Final Report

Development of an Assay Using the H295R Cell Line to Identify Chemical Modulators of Steroidogenesis and Aromatase Activity

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Submitted to:

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Offices of Science Coordination & Policy
1200 Pennsylvania Avenue, NW
Washington, DC. 20460

Tentative submission date:
May 2006

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1.0 EXECUTIVE SUMMARY

This report summarizes the work completed to date on Project No. GS-10F-0041 "Development of an Assay Using the H295R Cell Line to Identify Chemical Modulators of Steroidogenesis and Aromatase Activity". In the proposal, of the six Tasks that were identified, Tasks 1 and 2 have been completed while work is still being conducted on Task 3. In Task 3, there were 29 subtasks identified relative to the development and validation of the H295R assays relative to using the production of sex hormones as endpoints. These subtasks were reorganized into 13 specific tasks or "protocols" that combined the subtasks into groups that address similar issues in the protocol development and validation. A listing of each task and subtask along the status and/or conclusions for each work product is given (Table 1.1).

Currently, Tasks 1 and 2 have been completed and significant progress has been made to complete the subtasks that have been outlined for Task 3. Specifically, commercial and ATL-MSU ELISA kits have been evaluated and as a result, ELISA kits have been selected the measure of testosterone, estradiol, and progesterone in H295R cell media. In addition, the media extraction methods as well as cell culture methods have been optimized (subtasks 2 and 3). These methods have been used to determine basal hormone production in H295R cell media in different plate designs and over time (subtask 7). Pre-testing of cell media from previous chemical exposures has been completed (subtask 8) and the results show that chemicals can both increase and decrease the production of hormones by H295R cells. As a result, work is ongoing (subtask 11) to characterize the dose and time response relationships of model chemicals to alter the production of hormones by H295R cells. The results from these experiments are also being use to finalize all exposure procedures (subtask 11) that will be used to develop the final SOPs (subtask 6). This work should be completed by the end of November. Once completed, test chemical evaluations (subtask 12) will be initiated. The work completed to date has been summarized in a Power point presentation that was developed to be presented to the OECD. Overall, significant progress has been made on all phases of this project and the initial results indicate that exposure of this cell line to chemicals can result in measurable increases and decreases of testosterone, progesterone and estradiol.

Table 1.1. Tasks and current status of work completed for Project No. GS-10F-0041 "Development of an Assay Using the H295R Cell Line to Identify Chemical Modulators of Steroidogenesis and Aromatase Activity" [a]

Task	Description	Status	Conclusion
1	Quality management/Quality Assurance Plan	Completed	Report sent to EPA
2	Protocol Development	Completed	Report sent to EPA
3	Method Testing and Refinement		
	Subtask 1. Evaluation of ELISA Kits	Completed	Commercial ELISA kits and MSU-ATL kits evaluated to measure testosterone, estradiol, and progesterone.
	Subtask 2. Optimization of Extraction Methods	Completed	Extraction method optimized for all three hormones
	Subtask 3. Cell Culture Optimization	Completed	Cell culture conditions optimized
	Subtask 4. Development of GC/MS Analytical Methods	Ongoing	Method has been selected and established. Validation of method is ongoing.
	Subtask 5. Validation of ELISA Test Kits	Completed	ELISA kits for each hormone were selected and validated in terms of precision, accuracy and reproducibility.
	Subtask 6. SOP Preparation	Ongoing	Initial preparation of SOPs for cell culture and exposure, hormone extraction, and ELISAs
	Subtask 7 Evaluation of Basal Hormone Production	Completed	Basal production of testosterone, estradiol, and progesterone has been characterized
	Subtask 8. Model Compound Pre-testing	Completed	First chemicals have been tested and evaluated for all three hormones.
	Subtask 9. H295R Cell Viability/Cytotoxicity Evaluation	Ongoing	Testing conducted for each chemical prior to hormone evaluation tests
	Subtask 10. Optimization of Exposure Conditions (Model chemicals)	To be initiated	
	Subtask 11. Model Compounds: Dose and Time Response Experiments	Ongoing	First chemicals have been tested and results evaluated for all three hormones
	Subtask 12. Test Chemical Evaluations	To be initiated	
	Subtask 12. Statistical Analysis	On going	Data analyzed after each test/experiment
4	Determine the Specificity and Intra-laboratory Variability of Assay	To be initiated	
5	Demonstrate Transferability of the Assay	To be initiated	
6	Presentations and Final Report	Ongoing	Preparation of OECD presentation

[A] No all work completed for each Task and Subtask has been included in this data report but will be submitted in subsequent reports.

