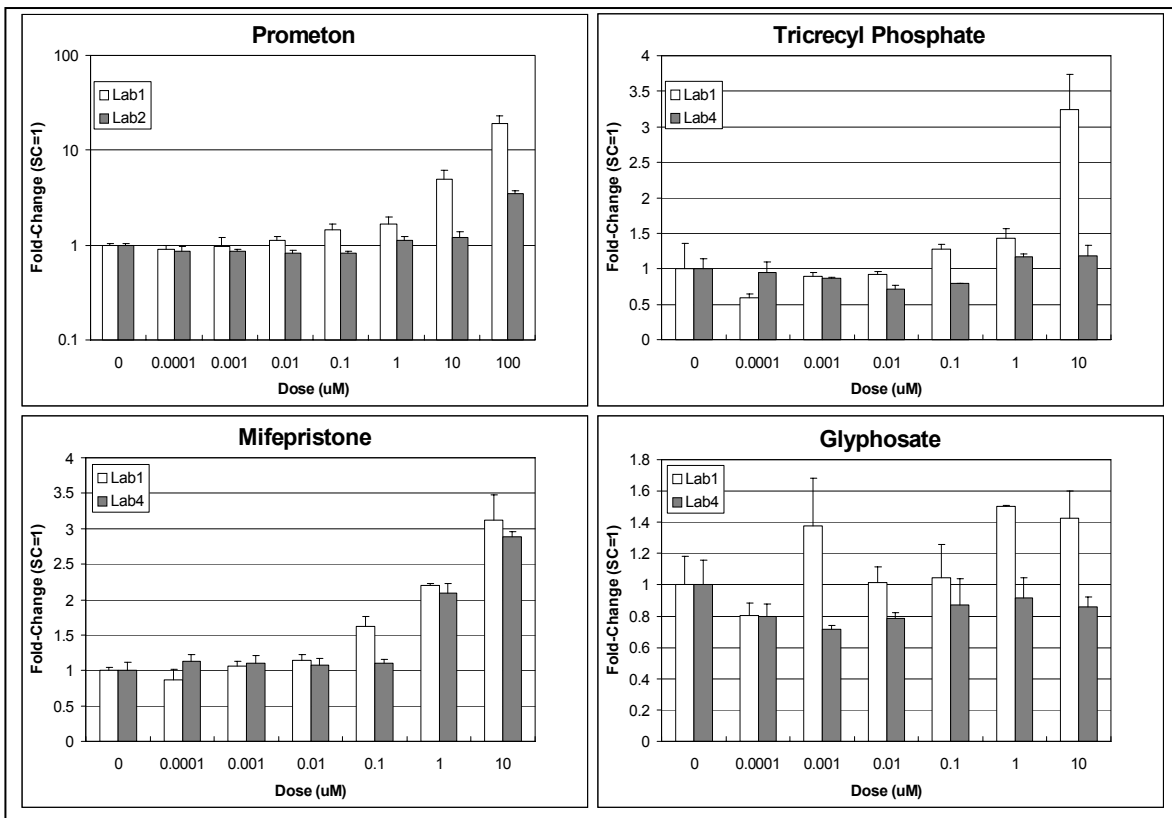


Figure 11.10: Changes in the concentrations of estradiol (E2) relative to the solvent controls (SC=1) after exposure to prometon, tricresyl phosphate, mifepristone, and glyphosate. Error bars = 1x standard deviation.

Table 11.1: Lowest observed effect concentrations (LOECs [μM]; measured by Dunnett’s test), strength and direction of change (arrows; see Table 10.1 for explanation) observed for the 16 test chemicals. Increasing intensities of yellow/red indicate increasing inhibiting strength/potency of a chemical; Increasing intensities of blue indicate increasing inducing strength/potency of a chemical. Light blue represents negative testing compound.

	Testosterone			
	LOEC		Max Change	
	1 st Lab	2 nd Lab	1 st Lab	2 nd Lab
Ketoconazole	1	1	↓↓↓	↓↓↓
Bisphenol A	10	10	↓↓	↓
Piperonyl butoxide	10	10	↓	↓
Genistein	10	10	↓↓	↓↓↓
Finasteride	10	100	↓↓	↓↓
Dinitrophenol	0.0001	100	↓	↓↓
Spironolactone	1		↓↓↓	
Fenarimol		10		↓↓
Danazol				
DEHP				
Dimethoate				
Flutamide				
Glyphosate				
Prometon				
Tricreyl phosphate	10		↑	
Mifepristone	0.1		↑	

	Estradiol			
	LOEC		Max Change	
	1 st Lab	2 nd Lab	1 st Lab	2 nd Lab
Danazol	1	10	↓↓↓	↓↓
Ketoconazole	10	10	↓↓	↓↓
Fenarimol		1		↓↓
Finasteride		100		↓
Glyphosate				
Dinitrophenol				
Spironolactone				
Piperonyl butoxide				
Dimethoate	10		↑↑	
Tricreyl phosphate	10		↑↑↑	
Flutamide	10		↑↑	
Prometon	100	100	↑↑↑↑	↑↑
Bisphenol A	10	1	↑↑	↑↑
DEHP	1 ^a	1	↑↑	↑↑
Mifepristone	0.1	1	↑↑	↑↑
Genistein	10	10	↑↑↑↑	↑↑↑↑

^a considered because clear concentration-response at all but the greatest concentration

<p>Inducers</p>	<p>≤ 1 μM or ↑↑↑↑</p> <p>≤ 10 μM or ↑↑↑</p> <p>≤ 100 μM or ↑↑</p> <p>Max concentration (>20%) or ↑</p> <p>Max concentration (<20%)</p> <p>No effect</p>	<p>Inhibitors</p>	<p>≤ 0.01 μM or ↓↓↓↓</p> <p>≤ 1 μM or ↓↓↓</p> <p>≤ 10 μM or ↓↓</p> <p>≤ 100 μM or ↓</p> <p>Max concentration (> 20%)</p>
------------------------	---	--------------------------	---

12 IN VIVO PREDICTIVITY OF THE H295R STEROIDOGENESIS ASSAY

In addition to the ability of an assay to produce reliable and transferable results as assessed in this validation effort, the potential of data obtained with an *in vitro* test such as the H295R Steroidogenesis Assay to be predictive of effects at higher organizational levels such as organisms is one of the key parameters of relevance for its use as a screening tool. Comparisons of *in vivo* and *in vitro* effects of prochloraz, ketoconazole, fenarimol, prometon, and aminoglutethimide have been made previously (Hecker et al. 2006; Villeneuve et al. 2007), and the findings reported in this study were similar to those reported by these authors. In brief, while not necessarily directly predictive of responses *in vivo*, the H295R always captured an effect if there was an alteration in hormone profiles *in vivo*. For example, the trends in changes observed for E2 after the exposure to fenarimol (fish: Ankley et al. 2005), prochloraz (amphibian: Brande-Lavritsen et al. 2008), ketokonazole (fish: Monteiro et al. 2000) and aminoglutethimide (fish: Monteiro et al. 2000; rat: Bergman and Laskey 1993), and T after the exposure to prochloraz (amphibian: Brande-Lavritsen et al. 2008; rat: Vinggaard et al. 2005) and ketokonazole (fish: Monteiro et al. 2000; rat: O'Connor et al. 2002) were comparable to those reported for the H295R cells in this study.

Changes in plasma levels of T after exposure to fenarimol (fish: Ankley et al. 2005) and aminoglutethimide (fish: Monteiro et al. 2000; rat: Bergman and Laskey 1993), and of E2 after exposure to ketoconazole (rat: O'Connor et al. 2002) were opposite to data obtained with the H295R Steroidogenesis Assay. While there were no effects of prometon on plasma T and E2 concentrations in fish (Villeneuve et al. 2007), the increase of E2 concentrations observed with the H295R cells may have been an indicator for the decrease in the expression of secondary sex characteristics in male fish. When comparing the effects described for both inducers and inhibitors of E2 production between the here presented H295R data and findings of *in vivo* studies they were comparable in 5 out of 10 and 5 out of 6 cases, respectively. These included the effects of atrazine (Wetzel et al. 1994; Spano et al. 2004), mifepristone (Fassett et al. 2008; Wang et al. 1994), danazol (Peters et al. 1980), tricresyl phosphate (Latendresse et al. 1995), genistein (Harrison et al. 1999) for the inducers, and the effects of letrozole (Kumru et al. 2007), aminoglutethimide (Bergman and Laskey 1993; Monteiro et al. 2000), prochloraz (Vinggaard et al. 2005; Brande-Lavritsen et al. 2008), ketoconazole (Monteiro et al. 2000), and fenarimol (Ankley et al. 2005) for inhibitors. In only two cases there were opposite trends among results for E2 production obtained with the H295R Steroidogenesis Assay and *in vivo* tests. Exposure to DEHP and prometon *in vivo* resulted in an inhibition (Davis et al. 1994) and no effect (Villeneuve et al. 2006) in E2 concentrations, respectively, while both chemicals caused a significant increase in E2 *in vitro* (H295R).

Three chemicals, benomyl, dimethoate and glyphosate, that tested negative for E2 effects *in vitro* (H295R) also did not cause any changes in serum E2 concentrations *in vivo* (Spencer et al. 1998; Rawlings et al. 1998; Soso et al. 2006). For all other chemicals tested no studies describing *in vivo* effects on the production of E2 were found. However, given the general toxic properties of compounds such as nonoxynol-9 (spermicide), EDS (cytotoxicant to leydig cells; Cooper and Jackson 1970; Kerr et al. 1985), and DNP (metabolic poison uncoupling oxidative phosphorylation), no specific interactions with the steroidogenic pathway would be expected. For T there were in general more discrepancies between the observed changes when compared between this and other *in vivo* studies. Only one out of five (mifepristone: Wang et al. 1994), and four out of seven (prochloraz: Vinggaard et al. 2005; Brande-Lavritsen et al. 2008; ketoconazole: O'Connor et al. 2002; Monteiro et al. 2000; genistein: Ohno et al. 2003; spironolactone: Canosa and Ceballos, 2001) chemicals revealed comparable trends in *in vivo* studies when compared to inducers and inhibitors of T production *in vitro*, respectively. However, only for four substances opposite trends between in results obtained with the H295R cells and in *in vivo* studies were observed. These were the inducers atrazine (Wetzel et al. 1994; Spano et al. 2004) and trilostane (Jungman et al. 1983), and the inhibitors letrozole (Kumru et al. 2007) and aminoglutethimide (Bergman and Laskey 1993; Monteiro et al. 2000). Six out of the 11 chemicals that tested negative for changes in T production

in the H295R Steroidogenesis Assay were also reported as not exhibiting significant alterations in concentrations of this hormone in vivo. These compounds were flutamide (Adamson et al. 2008; Mikkilä et al. 2006), glyphosate (Soso et al. 2006), DEHP (Noriega et al. 2009), finasteride (Canosa and Ceballos 2001), benomyl (Carter and Laskey 1982), and molinate (Ellis et al. 1998). For none of the other chemicals studies were found that described effects on the production of T in vivo. The reason for the increased number of chemicals with different effects on the production of T between in vivo studies and this work when compared to E2 is likely due to the intermediate role of T in the steroidogenesis pathway. Thus, it is possible that changes in T can be better compensated by the cells than those in E2. Overall, there was not a single chemical which would have been characterized as a false negative by the H295R Steroidogenesis Assay. Also, in some cases where no effects on hormone production were observed in vivo but other anti-androgenic responses were described such as for spironolactone as determined by the Hershberger assay (Canosa and Ceballos 2001) the H295R assay detected an alteration in hormone homeostasis that would have been characteristic of such an effect (e.g. increase in E2 or decrease in T). There were no studies describing the effects of the model inducer forskolin on hormone homeostasis in vivo. However, considering the rapid metabolism of forskolin by an organism no marked effects would be expected. Overall, these results indicate that, while not necessary always directly predictive of a specific type of response in an organism, the H295R would always flag a chemical as a potential disruptor of steroidogenic processes or a reproductive toxicant. Furthermore, there was only one chemical for which both in vivo and in vitro data were available that would have been wrongly characterized as inducers and inhibitors of T (atrazine; Wetzel et al. 1994) and E2 (prometon; Villeneuve et al. 2006) production by the H295R Steroidogenesis Assay, respectively.