2.0 OVERVIEW OF PROGRESS IN H295R HORMONE STUDY

This report summarizes the work that has been completed that evaluates the utility of the H295R cell line as an *in vitro* assay for to screen for potential effects of chemicals on steroidogenesis. The H295R cell system is a (a subpopulation of H295 that forms a monolayer in culture) human adrenocortical carcinoma cell line that has the ability to express most of the key enzymes necessary for steroidogenesis. In addition, the cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells, with the ability to produce the steroid hormones of each of the three phenotypically distinct zones found in the adult adrenal cortex. Specifically, the focus of this project is to develop and test a screening protocol based on the H295R cell line that measures the end production of steroid hormones, testosterone (T), 17 β-estradiol (E2), and progesterone (P). The assay will integrate possible effects on many parts of the steroidogenic pathway including:

1. Steroidogenic signal transduction,
2. Transport of cholesterol from the cytoplasm to the mitochondria by steroid acute regulatory protein,
3. Conversion by various enzymes of cholesterol to testosterone, namely:
    - P450 cholesterol side-chain cleavage enzyme (P450scc)
    - 3β hydroxysteroid dehyrogenase/Δ5-4 isomerase (3β HSD)
    - P450 17α-hydroxylase/C_{17-20} lyase (P450c17)
4. Conversion of testosterone to estrogen by aromatase.

As part of this effort, work has been initiated to optimize culture conditions so that the H295R cells are a sensitive reporter system for any of these effects on steroidogenesis. Concentrations of the three steroid hormones were measured by Enzyme-Linked-Imuno-Sorbent-Assay (ELISA). The project is on track to be completed on time. Specifically the project has:

Tasks Completed:

1) Developed an optimized protocol for an assay using the H295R cell line that can detect interference with the production of progesterone and testosterone from cholesterol and with 17β estradiol from testosterone. This phase of the project has tested a number of ELISA methods to measure the steroid hormones. The assay methods have been optimized and the validated. A standard operating procedure has been developed for the selected methods. The optimized methods were then used to determine the minimum detectable concentrations and variability of the assay. In addition, the level of release of the three hormones to the medium was determined under normal culture conditions. The completion of Phase I has resulted in a well characterized system that can be used to complete the next phases of the project.
2) An initial study, using several model compounds with known mechanisms of action was completed to prove the concept of using the H295R cells as a model screening system. The results of this study have indicated that the H295R system will be useful for this purpose.

Tasks Remaining

1) Obtain data demonstrating the performance of the assay with known stimulators and inhibitors of steroidogenesis and substances that are known to up- or down-regulate enzymes in the steroidogenic pathway,
2) Demonstrate transferability of the protocol and obtain preliminary measures of variability of the assay within the same laboratory and between laboratories.

Progress to date that has been completed in Phase I of the project includes the characterization of several commercial ELISA kits for the analysis of testosterone, estradiol and progesterone. Characterization of each ELISA kit included an evaluation of the accuracy, precision and sensitivity of the assays to detect hormones in H295R cell medium. This work has resulted in the selection of ELISA kits that will be used to further characterize the production of steroid hormones in H295R cell medium. In

addition, work has been initiated to investigate the effect of chemical exposure on the production of steroid hormones by H295R cells.

Work conducted to optimize and validate H295R cell protocols included:

1. Identified several commercial sources of ELISA test kits to measure steroid concentrations in the medium of H295R cells.
2. Tested the accuracy, precision, and sensitivity of ELISA kits with cell culture medium spiked with known amounts of steroid hormones. The results of this work have been used to select ELISA kits that will be used in future assays to develop and characterize the production of steroid hormones by H295R cells.
3. Tested different plate designs and cell exposure times to identify optimal conditions for the conduct of chemical exposures.
4. Tested effect of chemical on steroid hormone production by H295R cells under standard culture conditions.

Actions initiated within study plan:

1. Used established cell culture protocols to generate cell medium for use in the selection and validation of steroid hormone ELISA kit assays.
2. Identified commercial sources of ELISA kits for the quantitation of steroid hormones.
3. Characterized the accuracy, precision and sensitivity of ELISA kits to measure testosterone, estradiol, and progesterone in H295R cell medium.
4. Initiated work to identify optimal plate layouts for possible synchronization of experiments to quantify changes in hormone production with alterations of steroidogenic gene expression in H295R cell exposed to chemicals.
5. Initiated work to develop and validate a GC/MS/MS to quantify steroid hormones in cell medium. This work will be used to validate ELISA results.
6. Initiated work to characterize the temporal production of steroid hormones in cell medium from H295R cells under standard culture conditions. Work has also been started to evaluate the production of steroid hormones in medium from exposed cells.

7. Initiated several preliminary tests to measure the concentrations of testosterone, estradiol, and progesterone in H295R cell that had been exposed to chemicals. This initial work was conducted to show that chemicals can alter the production of steroid hormones but the assays have not yet been completely validated.
8. Preparation of a presentation for the EPA of work completed to date on the development and optimization of the H295R cell assay to screen of endocrine disrupters. This presentation will also include the results of several chemical exposures that demonstrate the potential of this assay to detect changes in hormone production.

Ongoing Actions:

1. Final optimization of E2 ELISA assay conditions. The Oxford Biomedical Research procedure has been selected as the E2 quantification method but additional work is needed to determine method detection limits that will be used in assays to characterize the effect of chemicals on E2 production.
2. Continue evaluation of H295R assay conditions including microtiter plate design, exposure times and appropriate dose selection. Some of this work has been completed but new studies have been initiated to better characterize temporal changes in hormone production under basal and exposed conditions.
3. Development of a list of model and test chemicals to be used in validation studies. This list will be completed in consultation with EPA and will be compared to a list of chemicals that have already been tested with H295R cells as part of the steroidogenic gene expression studies.

3.0 EXPERIMENT 1A

3.1 Protocol

Experiment #: 1A **Date:** 5/1/-9/16/04 **Expt. Leader:** MM/MH

Title: Background hormone concentrations in culture medium for H295R cells.

Questions: What are the background concentrations of testosterone (T) and estradiol (E2) and progesterone (P) in culture medium for H295R cells?

Experimental Design:

Cell culture medium (stock medium and supplemented medium) will be tested for the presence of the steroid hormones T, E2 and progesterone. In addition, a sub-sample of the stock and supplemented medium will be striped over activated charcoal to remove any possible steroid hormone residues from the solutions prior to analysis. The four different medium types will be extracted and analyzed for their concentrations of T, E2 and progesterone using commercially available ELISA test kits. In addition, an ELISA method developed at the Aquatic Toxicology Laboratory (SOP253) will also be used to measure T. To determine steroid recovery rates from unused medium, both stock and supplemented medium will be spiked with E2, T and progesterone prior to extraction. Concentrations of each hormone will be calculated parallel to the linear portions of the standard curves. Three to four different steroid concentrations will be tested. Appropriate statistical methods will be used to evaluate differences in hormone concentrations and assay variabilities.

Methods:

Cell culture medium will be prepared as outlined in ATL-SOP (Culturing of the H295R human adrenocortical carcinoma cell line (ATCC CLR-2128)). Supplemented medium will consist of stock medium + Dulbecco's modified Eagle medium nutrient mixture F-12 Ham and Ham's F-12 Nutrient mixture. Activated charcoal (1% of total medium volume) will be added to stripe the medium of possible steroid hormone residues. Extraction of medium will be conducted following the procedures described in ATL-SOP

253. Hormone ELISAs will be performed as outlined by the manufacturers or in ATL-SOP 253.

Statistics:

Descriptive statistics (averages, +/- SD, CV's); Comparison of hormone concentrations in different types of medium using basic parametric tests.

Expected Outcome:

As the supplemented medium contains low amounts of fetal FBS, it cannot be excluded that this medium contains measurable residues of T, E2 and progesterone. These hormones should not be detectable in stock medium. Striping with activated charcoal will remove any hormone residues from the test solution. Hormone spikes will be detected accurately and depending on the concentration spiked into medium, recovery rates will be between 70 and 130% percent of nominal concentrations. Low spike concentrations are likely to be less precise since they will be near the MDL while the higher spike concentrations will be more precise.

3.2 Summary of Results

Two commercial ELISA kits and one ELISA developed and validated at Michigan State University Aquatic Toxicology Laboratory (MSU-ATL) were evaluated relative to the detection and quantitation of Testosterone and estradiol in the medium of H295R cells. In addition, two commercial kits were evaluated relative to their ability to detect and quantitate progesterone in H295R cell medium. The results of these tests are summarized below while the raw data sheets and Excel spreadsheets can be found in Appendix A.

3.2.1 Testosterone

Testosterone was detectable at low concentrations in both the supplemented and stock medium using the Cayman and OB kits. However, with the SOP253 kit, T concentrations were not detectable in most of the extracts from both types of medium (Table 3.1). T concentrations in the medium that were measured by the OB ELISA kit were less than those determined by the Cayman assay. In addition, supplemented culture

medium had slightly greater T titers than the non-supplemented stock medium. Stripping with activated charcoal significantly reduced T concentrations in supplemented medium (t-test, p=0.014). The greatest average concentrations of T was measured in supplemented medium using SOP253 and was 471 ± 268 pg/ml. Serial dilutions of the extracts resulted in T concentrations that were parallel to the linear range of the standard curve.

Table 3.1: Background T concentrations (mean ± sd) in supplemented cell culture medium (sup) and stock medium (stock).

		T concentration (pg/ml medium)			
Test	Date	Stock	Sup	cs[a] stock	cs[a] sup
Cayman	8/28/04	60.2 ± 14.4	129.9 ± 20.9	39.8 ± 4.6	37.8 ± 3.7
	9/1/04	211.5 ± 4.9	235.0 ± 56.7	n/a	n/a
	9/10/04	n/a	181.3 ± 16.7	n/a	n/a
OB	9/7/04	34.2 ± 9.0	119.1 ± 21.1	n/a	n/a
	9/10/04	n/a	85.3 ± 13.7	n/a	n/a
	9/24//04	n/a	102.6 ± 1.2	n/a	n/a
SOP253	8/28/04	nd	nd	nd	nd
	9/1/04	nd	nd	n/a	n/a
	9/7/04	nd	470.9 ± 267.8	n/a	n/a

[a] medium was charcoal striped before extraction
nd indicates not detected due to concentration being less than detection limit
n/a indicates not analyzed