Table 12.1: Comparison of data obtained with the H295R Steroidogenesis Assay (this study) with in vivo data. ↑ Increase; ↓ Decrease; - No effect; n.d. no data/study available.

Chemical	Testosterone		Estradiol		Species	Reference
	H295R	In Vivo	H295R	In Vivo		
Aminoglutethimide	↓	↑	↓	↓	Fish; Rat	Bergman and Laskey 1993; Monteiro et al. 2000
Atrazine	↑ / -	-	↑	↑	Fish; Rat	Spano et al. 2004; Wetzel et al. 1994
Benomyl	-	-	-	-	Rat	Carter and Laskey 1982; Spencer et al. 1998
Bisphenol A	↑	n.d.	↓	n.d.	-	-
Butyl paraben	-	-	↑	n.d.	Rat	Taxvig et al. 2008
Danazol	-	n.d.	↑	↑	Human	Murakami et al. 1993; Peters et al. 1980
DEHP	-	-	↑	↓	Rat	Davis et al. 1994; Noriega et al. 2009
Dimethoate	-	n.d.	-	-	Sheep	Rawlings et al. 1998
Dinitrophenol	-	n.d.	-	n.d.	-	-
EDS	-	n.d.	-	n.d.	-	-
Fenarimol	↓	↑	↓	↓	Fish	Ankley et al. 2005
Finasteride	-	-	-	n.d.	Amphibian	Canosa and Ceballos 2001
Flutamide	-	-	↑	n.d.	Rat	Adamson et al. 2008; Mikkilä et al. 2006
Forskolin	↑	n.d.	↑	n.d.	-	-
Genistein	↓	↓	↑	↑	Monkey; Rat	Harrison et al. 1999; Ohno et al. 2003
Glyphosate	-	-	-	-	Fish	Soso et al. 2006
HCG	-	- ^d	-	- ^d	- ^d	- ^d
Ketoconazole	↓	↓	↓	↓	Fish; Rat	Monteiro et al. 2000; O'Connor et al. 2002
Letrozole	↓	↑	↓	↓	Rat	Kumru et al. 2007
Mifepristone	↑	↑	↑	↑	Human	Fassett et al. 2008; Wang et al. 1994
Molinate	↑	n.d. ^c	-	-	Rat	Ellis et al. 1998
Nonoxynol-9	-	n.d.	-	n.d.	-	-
Piperonyl butoxide	↓	n.d.	-	n.d.	-	-
Prochloraz	↓	↓	↓	↓	Amphibian; Rat	Vinggaard et al. 2005; Brande-Lavritsen et al. 2008
Prometon	-	-	↑	- ^b	Fish	Villeneuve et al. 2006
Spirolactone	↓	↓	-	n.d. ^b	Amphibian; Rat	Canosa and Ceballos 2001; Yamasaki et al. 2004
Tricrescyl phosphate	↑ / -	n.d.	↑	↑	Rat	Latendresse et al. 1995
Trilostane	↑ ^a	↓ / -	- ^a	-	Fish; Human; Rat	Jungmann et al. 1983; Villeneuve et al. 2008

^a Corrected for cross-reactivity

^b Identified as reproductive toxicant in vivo

^c Data not considered because inconclusive results

^d H295R Assay does only capture effects downstream of LH/FSH

13 ADJUSTMENTS MADE TO THE DRAFT GUIDELINES BASED ON THE VALIDATION STUDY

In order to address some of the remaining uncertainties of the H295R Steroidogenesis Assay a number of minor changes have been made to the protocols, and which are reflected in the draft test guideline submitted to the OECD VMG NA. These changes are the inclusion of a laboratory proficiency test to be able to better evaluate the capabilities of a laboratory to grow the cells and conduct the test. This test is to be conducted prior to the first conduct of the assay of a laboratory or after any changes such as facilities or personnel occur. Furthermore, based on the results obtained with regard to the adjustment of hormone data for cell viability in each well it was decided to not include this procedure in the guideline given the uncertainty of the true relation between the biochemically detected cell viability and the true number of cells present in a well. Instead, it is proposed to take photographs of the control wells and the wells containing the greatest exposure concentrations to enable a retrospective assessment of the true cell number in case questions arise based on the cell viability data. Also, the dosing regime for prochloraz in the QC plates has been changed from 0.3 and 3 μM to 0.1 and 1 μM to better reflect the active dose range for this chemical with respect to its effect on E2 and T production. Finally, there have been a number of small changes made with respect to the QA criteria set forth in the protocols. Briefly, these include the minimum basal production of the cells prior to use in the assay, allowances for between and within experiment variation, response criteria after exposure to model chemicals on the QC plate, and hormone detection system sensitivities.

13.1 Laboratory Proficiency Test

Before testing unknown substances, a laboratory should demonstrate that it is capable of achieving and maintaining appropriate cell culture and test conditions required for the successful conduct of the assay. As the performance of an assay is directly linked to the laboratory personnel conducting the assay, these procedures should be repeated if a change in laboratory personnel occurs.

This qualification test will be conducted under the same conditions listed in paragraphs 37 through 39 by exposing cells to six increasing concentrations of the model inducer forskolin and the model inhibitor prochloraz as shown in Table 2. Separate plates are run for forskolin and prochloraz. These qualification tests will be done under a somewhat different exposure regime and plate layout compared to the standard test chemical exposure procedure in that the concentrations are more closely distributed about the expected values of the EC_{50} of each of the control chemicals than would be the case for an initial run with an unknown chemical.

Table 13.1: Dosing schematic for the laboratory proficiency study.

	1	2	3	4	5	6
A	DMSO 1µL	DMSO 1µL	DMSO 1µL	For 10 or Pro 3	For 10 or Pro 3	For 10 or Pro 3
B	For 0.03 or Pro 0.01	For 0.03 or Pro 0.01	For 0.03 or Pro 0.01	For 1 or Pro 0.3	For 1 or Pro 0.3	For 1 or Pro 0.3
C	For 0.3 or Pro 0.1	For 0.3 or Pro 0.1	For 0.3 or Pro 0.1	For 0.1 or Pro 0.03	For 0.1 or Pro 0.03	For 0.1 or Pro 0.03
D	For 3 or Pro 1	For 3 or Pro 1	For 3 or Pro 1	Blank	Blank	Blank

Exposure of H295R to either forskolin (For) or prochloraz (Pro) in a 24 well plate during the laboratory proficiency test. Dosing is in µM for all test chemical doses. Note: Doses will be administered in DMSO at 0.1% v/v per well. The DMSO solvent control will receive 1 µL of DMSO only. Nothing is added to the Blank wells. Separate plates are run for forskolin and prochloraz

13.2 QC Plate Prochloraz Concentration Adjustment

There are two uses of the quality control (QC) plate:

1. H295R cell performance should be assessed for possible changes in hormone production as a function of cell age prior to using a new ATCC batch or after using a previously frozen stock of cells for the first time, unless the laboratory proficiency test (paragraphs 31-33) has been run with that batch of cells. To verify that the performance of H295R Cells under Standard Culture Conditions is meeting the QC requirements, a subset of passage five (5.0) cells is run in a QC plate. Laboratories that have already frozen passage five (5.0) cells can thaw one of the frozen batches, grow it for three (3) passages (passage # 3.5), and use these cells for the QC run.
2. A QC plate provides the positive controls for the assay when testing chemicals and should be part of each test run.

The QC test is conducted in a 24 well plate and follows the same incubation, dosing, cell viability/cytotoxicity, hormone extraction and hormone analysis procedures described in paragraphs 37 through 44 for testing chemicals. The QC plate contains two concentrations of a known inducer (forskolin) and inhibitor (prochloraz) of E2 and T synthesis. The minimum basal hormone production should be met in both the solvent control and blank wells.

Table 13.2: Quality control plate layout for testing performance of unexposed H295R cells and cells exposed to known inhibitors (PRO = prochloraz) and stimulators (FOR = forskolin) of E2 and T production. A 70% methanol solution will be added to all MeOH wells after termination of the exposure experiment and removal of medium.

	1	2	3	4	5	6
A	Blank ^a	Blank ^a	Blank ^a	Blank ^a + MeOH ^b	Blank ^a + MeOH ^b	Blank ^a + MeOH ^b
B	DMSO 1µL	DMSO 1µL	DMSO 1µL	DMSO 1µL + MeOH ^b	DMSO 1µL + MeOH ^b	DMSO 1µL + MeOH ^b
C	FOR 1µM	FOR 1µM	FOR 1µM	PRO 0.1µM	PRO 0.1µM	PRO 0.1µM
D	FOR 10µM	FOR 10µM	FOR 10µM	PRO 1µM	PRO 1µM	PRO 1µM

^a Blank wells receive medium only.

^b Methanol (MeOH) will be added **after** the exposure is terminated and the medium is removed from these wells.

Table 13.3: Performance criteria for the Quality Control Plate

	T	E2
Minimum Basal Production	500 pg/mL	40 pg/mL
Basal Production of hormone in the solvent control (SC)	≥ 5-times the minimum detection limit (MDL)	≥ 2.5-times the MDL
Induction (10 µM forskolin)	≥ 2 times the SC	≥ 7.5 times the SC
Inhibition (3µM prochloraz)	≤ 0.5 times the SC	≤ 0.5 times the SC

13.3 Cell Viability Assessment

A cell viability/cytotoxicity assay of choice can be used to determine the potential impact of the test chemical on cell viability. The assay should be able to provide a true measure of the percentage of viable cells present in a well, or it should be demonstrated that it is directly comparable to (a linear function of) the Live/Dead® Assay (Molecular probes, Eugene OR, USA, Cat # L-3224)(see Annex 3 section 6). An alternative assay that has been shown to work equally well is the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] test (Mosman 1983). The assessment of cell viability using the above methods is a relative measurement that does not necessarily exhibit linear relationships with the absolute number of cells in a well. Therefore, a subjective parallel visual assessment of each well by the analyst should be conducted, and digital pictures of the SCs and the two greatest non-cytotoxic concentrations are to be taken and archived to enable later assessment of true cell density if this should be required.