The magnitude of variability observed in the recovery rates from the spiked samples was dependent not only on the assay kit used to measure T, but it also depended on the absolute amount of T spiked into the medium (Table 3.2). Recoveries of T from stock medium were consistently lower than those observed with supplemented culture medium. One likely reason for this difference was that the stock medium did not contain proteins, which may have facilitated the dissolution of hormones. In the stock medium, the absence of proteins may have resulted in an increased adsorption of hormone to the walls of the test vials and tubes used in the extraction and assays and thus, resulted in poorer recoveries. Most consistent recoveries of T were measured with the Cayman kit where samples spiked with T over the concentration range of 100 to 3000 pg/ml were close to nominal concentrations (Figure 3.1). However, 250 pg/ml spiked samples had recoveries that were distinctly greater than 100% (195.7%). Recoveries for T as determined by the OB kits were variable and inconsistent when the results of three separate experiments

were compared. However, in experiments conducted on 9/7/04 and 9/24/04, the measured concentrations of T in the spiked medium approximated the nominal concentrations with the greatest accuracy as compared to the other kits (Figure 3.2). While recoveries measured using SOP253 were close to nominal concentrations for the 500 pg/ml spike recoveries for the 3000 pg/ml spike were all greater than nominal and ranged from 110% (stock media) to 174% (supplemented media). In the supplemented medium, when the results from all test kits and all spiked concentrations are compared the CVs for most of the tests were less than 25%. CVs greater than 25% were only observed for background T concentrations in the stock and supplemented medium as determined by the SOP253 kit (56.9%).

Table 3.2: Average recoveries (%) of T spiked into supplemented culture medium (sup) and stock medium (stock) before extraction.

			Recovery (%)			
Test	Date	Medium	100pg/ml	250pg/ml	500pg/ml	3000pg/ml
Cayman	9/1/04	Stock	n/a	n/a	58.8%	52.5%
		Sup	n/a	n/a	119.9%	101.2%
	9/10/04	Sup	100.4%	195.7%	157.2%	134.7%
SOP253	9/1/04	Stock	n/a	n/a	115.2%	110.1%
		Sup	n/a	n/a	118.4%	171.5%
	9/7/04	Stock	n/a	n/a	78.4%	141.3%
		Sup	n/a	n/a	112.5%	173.0%
OB	9/7/04	Stock	n/a	n/a	65.3%	73.7%
		Sup	n/a	n/a	98.9%	111.3%
	9/10/04	Sup	110.7%	28.9%	7.0%	32.8%
	9/24/04	Sup	104.8%	114.1%	109.1%	105.9%

n/a indicates not analyzed

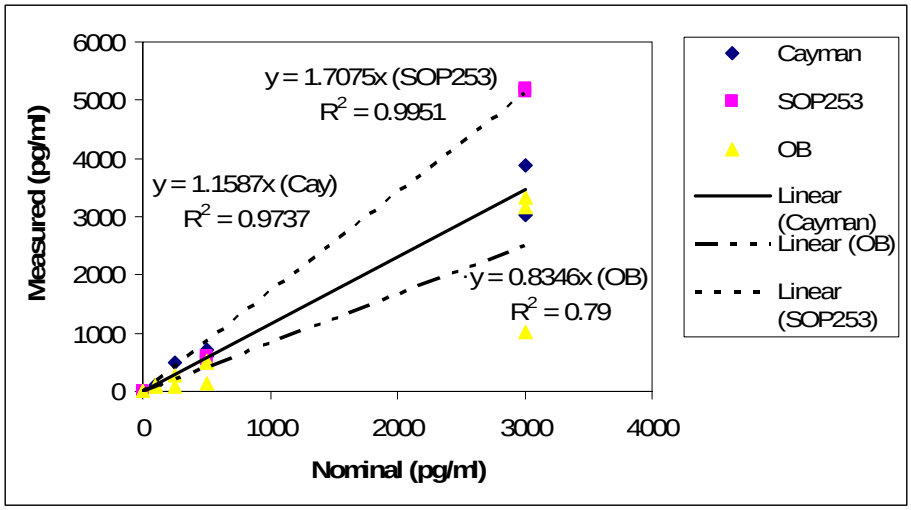

Figure 3.1: Linear regression of measured T concentrations in supplemented cell culture medium vs nominal T concentrations.

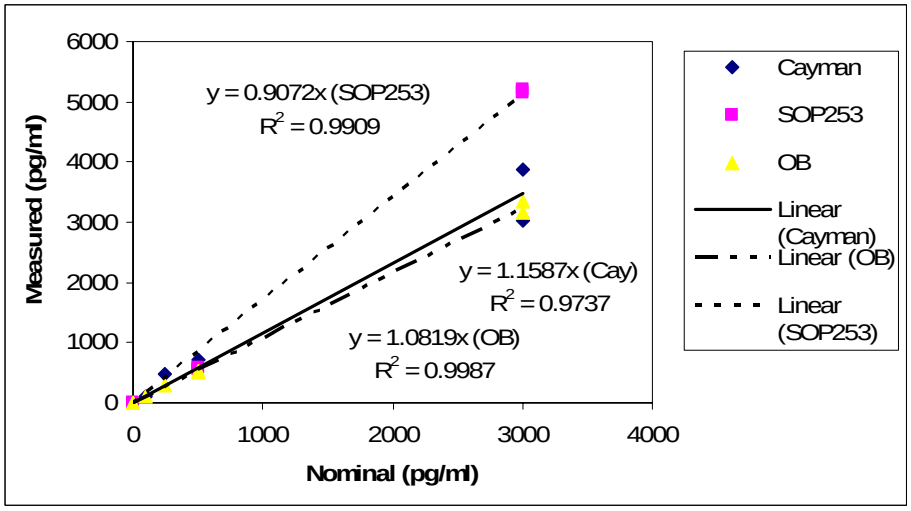

Figure 3.2: Linear regression of measured T concentrations in supplemented cell culture medium vs nominal T concentrations (without OB test 2 [9/10/04]).