All concentrations that exhibit cytotoxicity greater than 20% are to be omitted for further evaluation. If by visual inspection of the well and the digital photographs there appears to be an increase in cell number, the apparent increase needs to be verified. If an increase in cell numbers is verified, this should be stated in the test report, and the hormone data should be normalized by dividing hormone concentration by the relative change in the number of viable cells.

14 CONCLUSIONS AND SUMMARY

Considering the data obtained during the validation studies with the 12 core chemicals, the H295R Steroidogenesis protocol successfully detected inducers and inhibitors of T and E2 production as well as negative chemicals. For each hormone, there were only two cases in which a laboratory did not confirm the trend that was observed at the other groups. In all situations such inconsistencies only occurred for weak inducers/inhibitors, and only in two cases would have resulted in a false negative response. However, even in these two cases, a chemical would have been identified as a positive testing compound by the H295R Steroidogenesis Assay because there was a significant effect for the other hormone measured. In most of the cases (three out of four), these differences occurred at groups for which only data from one experiment for the chemical was available, indicating the need for replicate testing.

In addition, the assay successfully identified inducers and inhibitors of the production of T or E2, as well as negative chemicals from a list of 16 additional compounds with largely unknown modes of interaction with sex steroid synthesis. However, there was a greater variation in the responses among laboratories for these supplementary chemicals. Most of this variation was associated with one laboratory, and it is possible that there was an issue related to dosing given the lack of cytotoxic responses for two out of three compounds that were identified by the lead laboratory. When the data from this laboratory was eliminated from the analysis, there were only three out of 22 different experiments for which the response pattern did not match between labs, indicating that the assay seems to provide a reliable means to identify inducers and inhibitors of T and E2 production *in vitro*. In this context, it could be demonstrated the following stringent protocols for culturing the cells and conducting the exposure experiments are important for the successful conduct of the assay. In this context, one of the key issues observed was either insufficient or too great (e.g. supplemental chemical experiment at Lab 4) basal production of E2 due to changes as a function of cell age. The draft test guideline has been adjusted considering these factors (see section 12).

The assay also reliably characterized both inducers and inhibitors based on the strength and potency of their effect. However, given the nature of the dosing regime that bracketed a wide range of concentrations in an attempt to screen chemicals that differed in their potency and modes of action, there are some limitations that preclude the precise description of weak inducers and inhibitors. Therefore, it is recommended that the H295R Steroidogenesis assay ideally should be used with other *in vitro* or *in vivo* assays, and thus, even in the rare occasions where there might be uncertainties as to how a chemical will be characterized basing on the data obtained with the H295R assay, so that these chemicals would be further evaluated in other tests. An alternative approach to better categorize weak inducers or inhibitor of steroidogenesis would be to revise the dosing regime such that less spacing between doses occurs, allowing for the description of more precise dose-response relationships. Considering that with few exceptions (letrozole and prochloraz) effects only occurred at concentrations greater than 0.1 μM , the following four-fold increment scale should be sufficient: 0.1 μM , 0.3 μM , 1 μM , 3 μM , 10 μM , 30 μM , and 100 μM . It should also be noted that the sometimes elevated levels of inter-laboratory variability are the reason that the assay is recommended to be used for qualitative assessment of interference with steroidogenesis rather than a quantitative one at this time. This refinement of the dosing regime will be another step towards the reduction of such variation, rendering the assay a more accurate tool for the screening of chemicals.

A further issue that was observed during the validation studies was that some chemicals had the potential to interact directly with the hormone detection system utilized to measure hormone concentrations in the medium. However, for this portion of the validation study cross-reactivity of the assay system would have mischaracterized only one chemical as an inducer of E2 production. In such instances the samples should be re-analyzed using alternative hormone detection methods that are not confounded by the chemical of interest. As stated above, the incidence of such interference by a chemical occurred only in rare occasions and is not considered to be of concern relative to the overall performance of the assay. This emphasizes, however, the importance of evaluating the cross-reactivity of a chemical prior to actually conducting the assay that will objectively evaluate its hormone altering potential.

Additional factors that must be considered when evaluating chemicals using the H295R Steroidogenesis Assay are solubility and effects on cell viability. Chemicals that are not soluble at the highest concentration to be tested (typically 100µM) will need to be diluted to their solubility limit in whatever solvent (DMSO, ethanol, etc.) that is being used in the assay. Since non-soluble concentrations are not considered to be relevant to the specific mechanism of chemical interaction tested for by the H295R Steroidogenesis Assay, interaction with E2 and T production and will give misleading results, they should not be used in evaluating the potency and efficacy of the chemical in the assay. Furthermore, although we acknowledge that chemicals that are not completely in solution may have the potential to interact with cells or organisms, these effects are likely to be primarily associated with alterations of cell membranes and their associated function, and not due to the specific effect on endocrine function. Similarly, effects of chemicals on cell viability are to be considered as “general toxic” interactions that are not relevant to the specific aim of this assay: identifying effects on the production of T and E2.

The results of the validation study demonstrated that the H295R Steroidogenesis Assay protocol successfully identified both confounders, interference with the hormone detection assay and cytotoxicity, and thus, allowed to distinguish between “true inducers/inhibitors” and effects that were due to other factors such as cytotoxicity and hormone assay interference. Also, it could be shown that the assay seems to have a good predictive potential with regard to identifying chemicals that interact with steroidogenic or reproductive processes in vivo.

15 REFERENCES

- Ankley GT, Jensen, KM, Durhan EJ, Makynen EA, Butterworth BC, Kahl MD, Villeneuve DL, Linnum A, Gray LE, Cardon M, Wilson VS. (2005) Effects of two fungicides with multiple modes of action on reproductive endocrine function in the fathead minnow (*Pimephales promelas*). *Toxicol Sci* 86:300-308.
- Battelle (2005) Sliced Testis Assay Prevalidation: Baseline/Positive Control Study.
- Berman E and Laskey JW. (1993) Altered steroidogenesis in whole-ovary and adrenal culture in cycling rats. *Reprod Toxicol* 7:349–358.
- Brande-Lavridsen N, Christensen-Dalsgaard J, Korsgaard B. (2008) Effects of prochloraz and ethinylestradiol on sexual development in *Rana temporaria*. *J Exp Zool* 309A:389–398.
- Canosa LF and Ceballos NR. (2001) Effects of different steroid-biosynthesis inhibitors on the testicular steroidogenesis of the toad *Bufo arenarum*. *J Comp Physiol B* 171:519-526.
- Cooper ERA and Jackson H. (1970) Comparative Effects of Methylene, Ethylene and Propylene Dimethane-Sulphonates on the Male Rat Reproductive System. *J Reprod Fertil* 23:103-108.
- Davis BJ, Maronpot RR, Heindel JJ. (1994) Di-(2-ethylhexyl) phthalate suppresses estradiol and ovulation in cycling rats. *Toxicol Appl Pharmacol* 128:216-223.
- ECVAM (1995) Practical Aspects of the Validation of Toxicity Test Procedures: The Report and Recommendations of ECVAM Workshop No. 5. *ATLA* 23:129-147.
- Ellis MK, Richardson AG, Foster JR, Smith FM, Widdowson PS, Farnworth MJ, Moore RB, Pitts MR, Wickramaratne GA de S. (1998) The Reproductive Toxicity of Molinate and Metabolites to the Male Rat: Effects on Testosterone and Sperm Morphology. *Toxicol Appl Pharmacol* 151:22–32.
- Endocrine Disruptor Methods Validation Advisory Committee (2005) Letter from the EDMVAC to Stephen L. Johnson, Administrator of EPA, May 5, 2005.
- Fassett MJ, Lachelin GCL, McGarrigle HHG, Wing DA. (2008) Alterations in saliva steroid hormone levels after oral mifepristone administration in women with pregnancies of greater 41 weeks' gestation. *Reproductive Sciences* 15:394-399.
- Gazdar AF, Oie HK, Shackleton CH, Chen, TR, Triche, TJ, Myers CE, Chrousos GP, Brennan MF, Stein CA., La Rocca RV (1990) *Cancer Res* 50:5488-5496.