3.2.2 Estradiol

Estradiol (E2) was detected at concentrations between 189 ± 43 pg/ml (SOP253) and 504 ± 101pg/ml (Cayman) in the stock medium (Table 3.3). There were no consistent differences between hormone concentrations in supplemented culture medium and stock medium as was observed for T. In addition, stripping of the medium with activated charcoal before extraction did not significantly reduce E2 concentrations. There were no marked differences in E2 hormone concentrations between the different kits.

Table 3.3: Background E2 concentrations (mean ± sd) in supplemented cell culture medium (sup) and stock medium (stock).

		E2 concentration (pg/ml medium)			
Test	Date	Stock	Sup	CS[a] stock	CS[a] sup
Cayman	8/28/04	504 ± 101	445 ± 56	351 ± 53	384 ± 40
	9/1/04	338 ± 65	327 ± 99	n/a	n/a
OB	9/7/04	311 ± 67	334 ± 94	n/a	n/a
SOP253	8/28/04	189 ± 43	285 ± 59	180 ± 26	214 ± 40
	9/1/04	397[b]	341[b]	n/a	n/a
	9/7/04	382 ± 93	581 ± 7	n/a	n/a

[a] Charcoal striped medium, prior to extraction
[b] only one data point, other dilutions were less than detection limit
nd indicates not detected due to concentration being less than detection limit
n/a is not analyzed

Recovery rates from samples spiked with different concentrations of E2 prior to extraction varied when the results obtained with the different assay kits were compared (Table 3.4). With the exception of the experiments conducted on 9/7/04 with SOP253 and the OB kits, recoveries from stock medium were consistently lower than those in determined in the supplemented culture medium. This phenomenon was also observed for testosterone and is likely due to the lack of proteins in stock medium that may have facilitated the dissolution of hormones in the cell medium. Except from SOP253 recoveries from the 1000 pg/ml spike of supplemented medium were relatively consistent among the different ELISA kits (Table 3.4). In contrast, recoveries from supplemented medium spiked with 100 pg/ml were highly variable and ranged between 69.6% and 367.9% (Table 3.4). When the results from all test kits and all spiked concentrations tested with supplemented medium were evaluated, the CVs in the majority of the tests were less than 25% (Table 3.5). The greater CVs (> 25%) associated with the measurement of E2 in supplemented samples were observed only in tests conducted with the Cayman kit (30%) and the OB kit (28%) whereas the results obtained with the SOP353 kit had CVs consistently less than 20%.

Table 3.4: Average recoveries (%) of E2 spiked into supplemented culture

medium (sup) and stock medium (stock) before extraction. [a]

Test	Date	Medium	Recovery (%)	
			100pg/ml	1000pg/ml
Cayman	9/1/04	Stock	10.3%	45.8%
		Sup	175.6%	93.5%
SOP253	9/2/04	Stock	-18.1%	85.83%
		Sup	88.7%	143%
	9/7/04	Stock	65.4%	183.4%
		Sup	69.6%	163.4%
OB	9/7/04	Stock	124.0%	99.6%
		Sup	367.9%	92.4%

[a] recoveries were based on nominal spiked concentrations

Table 3.5: Coefficients of Variation (CVs) for the spike experiments with the different test kits in supplemented culture medium (sup) and stock medium (stock).

Test	Date	O pg/ml spike		100pg/ml spike		1000pg/ml spike	
		sup	stock	sup	Stock	sup	stock
Cayman	9/1/04	30%	19%	11%	14%	18%	21%
SOP253	9/1/04	a	a	14%	a	19%	12%
	9/7/04	1%	24%	18%	11%	14%	10%
OB	9/7/04	28%	22%	17%	22%	15%	22%

[a] only one data point in the linear range of the assay => calculation of CV not possible