- Giesy JP, Hilscherova K, Newsted JL, Mehrle PM (2002) Optimization of the H295R cell line for use in evaluating toxicant induced effects of steroidogenesis. Progress Report to the US-EPA ORD Service Centre/NHEERL. July 2002.
- Harrison RM, Phillippi PP, Swan KF, Henson MC. (1999) Effect of genistein on steroid hormone production in the pregnant rhesus monkey. *Exp Biol Med* 222:78-84.
- Hartung T et al. (2004) *ATLA* 32, 467-472.
- Hecker M, Newsted JL, Murphy MB, Higley EB, Jones PD, Wu R, Giesy JP (2006a) *Toxicol Appl Pharmacol* 217:114-124.
- Hecker M, Giesy, JP, Jones PD, Higley EB, Newsted JL, Mehrle P (2006b) Influence of cell passage and freeze/thaw events on basal production of 17 β -estradiol and testosterone by H295R cells. Interim Draft Report to the US-EPA. Contract code: GS-10F-0041L. September 2006.
- Hecker M, Hollert H, Cooper R, Vinggaard A-M, Akahori Y, Murphy M, Nellesmann C, Higley E, Newsted J, Wu R, Lam P, Laskey J, Buckalew A, Grund S, Nakai M, Timm G, Giesy J (2007a) *Env Sci Pollut Res* 14(Special Issue 1):23-30.
- Hecker M, Giesy, JP, Jones PD, Higley EB, Newsted JL, Mehrle P (2007b) Standardization and refinement of the H295R cell based assay to identify chemical modulators of steroidogenesis and aromatase activity - Repeat of Inter-Laboratory Study. Report to the US-EPA. Contract code: GS-10F-0041L. March 2007.
- Hilscherova K, Jones PD, Gracia T, Newsted JL, Zhang X, Sanderson JT, Yu RMK, Wu RSS, Giesy JP (2004) *Toxicol Sci* 81:78-89.
- ICCVAM (1997). Validation and Regulatory Acceptance of Toxicological Test Methods. A report of the Ad Hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Report No. 97-3981, National Institute of Environmental Health Sciences, Research Triangle Park, NC.
- Jungmann E, Althoff PH, Balzer-Kuna S, Magnet W, Rottmann-Kuhnke U, Sprey R, Schwedes U, Usadel KH, Schöffling K. (1983) The inhibiting effect of trilostane on testosterone synthesis. Hormonal and morphological alterations induced by subchronic trilostane treatment in rats and healthy volunteers. *Arzneimittelforschung* 33:754-756.
- Kavlock RJ, Daston GP, De Rosa C, Fenner-Crisp P, Gray LE, Kaattari S, Lucier G, Luster M, Mac MJ, Maczka C, Miller R, Moore J, Rolland R, Scott G, Sheehan DM, Sinks T., Tilson HA. (1996) *Environ Health Perspect* 104:715-740.
- Kerr JB, Donachie K, Rommerts FFG. (1985) Selective destruction and regeneration of rat Leydig cells in vivo: A new method for the study of seminiferous tubular-interstitial tissue interaction. *Cell Tissue Res* 242:145-156.
- Kumru S Yildiz AA, Yilmaz B, Sandal S, Gurates B. (2007) Effects of aromatase inhibitors letrozole and anastrozole on bone metabolism and steroid hormone levels in intact female rats. *Gynecological Endocrinology* 23:556—561.
- Latendresse JR, Brooks CL, Capen CC. (1995) Toxic effects of butylated triphenyl phosphate-based hydraulic fluid and tricresyl phosphate in female F344 rats. *Veterinary Pathology* 32:394-402.
- Mikkilä TFM, Toppari J, Paranko J. (2006) Effects of neonatal exposure to 4-tert-octylphenol, diethylstilbestrol, and flutamide on steroidogenesis in infantile rat testis. *Toxicol Sci* 91:456-466.
- Monteiro PRR, Reis-Henriques MA, Coimbra J. (2000). Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (*Platichthys flesus* L.). *Aquat Toxicol* 48:549-559.
- Noriega NC, Howdeshell KL, Furr J, Lambright CR, Wilson VS, Gray Jr. LE. (2009) Pubertal administration of DEHP delays puberty, suppresses testosterone production, and inhibits reproductive tract development in male Sprague-Dawley and Long-Evans rats. *Toxicol Sci* 111:163-178.
- O'Connor JC, Frame SR, Ladies GS. (2002) Evaluation of a 15-day screening assay using intact male rats for identifying steroid biosynthesis inhibitors and thyroid modulators. *Toxicol Sci* 69:79-91.
- OECD (1996) Final Report of the OECD Workshop on the Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods (Solna Report) as presented to the Seventh Meeting of the National Coordinators of the Test Guidelines Program, 18-19 September 1996. ENV/MC/CHEM/TG(96)9.

- OECD (1998) Report of the First Meeting of the OECD Endocrine Disrupter Testing and Assessment (EDTA) Task Force, 10-11 March 1998. ENV/MC/CHEM/RA(98)5
- OECD (2002). Report of the Sixth Meeting of the Endocrine Disrupters Testing and Assessment Task Force, 24-25 June 2002. ENV/JM/TG/EDTA/M(2002).
- OECD (2005). Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Guidance Document No. 34. June 2005
- Ohno S, Nakajima Y, Inoue K, Nakazawa H, Nakajin S (2003) Genistein administration decreases serum corticosterone and testosterone levels in rats. *Life Sciences* 74:733-742.
- Peters F, Reck G, Zimmermann G, Breckwoldt M. (1980) The effect of danazol on the pituitary function, thyroid function, and mastodynia. *Arch Gynecol* 230:3-8.
- Puddefoot, J.R., Barker, S., Glover, H.R., Malouitre, S.D.M., Vinson, G.P. (2002) Non-competitive steroid inhibition of oestrogen receptor functions. *Int J Cancer* 101:17-22.
- Rainey, W. E., Bird, I. M., Sawetawan, C., Hanley, N. A., McCarthy, J. L., Mcgee, E. A., Wester, R., and Mason, J. I. (1993) *J. Clin. Endocrinol. Metab.* 77, 731-737.
- Rawlings NC, Cook SJ, Waldbillig D. (1998) Effects of the pesticides carbofuran, chlorpyrifos, dimethoate, lindane, triallate, trifluralin, 2,4-D, and pentachlorophenol on the metabolic endocrine and reproductive endocrine system in ewes. *J Toxicol Environ Health A* 54:21-36.
- Sanderson, J. T., Boerma, J., Lansbergen, G., and Van den Berg, M. (2002) *Toxicol. Appl. Pharmacol.* 182, 44-54.
- Shapiro R., and Page L.B. (1976) Interference by 2,3-dimercapto-1-propanol (BAL) in agiotensim I radioimmunoassay. *J. Lab. Clin. Med.* 2, 22-31.
- Soso AB, Barcellos LJG, Ranzani-Paiva MJ, Kreutz LC, Quevedo RM, Anziliero D, Lima M, da Silva LB, Ritter F, Bedin AC, Finco JA. (2007) Chronic exposure to sub-lethal concentration of a glyphosate-based herbicide alters hormone profiles and affects reproduction of female Jundi'a (*Rhamdia quelen*). *Environ Toxicol Pharm* 23:308-313.
- Spanò L, Tyler CR, van Aerle R, Devos P, Mandiki SNM, Silvestre F, Thoméc J-P, Kestemont P. (2004) Effects of atrazine on sex steroid dynamics, plasma vitellogenin concentration and gonad development in adult goldfish (*Carassius auratus*). *Aquat Toxicol* 66:369-379.
- Spencer F, Chi L, Zhu M-X. (1996) Effect of benomyl and carbendazim on steroid and molecular mechanisms in uterine decidual growth in rats. *J Appl Toxicol* 16:211-214.
- Takeyoshi M. 2007. Prevalidation and Inter-laboratory Validation of Stably Transfected Transcriptional Activation Assay to Detect Estrogenic Activity: The Human Estrogen Receptor Alpha Mediated Reporter Gene Assay Using hER-HeLa-9903 Cell Line. Chemical Evaluation and Research Institute, Japan. October, 2007.
- Taxvig C, Vinggaard AM, Hass U, Axelstad M, Boberg J, Hansen PR, Frederiksen H, Nellemann C. (2008) Do parabens have the ability to interfere with steroidogenesis? *Toxicol Sci* 106:206-213.
- Villeneuve DL, Blankenship AL, Giesy JP (1998) Estrogen receptors - environmental xenobiotics. In: Denison MS, Helderich WG (eds) *Toxicant-receptor interactions and modulation of gene expression*. Lippincott-Raven Publishers, Philadelphia.
- Villeneuve DL, Ankley GT, Makynen EA, Blake LS, Greene KJ, Higley EB, Newsted JL, Giesy JP, Hecker M. (2007) Comparison of fathead minnow ovary explant and H295R cell-based steroidogenesis assays for identifying endocrine-active chemicals. *Ecotoxicol Environ Saf* 68:20-32.
- Villeneuve DL, Blake LS, Brodin JD, Cavallin JE, Durham EJ, Jensen KM, Kahl MD, Makynen EA, Martinovic D, Mueller ND, Ankley GT (2008) Effects of 3 β -hydroxysteroid dehydrogenase inhibitor, trilostane, on the fathead minnow reproductive axis. *Toxicol Sci* 104:113-123.
- Vinggaard AM, Christiansen S, Laier P, Poulsen ME, Breinholt V, Jarfelt K, Jacobsen H, Dalgaard M, Nellemann C, Hass U 2005. Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring. *Toxicol Sci* 85:886-897.
- Wang J-D, Shi W-L, Zhang G-Q, Bai X-M. (1994) Tissue and serum levels of steroid hormones and RU 486 after administration of mifepristone. *Contraception* 49:245-253.

- Wetzel LT, Luempert III LG, Breckenridge CB, Tisdell MO, Stevens JT. (1994) Chronic effects of atrazine on estrus and mammary tumor formation in female Sprague-Dawley and Fischer 344 rats. *J Toxicol Environ Health* 43:169-182.
- Wilson, V. S., Bobseine, K., Lambright, C. R., and Gray, L. E. (2002) *Toxicol. Sci.* 66, 69-81.
- Wilson, V. S., Bobseine, K., and Gray, L. E. (2004) *Toxicol. Sci.* 81, 69-77.

16 APPENDICES

- APPENDIX I – H295R Steroidogenesis Assay Protocol – Cell identification scheme
- APPENDIX II – H295R Steroidogenesis Assay Protocol – Preparations of solutions and reagents
- APPENDIX III – H295R Steroidogenesis Assay Protocol – Supplemental procedures

Appendix I

Cell culture identification scheme

Cell culture diagrams are useful and necessary for the smooth operation of the cell culture laboratory. They are used for a variety of reasons including: (i) They allow for tracking the progress of a cell line and notice problems or incongruencies, which may arise over time (These are very important to note), and (ii), they also provide a platform for recording all of the work done with the cells.

METHOD, PROCEDURES AND REQUIREMENT

1. Start with a clean page in your laboratory notebook. At the top of the page, record your initials, the date and “Cell Culture Diagram for H295R”.
2. In a box at the top of the page record all of the information from the saved cryovial:
 - Cell Type
 - Date the cells were frozen
 - ATCC Lot number
 - Freeze Down ID / Passage # / Total # of Passages since original ATCC cell batch was started in cell culture / Plate ID
3. Each time you split the cells you will increase the passage number and total number of passages by one. Each time you freeze down the cells you will increase the freeze down ID (Greek letter) by one starting with A and continuing with B, Γ, Δ, E etc. (see Table 1 for key). When you start cells that have been frozen down you will reset the passage number to 1 and continue the total number of passages. Each different plate/flask will be labelled with another letter with the first being A, the second B, and so on.
4. Each data sheet in the study will contain the information on the cells used i.e. Cell Type, Date the cells were frozen, ATCC Lot number, Freeze Down ID, Passage #, Total # of Passages since original ATCC cell batch was started in cell culture, and Plate ID.
As the cells continue to grow, record everything that you do whether it is change the media, freeze cells down, split the cells etc. (See Figure 1 for a sample diagram).
5. Continue with the cell culture diagram until the cells are no longer being actively used in research or they are all frozen.

Note: For the specific purpose of the validation studies cells will be grown from an original ATCC batch for five (5.0) passages, and then frozen down resulting (given that cells from one culture plate were always split into three new plates as shown below) in a maximum of 162 batches. One of these batches will then be started up in culture again and grown for at least four (4) passages (passage # 4.5 = plate ID# B/4.5/9/X) prior to initiation of the exposure experiments. Only cells that have been frozen down one (1) time (B generation) will be used for these experiments. The passage number is not to exceed 7.5.

Name: _____ Date: _____
 Project: _____
 ATCC Lot Number: _____

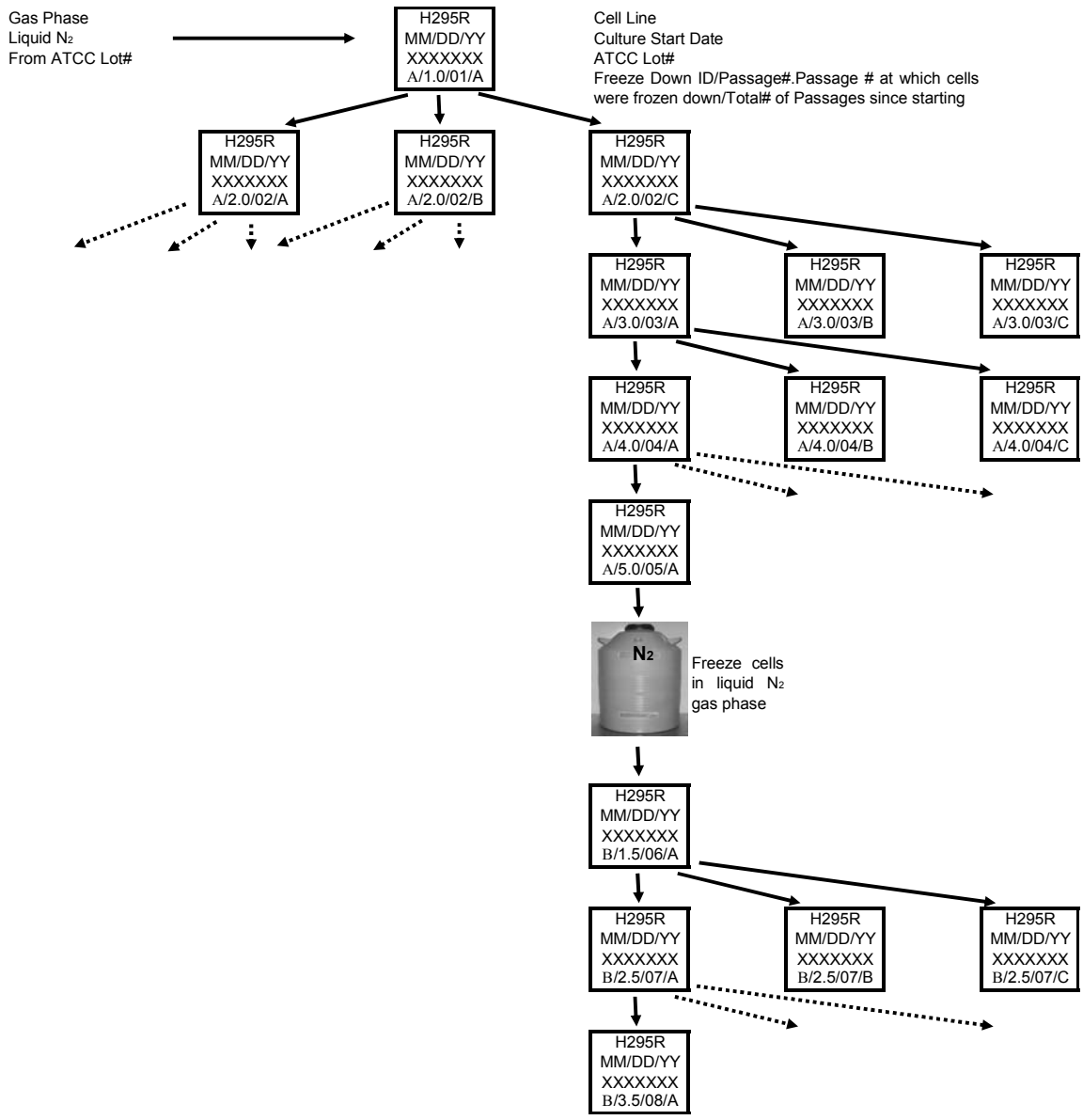


Table 1.

Symbol			Freeze Down Number	Symbol			Freeze Down Number
α	A	Alpha	0	ν	N	Nu	12
β	B	Beta	1	ξ	Z	Xi	13
γ	Γ	Gamma	2	\omicron	O	Omicron	14
δ	Δ	Delta	3	π	Π	Pi	15
ϵ	E	Epsilon	4	ρ	P	Rho	16
ζ	Z	Zeta	5	σ	Σ	Sigma	17
η	H	Eta	6	τ	T	Tau	18
θ	Θ	Theta	7	υ	Y	Upsilon	19
ι	I	Iota	8	ϕ	Φ	Phi	20
κ	K	Kappa	9	χ	X	Chi	21
λ	Λ	Lambda	10	ψ	P	Psi	22
μ	M	Mu	11	ω	Ω	Omega	

Appendix II

Preparations of solutions and reagents

17 1. DULBECCO'S PHOSPHATE BUFFERED SALINE (PBS)

17.1 1.2 EQUIPMENT, MATERIALS AND REAGENTS

- Equipment: Stir Plate; Analytical Balance; Pump for Filtration
- Materials: Weigh Boats; Chemical Spatula; Pasteur pipettes; Stir Plate; 1000 ml Graduated Cylinder; 1000 ml Autoclaved Amber Bottle; 500 ml Bottle Top Filter (0.22 micron Cellulose Acetate; Low Protein Binding Membrane; Sterilizing; Corning Inc Cat# 430513); 10L Carboy or Storage Container
- Reagents:
- KCl (potassium chloride); J.T. Baker Cat# 3040-01
 - KH_2PO_4 – monobasic, 99% (potassium phosphate);
 - Na_2PO_4 – 99+% (sodium phosphate);
 - NaCl – 100.2% (sodium chloride);

17.2

17.3 1.3 METHOD, PROCEDURES AND REQUIRMENTS

1. Measure out 9 L of ultra clean grade water (*e.g.* nanopure) into a carboy or storage container. (**Note:** As an alternative to the 10 L volume described here, smaller amounts of PBS buffer can be made with appropriate adjustments to the amounts of reagents used.)
2. Place the carboy on a stir plate and place the stir bar in the carboy. Turn on the stir plate.
3. Add 2.0 g of KCl, 2.0 g of KH_2PO_4 , 11.5 g of Na_2PO_4 , and 80 g of NaCl to the carboy. (**Note:** Add the chemicals slowly and wait for them to dissolve before proceeding. Remember to rinse any traces of chemical out of the weigh boat and into the solution using nanopure water.)
4. Bring the total volume up to 10 L with ultra clean grade water.
5. Adjust the pH to 7.4 using 10M HCl (if the pH needs to be decreased) or 10M NaOH (if the pH needs to be increased). (**Note:** Add the HCl or NaOH one drop at a time (using a Pasteur pipette) as the high concentration will change the pH considerably.)
6. Prior to use in the cell culture, sterilize the buffer by autoclaving or filtration using a sterile 0.22 micron pore bottle top filter in a 1L amber autoclaved bottle.
7. Label the bottle as follows:
 - PBS
 - H295R Cells
 - “Filter Sterilized” (only for the work solution to be used with cells)
 - pH = 7.4
 - Preparer’s initials
 - Expiration date
8. Store the buffer at room temperature.
9. PBS can be used for up to 6 months, after that new PBS buffer should be made. (**Note:** Alternatively, Dulbecco’s Phosphate Buffered Saline 10x, Modified, Without Calcium Chloride and Magnesium Chloride, Liquid, Sterile-Filtered, Cell Culture Tested can be Purchased from Sigma (Cat # D-1408).)

18 2 STOCK AND SUPPLEMENTED MEDIUM

2.1 EQUIPMENT, MATERIALS AND REAGENTS

- Equipment: Biosafety Cabinet; Stir Plate; Analytical Balance; Pump for Filtration

- Materials:** Weigh Boats; Chemical Spatula; Pasteur pipettes; Stir Plate; 1000 ml Graduated Cylinder; 1000 ml Autoclaved Amber Bottle; 500 ml Bottle Top Filter (0.22 micron Cellulose Acetate; Low Protein Binding Membrane; Sterilizing; Corning Inc Cat# 430513)
- Reagents:**
- Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham; Sigma Cat # D-2906; stored at 2-8°C (equals a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DME) and Ham's F-12 Nutrient mixture in 15 mM HEPES buffer without phenol red and sodium bicarbonate)
 - Na₂CO₃ (sodium bicarbonate);
 - ITS+ Premix; BD (What is BD?) Bioscience Cat # 354352; stored at 2-8 °C; stable for at least 3 months (the premix contains insulin, transferrin, selenium, bovine serum albumin (BSA) and linoleic acid)
 - BD Nu-Serum; BD Bioscience Cat # 355100 (=100 mL) or 355500 (=500 mL); stored at -20°C; stable for at least 3 months; upon arrival BD Nu-Serum should be aliquotted into 13 mL sterile storage vials under sterile conditions before freezing.

Final concentrations of components in supplemented medium:

- 15 mM HEPES buffer
- 6.25 ug/mL insulin
- 6.25 ug/mL transferrin
- 6.25 ng/mL selenium
- 1.25 mg/mL BSA
- 5.35 ug/mL linoleic acid
- 2.5 % Nu-Serum

2.2 METHOD, PROCEDURES AND REQUIRMENTS

Note: Due to possible varying hormone concentrations in different batches of Nu-Serum, each new serum batch should be tested for background hormone concentrations prior to use, and for completeness reasons background concentrations of T and E2 should be documented. . Furthermore, the same batch of Nu-Serum should be used for each set of experiments.

Stock Medium (1L):

1. Place approximately 900 mL of ultra clean grade water (*e.g.* nanopure) into the 1000 ml graduated cylinder.
2. Place the stir bar in the graduated cylinder and place the graduated cylinder on the stir plate. Turn the stir plate on.
3. As the water stirs add one bottle of DME/F-12 powder (bottle size for 1 L).
4. Rinse the empty DME/F-12 bottle with ultra clean grade water to remove all traces of chemical and add this to the solution once the initial powder added is totally dissolved.
5. Be sure to rinse the medium stuck on the sides of the graduated cylinder down into the solution using ultra clean grade water.
6. Add 1.2 g of sodium bicarbonate (Na₂CO₃) and rinse the weigh boat with water to remove all traces of chemical.
7. Adjust the pH of the solution to 0.1- 0.3 below the desired final pH of 7.4 with 1N HCl or 1N NaOH (whichever appropriate).
8. Bring the final volume of the solution up to 1000 ml using ultra clean grade water.
9. Filter the medium using a sterile 0.22 micron pore bottle top filter into an amber autoclaved bottle. This should be done in the bio-safety cabinet because to maintain the sterility of this solution.
10. Label the bottle as follows:
 - DME/F-12 Stock Medium

- H295R Cells
 - “Filter Sterilized”
 - pH = 7.4
 - Preparer’s initials
 - Date
 - Expiration date
11. Medium should be stored at 4°C.
 12. Stock medium can be used for up to 3 months, after that new medium should be made.