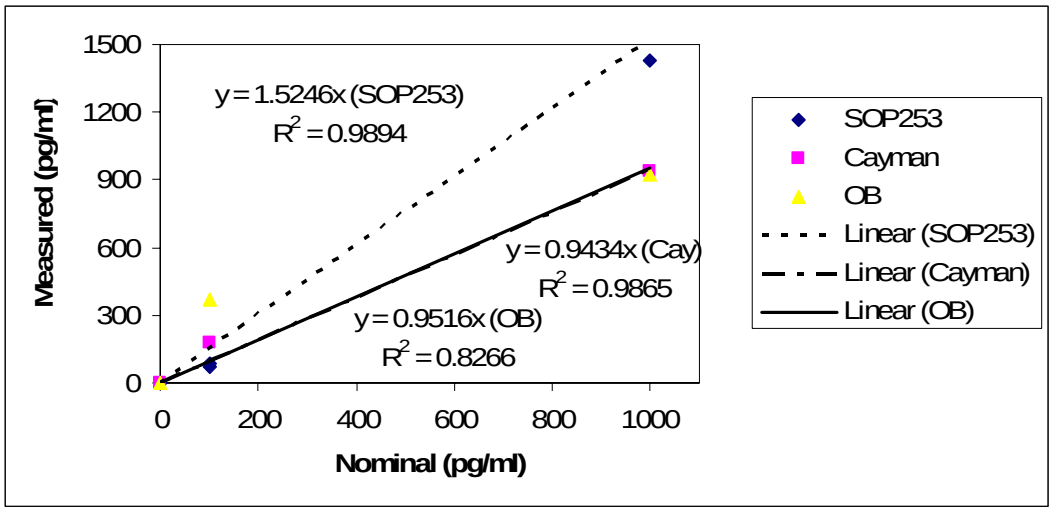

Figure 3.3: Linear regression of measured E2 concentrations in supplemented cell culture medium vs nominal E2 concentrations.

3.2.3 Progesterone

Except for the experiment conducted on 9/13/04, background progesterone concentrations measured by the Cayman test kit ranged between 38 and 81 pg/ml in the culture medium (Table 3.6). In contrast, progesterone concentrations determined in the supplemented medium on 9/13/04 with the Cayman kit were approx. 6- to 10-fold greater than those measured in tests conducted during all other experiments. Unlike T and E2, progesterone was not be detected in the stock or non-supplemented medium. In addition, progesterone was not detected in either stock or supplemented medium with the OB ELISA kit. CVs for progesterone concentrations in the supplemented medium ranged between 2.8 and 20.3%.

Table 3.6: Background progesterone concentrations (mean ± sd) in supplemented cell culture medium (sup) and stock medium (stock).

Test	Date	T concentration (pg/ml medium)			
		Stock	Sup	cs[a] stock	cs[a] sup
Cayman	9/2/04	nd	37.7 ± 5.4	8.3[b]	9.5[b]
	9/13/04	n/a	398.2 ± 11.2	n/a	n/a
	9/15/04	n/a	80.7 ± 16.4	n/a	n/a
	9/16/04	n/a	65.5 ± 3.3	n/a	n/a
OB	9/3/04	nd	nd	nd	nd
	9/16/04	n/a	nd	n/a	n/a

[a] medium was charcoal striped before extraction
[b] only one data point in the linear range of the assay
nd is not detected due concentrations being less than detection limit of the assay
n/a is not analyzed

Recovery rates of progesterone from medium were variable over spiked concentration range evaluated in this study as measured by the different kits (Table 3.7). While the recovery rates as determined with the OB kit over the spiked concentration range were highly variable (89-246%), there was a greater consistency in the recovery of the progesterone from the spiked samples with the Cayman that ranged from 82.5% to 157% over three different experiments. The only exceptions to this observation were seen in the two lowest spiked samples that were evaluated on 9/13/04. The relatively high background measured on 9/13/04 seemed to have had an influence on the recoveries at the lowest concentrations (-10% for the 250 pg/ml and 58% for the 500 pg/ml spiked

samples). However, in subsequent experiments, similar recoveries at these spiked progesterone concentrations were not observed in the medium samples. This result indicates that the results collected on 9/13/04 were not representative of the actual recoveries of progesterone and that recoveries were most likely much greater. In general, the recoveries of progesterone in spiked samples tended to overestimate progesterone concentrations regardless of the ELISA kits used to quantitate progesterone in the cell medium. In particular, the Cayman kit overestimated progesterone concentrations where on average, the measured concentrations were 125% of the nominal concentration with a range of 108 to 157%. In contrast, the OB kit was much more variable with recoveries ranging from 89 to 245% over the same spiked concentration range evaluated with the Cayman kit. Overall, there was good agreement between the measured and the nominal concentrations in the spiked samples evaluated with the Cayman kit (Figures 3.4 and 3.5). For the Cayman kits, most of the CV values were less than 21% with the exception of the 5000 pg/ml spike measured on 9/13/04 (CV=37.8%).

Table 3.7. Average recoveries (%) of progesterone spiked into supplemented culture medium (sup) before extraction. [a]

Test	Date	Medium	Recovery (%)			
			250pg/ml	500pg/ml	1000pg/ml	5000pg/ml
Cayman	9/13/04	Sup	-10.2%	58.0%	93.2%	82.5%
	9/15/04	Sup	121.3%	128.2%	156.8%	107.6%
	9/16/04	Sup	100.3%	140.6%	124.3%	122.4%
OB	9/16/04	Sup	193.7%	89.4%	114.0%	245.8%

[a] Recoveries were estimated based on spiked concentrations and adjusted for concentrations of progesterone in the medium.