Supplemented Medium (500 ml):

1. Thaw one vial (~13mL) BD Nu-Serum using either a 37°C water bath or incubator.
2. Allow the vial with 5 mL ITS+ premium mix to equilibrate to room temperature – swirl gently to mix content.
3. Place an autoclaved 500 mL graduate cylinder in the bio-safety cabinet, and add 450 mL of stock medium.
4. Add 5 mL of the ITS+ premium mix and 12.5 mL of the BD Nu-Serum to the cylinder containing the 450 mL of stock medium, and bring the total volume up to 500 mL.
5. Filter the medium in the bio-safety cabinet into an autoclaved 500 mL amber bottle using a 0.22 micron bottle top filter.
6. Label the bottle as follows:
 - DME/F12 Supplemented Medium
 - H295R Cells
 - “Filter Sterilized”
 - pH = 7.4
 - Preparer’s initials
 - Date
 - Expiration date
7. Medium should be stored at 4°C.
8. Supplemented mediums can be used for up to 3 weeks, after that new supplemented medium should be made.

19 3 FREEZE MEDIUM

3.1 EQUIPMENT, MATERIALS AND REAGENTS

- Equipment: Biosafety Cabinet
- Materials: 10 mL Strippettes; 15 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052); Autoclaved 100 mL Pyrex Bottle;
- Reagents: Sterile Stock Medium (see chapter 4.2); Dimethyl Sulfoxide (DMSO) (A.C.S Reagent Baker Analyzed Cat# 9224-01); BD Nu-Serum (see chapter 4.2).

3.2 METHOD, PROCEDURES AND REQUIRMENTS

1. All work is to be conducted under sterile conditions in a bio-safety cabinet.
2. The freeze media is made with H295R media supplemented with 7.5% Nu-Serum and 5% Dimethyl Sulfoxide (DMSO).
3. Add 7.5 mL Nu-Serum and 5.26 mL DMSO to 92.5 mL H295R stock medium in a sterile 100 mL Pyrex bottle.
4. Mix well and aliquot freeze media into 15 mL centrifuge tubes (about 12 mL per tube).
5. Label the tubes as follows:

- Freeze Medium
 - H295R Cells
 - “Filter Sterilized”
 - Preparer’s Initials
 - Expiration date
6. Store at –20 °C until needed.
 7. Freeze medium can be stored for up to 6 months.

20 4 TRYPSIN 1X

4.1 EQUIPMENT, MATERIALS AND REAGENTS

<u>Equipment:</u>	Biosafety Cabinet; Pipet-Aid
<u>Materials:</u>	10 mL Strippettes; 15 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052); 500 ml Bottle Top Filter (0.22 micron Cellulose Acetate; Low Protein Binding Membrane; Sterilizing; Corning Inc Cat# 430513); 100 mL Autoclaved Pyrex bottle
<u>Reagents:</u>	Trypsin-EDTA 10X (Life Technologies Inc. Cat # 15400-054); Sterile PBS (see chapter 4.1)

4.2 METHOD, PROCEDURES AND REQUIRMENTS

1. All work is to be conducted under sterile conditions in a bio-safety cabinet.
2. Filter 10X Trypsin-EDTA with a 0.22 micron filter and transfer 10 mL of the filtered solution into 15 mL polystyrene tubes.
3. Store unused filtered 10X trypsin at -20°C until needed.
4. Place 10ml of filtered 10X Trypsin-EDTA into a sterile 100 mL Pyrex bottle.
5. Bring the volume up to 100 mL using sterile PBS.
6. Aliquot the newly made 1X Trypsin into 15 mL polypropylene tubes.
7. Label the tubes with:
 - Trypsin 1X
 - Date
 - Preparer’s initials
 - Expiration date
8. Store in the freezer at -20 °C until needed.
Maximum storage time is 6 months

5. CONTROL AND TEST CHEMICAL SOLUTIONS

5.1 EQUIPMENT, MATERIALS AND REAGENTS

<u>Equipment:</u>	Pipet-Aid, Stir Plate; Analytical Balance
<u>Materials:</u>	Weigh Boats; Chemical Spatula; Pasteur pipettes; Waste Container, 2,10, 200 and 1,000 µL Pipettes, Sterile Pipette Tips, Sterile Amber Glass Vials (National Scientific Company; Cat# C4000-2W)

Reagents & Chemicals:

- Dulbecco’s Phosphate Buffered Saline (PBS) *
- Stock Medium *
- Supplemented Medium *
- Sterile 1x Trypsin *
- DMSO (Aldrich, Cat. No. D2438)
- Forskolin (MW = 410.50); 4.1050 mg

- Prochloraz (MW = 376.67); 3.7667 mg
- Test Chemicals

* The preparation and storage conditions for Dulbecco's Phosphate Buffered Saline (PBS), Stock and Supplemented Medium, and trypsin stock are described in detail in sections 1, 2 and 4 of this annex..

5.2 METHODS AND PROCEDURES

1. Prepare 100 mM stock concentrations of forskolin, prochloraz and test chemicals dosing solutions in DMSO. Weigh out the appropriate amount of forskolin (4.1050 mg), prochloraz (3.7667 mg) and test chemicals (MW x 0.00001) in tared vials. Add 100 μ L of DMSO to the forskolin, prochloraz and all test chemical vials, cap and vortex to dissolve each substance in the DMSO. This results in 100 mM stock solutions for forskolin and prochloraz. For all test chemicals this results in the Stock 1 Test Solution.
2. Dilute these stock solutions as follows:
 - *Forskolin:* Dilute 100 mM stock solution 1:10 (10 μ L of 100 mM Stock 1 + 90 μ L DMSO) to make 100 μ L of a 10 mM solution. Dilute 10 μ L of this 10 mM solution 1:10 to make 100 μ L of a 1 mM solution.
 - *Prochloraz:* Dilute 100 mM stock solution 1:10 (10 μ L of 100 mM Stock 1 + 90 μ L DMSO) to make 100 μ L of a 10 mM solution. Dilute 10 μ L of this 10 mM solution 1:10 to make a 1 mM solution. Dilute 10 μ L of this 1 mM solution 1:10 to make a 0.1 mM solution.
 - *Test Chemicals:* Dilute Stock 1 1:10 (10 μ L of 100 mM Stock 1 + 90 μ L DMSO) to make 100 μ L of Stock 2 solution. Continue diluting these stock solutions in similar manner with DMSO until a total of seven (7) dilutions have been made (Stock 1 – Stock 7). Dilutions should be made serially from the next greater concentration (e.g. a Stock 4 would be made by adding 10 μ L of a Stock 3 to 90 μ L of DMSO).
3. Label each vial as follows:
 - *Forskolin & prochloraz:* Chemical name, date the solution was made, concentration of chemical in mM, type of solvent (DMSO) and preparers initials.
 - *Test chemicals:* Chemical ID number, date the solution was made, Stock identifier (e.g. Stock 1), type of solvent (DMSO) and initial.
4. Store the stock solutions at 4°C.

Appendix III

Supplemental procedures

20.1 1.0 STARTING CELLS FROM THE FROZEN STOCK

20.1.1 1.1 EQUIPMENT, MATERIALS AND REAGENTS

<u>Equipment:</u>	Biosafety Cabinet; Pipet-Aid; Centrifuge
<u>Materials:</u>	10 mL Strippettes; Waste Container; 15 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052); 100 mm x 20 mm Cell Culture Dish (treated; polystyrene, non-pyrogenic; sterile; Corning Inc Cat# 430167) or Cell Culture Flask 75 cm ² (treated; polystyrene, non-pyrogenic; sterile; Corning Inc Cat# 430641)
<u>Reagents:</u>	Sterile Supplemented Medium (see chapter 4.2); H295R cells (ATCC Cat # CRL-2128)

1.2 METHOD, PROCEDURES AND REQUIRMENTS

1. Aliquot 10 mL of the supplemented medium to a 15 mL centrifuge tube. Use one centrifuge tube with medium for each H295R vial that is being thawed.
2. Remove a vial of H295R cells from liquid nitrogen storage.
3. Thaw the vial rapidly by agitation in a 37°C water bath or using the warmth of hands. Remember to be very careful, the vials are extremely cold and could cause damage to skin if held in one position for too long. Thawing should be rapid (within 40-60 seconds). As soon as the ice is melted, remove the ampoule/vial from water bath and wipe it down with 70% ethanol at room temperature and transfer to a bio-safety cabinet. All following steps should be done under aseptic conditions.
4. In the bio-safety cabinet, pipette the thawed cell solution into the medium that was aliquoted in step 1. (**Note:** The thawed cell solution should be placed into the aliquoted medium as quickly as possible. If the cells remain in the freezing medium for too long the viability will be poor.)
5. Centrifuge the cell suspension at 125g for 10 min, discard the supernatant and re-suspend the cells with 12 mL of supplemented media by gentle swirling of the tube.
6. Transfer the cell suspension to a 100 mm x 20 mm culture plate (further referred to as “plate”). (**Note:** Culture flasks can also be used as an alternative to culture plates. However, the amount of medium will need to be adjusted if using a different size plate/flask with a different surface area.)
7. Label the plate with:
 - Cell Type
 - Date
 - Initials of the person thawing the cells
 - The plates will be given a plate designation after their survival is assured.
8. Change the medium the next day (see following chapter on “Maintaining and Sub-culturing the Cells” for the description of H295R cell maintenance and medium change).

2.0 MAINTAINING AND SUB-CULTURING THE CELLS

2.1 EQUIPMENT, MATERIALS AND REAGENTS

<u>Equipment:</u>	Biosafety Cabinet; Pipet-Aid, Incubator (37°C, 5% CO ₂)
<u>Materials:</u>	10 mL Strippettes; 100mm x 20mm Cell Culture Dish (treated; polystyrene, non-pyrogenic; sterile; Corning Inc Cat# 430167); Waste Container; 15 and 50 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052 [15 mL] and 430290 [50 mL])
<u>Reagents:</u>	Sterile Supplemented Medium (see chapter 4.2); Sterile 1x Trypsin- EDTA (see chapter 4.4); Sterile PBS (see chapter 4.1)

2.2 METHOD, PROCEDURES AND REQUIRMENTS

H295R cells are cultivated in an incubator at 37°C with 5% CO₂ in air atmosphere.

Medium Renewal - Renew medium 2-3 times weekly:

1. Pre-warm supplemented medium to 37°C in a water bath or incubator.
2. Wipe all tubes/containers off with 70% ethanol and transfer cell culture plates and supplemented medium into a bio-safety cabinet.
3. Carefully pipette old medium off the culture plate without disturbing cells.
4. Add 12 mL of fresh supplemented medium to the plate.

Splitting of Cells - Split the cells when they are close to confluence (about 90% confluent):

1. Warm PBS and supplemented medium to 37°C in a water bath or incubator
2. Thaw and warm 1x Trypsin-EDTA at 37°C in a water bath or incubator.
3. Wipe all tubes/containers off with 70% ethanol and transfer cell culture plates and solutions into a bio-safety cabinet.
4. Measure 15 mL of PBS for each plate to be split into a 50 mL centrifuge tube (e.g. 45 mL for three plates; use 2nd tube for 4-6 plates, etc.).
5. Carefully pipette old medium off the culture plate without disturbing cells.
6. Rinse each plate with 5 ml of sterile PBS, and discard PBS. Rinse a total of 3 times. Make sure to change pipette tips between each rinsing.
7. Add 1.5 mL of sterile 1x trypsin/plate and gently swirl plate to distribute trypsin evenly (volume should be adjusted in accordance with plate/flask size).
8. Wait for the cells to detach from the bottom of the plate. **(Note: (i), Plate can be placed in the incubator if the cells do not separate easily, (ii) Remember that the trypsin will kill the cells if left on for too long.** Watch the cells closely and stop the trypsin action as soon as the cells have separated from the plate/flask (typically this should not take longer than 4-5 minutes). **Cells should not be exposed to trypsin for more than 10-15 minutes!**
9. Stop the trypsin action with 10.5 mL of supplemented medium (once again the volume will need to be adjusted for a different sized plate/flask).
10. Place the appropriate amount of cell solution in the new plate/flask. The amount of cell solution should be adjusted so that the cells are confluent within 5-7 days. The recommended sub-cultivation ratio is 1:3 to 1:4.
11. Label the plate with:
 - Cell Type
 - Date
 - Initials of the person splitting the cells
 - Unique identifier code containing ATCC Lot number, Freeze Down ID, Passage #, Total # of Passages since original ATCC cell batch was started in cell culture, Plate ID (see chapter 6 and APPENDIX I: Cell Culture Diagram)
 - Plate designation

3.0 FREEZING H295R CELLS (PREPARING CELLS FOR LIQUID NITROGEN STORAGE)

3.1 EQUIPMENT, MATERIALS AND REAGENTS

Equipment: Biosafety Cabinet; Pipet-Aid; Controlled Rate Freezing Container (1 Degree C Freezing Container; Nalgene Cat# 5100-0001); Centrifuge, Liquid Nitrogen Tank

Materials: 10 ml Strippettes, Sterile Cryrogenic Vials (Polypropylene, Biohit Inc. Cat# 4503-1); Waste Container, 15 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052)

Reagents: Supplemented Medium (See chapter 4.2); Sterile 1x Trypsin- EDTA (see chapter 4.4); Freeze Medium (see chapter 4.3); Sterile PBS (see chapter 4.1)

3.2 METHOD, PROCEDURES AND REQUIRMENTS

1. Follow the procedure for splitting cell lines (chapter 5.2) through step nine in the method (stopping of trypsin action with 10.5 mL supplemented medium).
2. Pipette all of the cell solution into a sterile 15 ml centrifuge tube that is labelled with the plate identifier code.
3. Centrifuge tube with cells for 5 minutes at 350g at room temperature.
4. Upon removal from the centrifuge there should be a pellet of cells in the bottom of the centrifuge tube. If not, spin again under the same conditions.
5. Pipette off the supernatant and place it into the waste container in the bio-safety cabinet. (**Note:** Be sure not to suck up the pellet. If this does happen, re-suspend the pellet in the medium and spin down in the centrifuge again.)
6. Re-suspend the pellet of cells in 1 ml of the appropriate cell freezing medium
7. Transfer the solution to a sterile cryogenic vial and label with:
 - Cell Type
 - Date
 - Initials of the person freezing the cell line down.
 - Unique identifier code containing ATCC Lot number, Freeze Down ID, Passage #, Total # of Passages since original ATCC cell batch was started in cell culture, Plate ID (see chapter 6 and APPENDIX I: Cell Culture Diagram)
8. Place the vial(s) into the controlled rate freezing container.
9. Put the container into a -80°C freezer for 24 hours.
10. After 24 hours in the freezer, transfer to liquid nitrogen for storage.
11. The storage in liquid nitrogen vapor phase is recommended instead of having the cryogenic vials submerged in the liquid nitrogen fluid.

4.0 PLATING AND PRE-INCUBATION OF CELLS

4.1 EQUIPMENT, MATERIALS AND REAGENTS

Equipment: Biosafety Cabinet; Pipet-Aid; Centrifuge; Microscope

Materials: 10 mL Strippettes; Waste Container; 15 and 50 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052 [15 mL] and 430290 [50 mL]); 24-well Tissue Culture Plates (Corning Inc. Costar Cat# 524); Sterile 1.5 mL Eppendorf tubes; 10 μ L, 100 μ L, 1 mL Pipettes; Sterile Pipette Tips; Hemocytometer

Reagents: Sterile Supplemented Medium (see H295R culture protocol, chapter 4.2); Passage 4.5 to 10.5. NCI-H295R cells (ATCC Cat # CRL-2128) cultured under standard conditions as described in the H295R culture protocol; Sterile 1x Trypsin- EDTA (see H295R culture protocol, chapter 4.4); Sterile PBS (see H295R culture protocol, chapter 4.1)

4.2 METHODS, PROCEDURES AND REQUIREMENTS

1. Pre-warm PBS and supplemented medium to 37°C in a water bath or incubator.
2. Thaw and pre-warm 1x Trypsin-EDTA at 37°C in a water bath or incubator.
3. Wipe all tubes/containers off with 70% ethanol and transfer cells and solutions into a bio-safety cabinet.

4. Remove a H295R cell culture plate cultured under standard conditions as outlined in the H295R culture protocol from incubator and place in bio-safety cabinet. (**Note:** The number of cell culture dishes that will be needed for an experiment depends on the number of plates needed for the exposure experiment and the confluency of the cells in the culture dishes. . General rule: use 1 cell culture dish (100 mm) of 80-90% confluent cells to plate two 24-well plates at a target density of 200,000 to 300,000 cells per mL of medium.)
5. Prepare centrifuge tube or small sterile glass bottle with about 11 mL media for every plate to be trypsinized.
6. Measure 15 mL of PBS for each plate to be split to a 50 mL centrifuge tube (e.g. 45 mL for three plates; use 2nd tube for 4-6 plates, etc.).
7. Carefully pipette old medium off the culture plate without disturbing cells.
8. Rinse plate with 5 mL of sterile PBS, and discard PBS. Rinse a total of 3 times. Make sure to change pipette tips between each rinsing. **Note:** Rinse gently to avoid detaching cells from the plate by adding PBS down the side of the well.
9. Add 1.5 mL of sterile 1x trypsin/plate and gently swirl plate to distribute trypsin evenly (volume should be adjusted in accordance with plate/flask size).
10. Wait for the cells to detach from the bottom of the plate. (**Note:** (i), Plate can be placed in the incubator if the cells do not separate from the plate easily. Also, need to be careful not to knock or shake plate to avoid clumping of cells'(ii), remember that the trypsin will kill the cells if left on for too long. Watch the cells closely and stop the trypsin action as soon as the cells have separated from the plate/flask (typically this should take not longer than 4-5 minutes). **Cells should not be exposed to trypsin for more than 10 minutes!**
11. Harvest the trypsinized cells and transfer them into the centrifuge tube or bottle with medium.
12. Thoroughly mix (aspirate using 10 mL stripette) the cell suspension of medium and trypsinized cells to make the content homogenous. (**Note:** This is important for accurate cell counts because the cells tend to clump.)
13. Take a small sub-sample (30-50 μ L) of the well-mixed cell suspension and transfer to an Eppendorf tube.
14. Clean the hemocytometer and the cover glass with 70% Ethanol. Add 10 μ L of the cell solution from the Eppendorf tube under the cover glass in the hemocytometer. Count the cells – at least 3 squares from each side of the hemocytometer. Calculate the mean of all the counts. The cell density is the mean cell count $\times 10^4$. For example if the mean count is 110, there are 1,100,000 cells/mL = 1.1×10^6 cells/mL
15. Calculate the volume of cell solution needed for the selected number of 24-well plates (calculate 1.2 mL media/well).
16. Cells need to be seeded at 300,000 cells/mL medium resulting in approximately 50-60% confluency in the wells.

Note: This is the preferred density of cells for optimal hormone production in the medium. At higher densities cells tend to be affected by hormonal feedback mechanisms and T as well as E2 production patterns are altered.

17. Dilute the cell solution to the desired plating density. Thoroughly mix the cell solution to assure homogenous cell density.

18. Plate the cells with 1 mL of the cell solution/well.

19. The new plates should be labelled with the cell type, preparer's initials and plating date. Individual wells (samples) should be labelled with sample name.

Note: All of the above steps with the exception of steps 14 & 15 (counting of cells) need to be conducted under sterile conditions in a bio-safety cabinet.

20. Incubate seeded plate in incubator at 37°C under a 5% CO₂ in air atmosphere for 24 hours.

5.0 CONDUCT OF CHEMICAL-HORMONE ASSAY INTERFERENCE TEST

Prior to initiation of the actual exposure experiments, each chemical has to be tested for potential interference with the hormone measurement system being utilized. This is of particular relevance for antibody based assays such as ELISAs and RIAs because it has been previously shown that some chemicals can interfere with these tests (Shapiro and Page, 1976). This "chemical interference test will be conducted as described in Section 6 of this appendix for the analysis of medium samples. Prior to extraction and analysis, chemical spiked medium needs to be prepared as follows.

5.1 EQUIPMENT, MATERIALS, AND REAGENTS

Equipment: Biosafety Cabinet; Pipet-Aid, Vortex

Materials: 10 mL or 25 mL Strippettes; Waste Container; 15 or 50 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052 [15 mL] or 430290 [50 mL]); 200 µL and 1000 µL Pipettes; Sterile Pipette Tips, Eppendorf Tubes (1.5 mL)

Reagents:

- Sterile Supplemented Medium (see H295R culture protocol, chapter 4.2)
- DMSO (Aldrich, Cat. No. D2438)
- Test chemical stock solutions (serial dilutions 1 – 6; see chapter 4.1)
- Testosterone (Sigma Cat# T1500)
- 17beta-Oestradiol (Sigma Cat# E1024)
- Ethanol (95%)

5.2 METHOD, PROCEDURES AND REQUIREMENTS

1. Prepare Stock Solutions:

1. Dissolve 1 mg of T and E2 each in 1 mL of 95% ethanol in Eppendorf tubes (**Primary Hormone Stocks**).
2. Dilute 10 µl of **Primary Hormone Stocks** from previous step each in 990 µl of a supplemented medium-ethanol solution (75 % EIA / 25 % ethanol) and vortex (**Secondary Hormone Stocks**)

3. Dilute 10 μ l of *Secondary Hormone Stocks* from previous step each in 990 μ l supplemented medium and vortex (*Tertiary Hormone Stocks*).
 4. Dilute 100 μ l of *Tertiary Hormone Stocks* from previous step each in 900 μ l supplemented medium and vortex, resulting in a hormone stock concentration of 10 ng / mL (*Final Hormone Stock Solution*).
2. Prepare four Eppendorf Tubes per test chemical for initial interference test.
 3. Add 999 μ L of supplemented medium to tubes 1 and 2 and 779 μ L of supplemented medium to tubes 3 and 4.