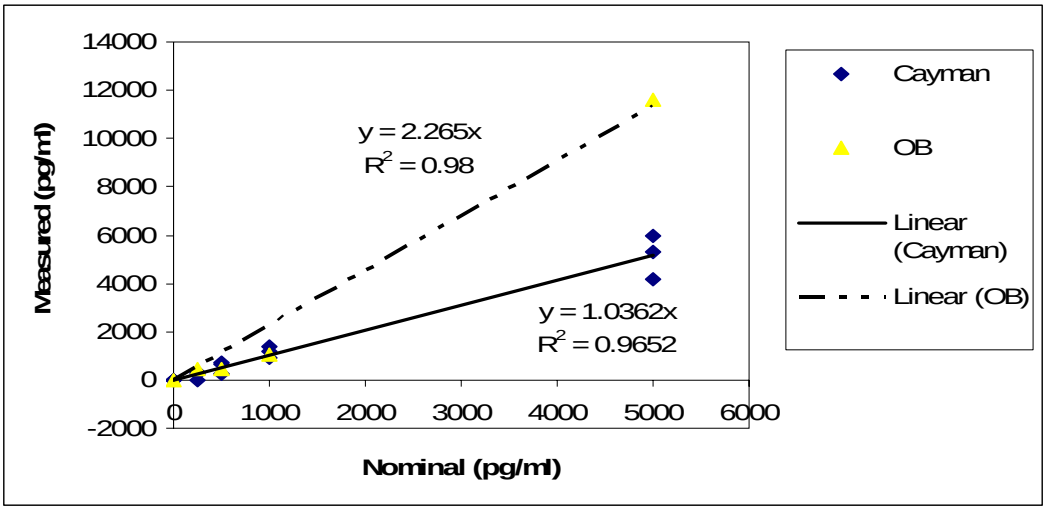

Figure 3.4: Linear regression of measured progesterone concentrations in supplemented cell culture medium vs nominal progesterone concentrations.

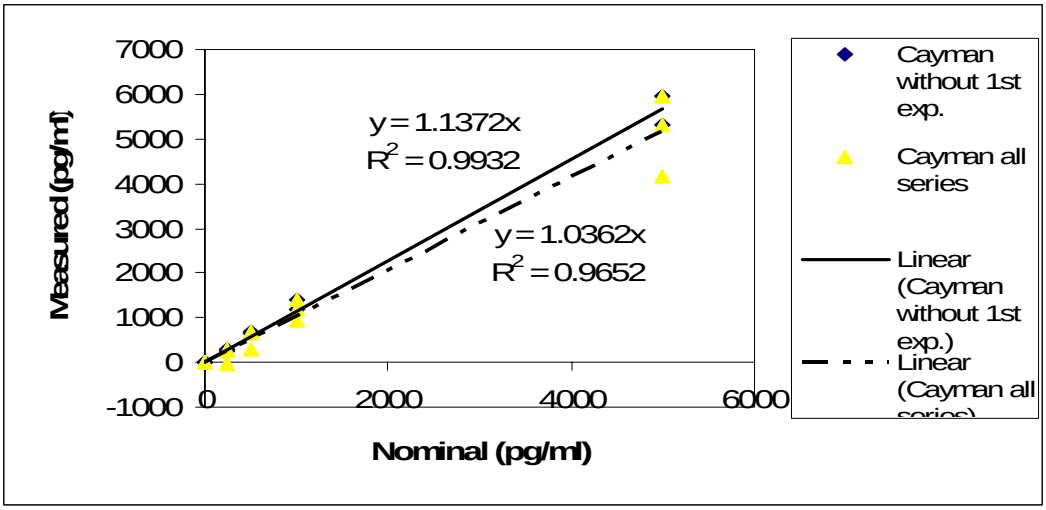

Figure 3.5: Linear regression of measured progesterone concentrations in supplemented cell culture medium vs nominal progesterone concentrations using the Cayman ELISA kit. Diamond data points: Results from experiments with low background progesterone concentrations; Triangular data points: Results are from all experiments.

3.3 Further Considerations:

3.3.1 Testosterone

Based on the results from tests conducted with both spiked and non-spiked medium, it was decided that the Cayman T ELISA kit gave the best results relative to accuracy and

precision and that it would used in further experiments to characterize the H295R hormone production. Of the kits tested, the Cayman kit had most consistent rates of recovery of testosterone from medium over the range of spike concentrations evaluated in the tests. However, it is important to note that if the results from Experiment 2 (9/10/04) with the OB kit are removed from consideration, the accuracy and precision of the OB kit was greater (slope=1.08; R^2=0.999) than that observed with either the Cayman or SOP253 kits. Concentrations as low as 100 pg/ml were detected with a high degree of precision with both the OB (Recovery =110.7%, CV=2.5%) and Cayman kits (Recovery =100.4%, CV=8.6%). These results indicate that both assays are sensitive and are capable of detecting small changes in testosterone production in the H295R cell medium. Although the Cayman ELISA tended to slightly overestimate absolute concentrations T in the medium, it was also able to determine the T concentrations with greater precision that was achievable with the two other kits (slope=1.16; R^2=0.974). While the accuracy and precision of the OB kit to measure testosterone in cell culture medium was similar to that observed with the Cayman kit, it was not selected due to the cost associated with the OB kit. Furthermore, the overall accuracy precision was not significantly greater than that observed with the Cayman kit. As a result, the Cayman kit was selected for future tests of the assay.