Add *Final Hormone Stock* Solutions, Test Chemical Stock Solution 1 (greatest concentration), and DMSO to the tubes as indicated below in Table A.

4.

Table A: Chemical-Hormone Interference Test*

	Tube 1	Tube 2	Tube 3	Tube 4
Supplemented Medium	999	999	779	779
T Final Hormone Stock	---	---	200	200
E2 Final Hormone Stock	---	---	20	20
Test Chemical Stock 1	---	1	---	1
DMSO	1	---	1	---

* All measurements are in micro liters (μ L)

5. Vortex thoroughly for >15 seconds.
6. Extract medium supplemented with test chemical and test chemical plus hormone stock solutions produced in previous steps and analyze for hormones as described in the section 7 of this Annex.

Note: If significant interference of chemical occurs as determined by hormone analysis (significant interference is defined as $\geq 20\%$ of basal hormone production for T and/or E2) the Chemical Hormone Assay Interference Test has to be repeated for all test chemical stock solution dilutions to identify the threshold dose at which significant interference occurs.

6.0 LIVE/DEAD® CELL VIABILITY/CYTOTOXICITY ASSAY

The *LIVE/DEAD* Viability/Cytotoxicity Kit (Molecular probes, Eugene OR, USA, Cat # L-3224) gives a simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability, intracellular esterase activity and plasma membrane integrity. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity as determined by the enzymatic conversion of the virtually non-fluorescent cell-permeate calcein AM to the intensely fluorescent calcein. The poly-anionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (Ex/Em ~ 495 nm/ ~ 515 nm). Ethidium homodimer 1 (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a

bright red fluorescence in dead cells (Ex/Em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually non-fluorescent before interacting with cells.

The purpose of this section is to provide a consistent format for cytotoxicity testing with the H295R assay using a *LIVE/DEAD* Viability/Cytotoxicity Kit. The cytotoxicity testing will be conducted in the chemical exposure plate and should be conducted immediately after termination of the exposure experiments.

The methods described here have been optimized based on the use of 24 well plates and the Fluoroskan Ascent plate reader.

6.1 EQUIPMENT, MATERIALS AND REAGENTS

Equipment: Vortex; Microscope; Eppendorf Multipipette; Plate reading fluorometer (note: this protocol has been tested with the Fluoroskan Ascent Fluorometric Microtiter Plate Reader (Thermo Electron Corporation))

Materials: 100-1000, 20-200, 2-20 and 0.5-2 \square Pipettes, Pipette Tips; 15 and 50 mL Centrifuge Tubes (polypropylene; sterile; plug seal cap; Corning Inc Cat# 430052 [15 mL] and 430290 [50 mL]); 24-well Tissue Culture Plates (Corning Inc. Costar Cat# 524) seeded at 300,000 cells per well with H295R cells and exposed for 48 hours

Reagents:

- Cell viability assay reagents; sold either as a kit from Molecular Probes (#L-3224; Eugene, OR) or as individual components:
 - Calcein AM (Molecular Probes #C-3100); MW = 994.87; made up as 4000x (2 mM) stock (50 μ g/12.56 μ L DMSO)
 - Ethidium homodimer I (Molecular Probes #E 1169); MW = 857; made up as 2000x (1 mM) stock in DMSO
- Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (PBS; see chapter 4.1.1)

6.2 METHOD, PROCEDURES AND REQUIREMENTS

Preparation steps (prior to assay)

1. Inspect plates visually with and without microscope - check degree of confluence, homogeneity from well-to-well, and any signs of cytotoxicity or altered morphology. Note all observations in laboratory notebook.
2. The assessment of cell viability using the above methods is a relative measurement that does not necessarily exhibit linear relationships with the absolute number of cells in a well. Therefore, a subjective parallel visual assessment of each well by the analyst should be conducted, and digital pictures of the SCs and the two greatest non-cytotoxic concentrations are to be taken and archived to enable later assessment of true cell density if this should be required.
3. Prepare viability assay reagent (refer to Supplies and Biochemicals Section): Each plate will need 16 mL (24 wells * 600 μ L) plus a little extra (1.6 mL). Dilute the appropriate amounts of calcein and Ethidium with the appropriate volume of of PBS with Ca^{2+} and Mg^{2+} :

Calcein stock (μ L): 0.5 μ L/mL

Ethidium bromide stock (μ L) 2.0 μ L/mL

CAUTION: Ethidium bromide homodimer is a powerful mutagen - handle with care and throw contaminated tips, etc., into biohazard bags.

4 Set up fluorometric plate reader following the manufacturer's specifications.

6.3 CELL VIABILITY ASSAY PROCEDURE (PROCESS ONE PLATE AT A TIME)

1. Remove plate from incubator and remove media for subsequent hormone analysis or extraction as described in Section 6 of this appendix and add 300 μ L of PBS with Ca^{2+} and Mg^{2+} . Then rinse 1 time with 300 μ L of PBS with Ca^{2+} and Mg^{2+} . **Note:** Remove medium from not more than 4 wells at a time and fill with PBS with Ca^{2+} and Mg^{2+} before removing medium from second set of wells to prevent cells from drying.
2. **QC plate only:** Remove the PBS from wells A4-6 and B4-6 and add 300 μ L methanol to these wells, and allow standing at room temperature for 30 minutes.
3. Remove the methanol and rinse those wells three times with 300 μ L PBS with Ca^{2+} and Mg^{2+} . Rinse the balance of the wells once with 300 μ L PBS with Ca^{2+} and Mg^{2+} . Leave the last 300 μ L rinse on the cells.
4. Add 600 μ L of viability assay reagent to all wells.

IMPORTANT CONSIDERATION: Since the staining is progressive up to at least 3 to 4 hours it is important to space the staining of each plate to the time it takes to read the fluorescence.

5. Incubate at room temperature for 1 hour (cover the plate with lid to prevent evaporation).
6. Scan plate in fluorometer using the above described settings.
7. Export/print data (check that values are appropriate, otherwise adjust sensitivity and rescan). Data analysis is discussed in Section 6.1.3 along with an example raw data file and a Microsoft Excel spreadsheet version of a sample data analysis.

6.4 DATA ANALYSIS

Average the three measurements for calcein AM and ethidium bromide. Divide the average calcein AM fluorescence for each sample by its ethidium bromide homodimer fluorescence to obtain a live to dead ratio. Graph the average calcein AM fluorescence and standard deviation for the negative control, solvent control, and each concentration tested. Examine the calcein AM data visually. If the blank has greater viability than the other treatments, the solvent may be toxic to the cells. If viability is adversely affected in the least dose and continues to decrease with increasing concentration of the test substance, the test substance may be toxic to the cells. In either of these cases, the hormone data should be regarded with great suspicion. If the solvent is toxic, try a different solvent or a lower concentration of solvent. If the test substance is toxic conclude that cytotoxicity is likely to preclude any other effects at this test concentration.

7.0 HORMONE EXTRACTION FROM MEDIUM

7.1 EQUIPMENT, MATERIALS, AND REAGENTS

Equipment: Vortex; Scintillation Counter (Beckman Coulter- LS6500 Multi-purpose SC); Nitrogen Evaporator

Materials: 100-1000, and 2-20 μ L Pipettes, Pipette Tips; Glass Scintillation Vials (Research Products International Corp. Cat#-211000); Glass Test Tubes (> 5 mL) with caps

Reagents:

- Scintillation cocktail Bio-Safe II (Research Products International Corp Cat# 111195)
- Ether, Anhydrous (J.T. Baker Cat# 9244-22)
- Testosterone(1,2,6,7-3H(N))-1mCi (PerkinElmer Cat# 370001)

7.2 METHOD, PROCEDURES AND REQUIREMENTS

1. Label two glass test tubes for each medium sample to be extracted.
2. Pipet 450 μ L medium into the test tube.
3. Spike medium samples with 10 μ L of $^3\text{H-T}$ (concentration = 0.0002 $\mu\text{Ci}/\mu\text{L}$) to test for extraction recoveries. (At this low concentration the radio-labeled hormone spike will be measurable in a liquid scintillation counter, but will not affect the end result of the hormone concentration in the ELISA test).
4. Briefly vortex medium samples after spiking.
5. Add 10 μ L of the ^3H -labeled hormone to a liquid scintillation vial containing 4 mL of scintillation cocktail (this tube is used as a reference for the calculation of DPM of the “DPM spike tube” used later for the derivation of extraction efficiencies (see paragraph 29 of the Test Guideline).
6. Bring all medium samples to 1 mL with nanopure water.
7. Add 2.5-mL ether to each test tube, and cap. Be careful not to remove test tube label in the process.
8. Vortex each tube for a minimum of 1 minute to allow the water and ether layers to mix.
9. Allow the ether and water fractions to separate or centrifuge for 10 min at 2,000 rpm. Carefully collect the ether fraction (supernatant) into a new test tube using a glass pipet without disturbing water fraction (*Alternative method*: Prepare a dry ice-acetone bath in a fume hood by placing several large chunks of dry ice in a large glass beaker and filling with acetone. Hold plasma samples in the dry ice bath for approximately 30 sec or until water fraction is completely frozen. Then collect non-frozen ether fraction as described above.)
10. Re-extract original water fraction by adding 2.5 mL ether to the tube, cap, vortex, and centrifuge as above.
11. Collect ether fraction into the same tube containing first ether fraction.
12. Wash ether with 1 mL nanopure water to remove hydrophilic contaminants that may be present. Cap, vortex, and centrifuge as above, and transfer ether to new glass vial. (**Note**: May not be necessary. Can be omitted if laboratory can demonstrate that there is no difference in analytical results between washed and non water treated samples).
13. Evaporate the ether fractions to dryness under a stream of nitrogen.
14. If samples are to analyzed immediately, reconstitute in 250 μ L assay buffer that is provided with each ELISA kit and vortex. If analyses are to be conducted at a later date, cap vial and store dry at -80°C for up to 8 weeks.

15. Remove 10 μL of the assay buffer extract from each sample and place in a liquid scintillation vial containing 4 mL of cocktail.
16. Test for extraction efficiency by running ^3H -labeled spike, ^3H -labeled plasma and a blank sample (10 μL of assay buffer) extracts in the liquid scintillation counter.
17. After reconstitution of the extract either use sample within 24 hrs in the ELISA (see next section) or freeze at $-80\text{ }^\circ\text{C}$ until further processing (maximum storage time of reconstituted samples should not exceed 4 weeks).
18. Calculate recoveries from scintillation counter readings (CPM) as follows:

$$\% \text{ recovery} = \frac{(\text{CPM}_{\text{Sample}} - \text{CPM}_{\text{Blank}}) \times 25}{(\text{CPM}_{\text{Spike}} - \text{CPM}_{\text{Blank}})} \times 100$$