The low background T concentrations measured in supplemented culture medium was not considered a problem in the use of any ELISA assay because basal hormone production by H295R cells is at least 20-30 times greater than these concentrations (see experiments 3&4). As a result, changes in testosterone production by H295R cell that have been induced by exposure to chemicals can be measured over a wide range. Furthermore, based on the accuracy and precision of the ELISAs, it will be possible to evaluate small changes in hormone concentrations that can be used will be defined the suppression or induction of testosterone production. These parameters will be more fully characterized in future experiments with culture medium from H295R cells exposed to different chemical classes and concentrations.

3.3.2 Estradiol

To date, none the kits that were evaluated have measured E2 with sufficient accuracy and precision over the concentration ranges tested in supplemented H295R cell medium. This issue has been particularly problematic for tests conducted with low concentrations of spiked E2. For instance, while the recoveries from the 1000 pg/ml spikes were relatively precise and accurate as measured with the OB and Cayman ELISA kits, the recoveries from the 100 pg/ml spiked samples were highly variable (175-368%). In contrast, recoveries measured using SOP253 were relatively precise for the 100 pg/ml spikes (69.7 – 88.7%) but this kit overestimated nominal concentration at the 1000 pg/ml spike (143 – 163.4%). One reason for this problem at low spiked concentrations was that the background E2 concentrations measured in all extracted samples was relatively great (up to 581 pg/ml) when compared to basal E2 concentration measured in medium collected from cell cultures. This background was observed in all extracted samples including distilled water. The rationale for the additional effort to evaluate the causes for the high background is that basal concentration of E2 in the medium of H295R cells is only slightly greater than the measured background concentrations measured with the assay kits. That is, the basal concentrations of E2 in H295R culture medium is 961 ± 392 pg/ml (mean ± SD) as determined in the Cayman, OB and SOP253 kits. Thus, while it will be possible to measure a reduction in E2 production as compared to control levels, it might not be possible to establish dose-response relationships since the range of measured concentrations is relatively small and the variability relatively great. The causes for this high background are unclear and several studies have been initiated to determine and quantify potential sources of high background. As part of the effort to identify sources of the relatively high E2 backgrounds, additional experiments will be conducted and the results will be summarized in Experiment 1B. These experiments will address the following possible issues:

- Extraction material (glassware, caps, etc.)
- Extraction solvent
- Medium composition
- Standard curve (e.g. dilution of standards)
- ELISA kit materials (e.g. buffers)

3.3.3 Progesterone

Based on the test results, the Cayman ELISA was selected as the kit that will be used for the measurement of progesterone in future studies to characterize hormone production by H295R cells. With the exception of the experiment conducted on 9/13/04, the Cayman ELISA demonstrated its ability to measured low concentrations of progesterone in the culture (38-81pg/ml) with sufficient accuracy and precision. In contrast, the OB kit did not detect progesterone in the cell culture medium at levels that were detected by the Cayman kit. This is due to the fact that the method detection limit of the OB kit method detection limit was approximately a factor 40 greater than that of the Cayman kit. In addition, the recoveries measured in spiked samples were more variability in the OB kit making this ELISA less desirable as compared to the Cayman kit.

With the exception of the experiment conducted on 9/13/04, recovery rates in spiked samples were good over the concentration range tested (slope=1.14; R^2=0.993). Subsequent tests showed that the results from 9/13/04 were an exception in that the results of this test could not be replicated. The Cayman test kit detected progesterone concentrations as low as 250 pg/ml with a high precision (100 and 121%). However, the Cayman ELISA did tend to overestimate absolute progesterone concentrations but all concentrations were within an acceptable range for the assay (70 to 130% of nominal). Overall, the Cayman progesterone ELISA was classified as a highly precise test system that can be used to determine small changes in progesterone concentrations. Furthermore, this assay can operate over a wide progesterone concentration range in such that both induction and suppression of progesterone production by H295R cells can be investigated. The accuracy and precision will be further characterized in future experiments with culture medium from H295R cells exposed to several chemical classes.

4.0 EXPERIMENT 1B

4.1 PROTOCOL

Experiment #: 1B **Date:** 9/10/-9/24/04 **Expt. Leader:** MM/MH

Title: Background E2 concentrations in culture medium for H295R cells.

Questions: How can the relatively high background concentrations of E2 in culture medium for H295R cells be reduced?

Experimental Design:
Cell culture medium (supplement medium) will be extracted in different ways to determine possible contamination routes with E2 or cross-reacting compounds. To determine if the elevated backgrounds may be due to the composition of the medium nanopure water will be co-extracted with each batch of medium samples. Factors to be tested are as follows:

- Extraction process (general) -> charcoal strip samples after extraction, run non-extracted and extracted (not charcoal striped) nanopure water parallel in ELISA
- Medium -> charcoal strip medium before extraction
- Extraction materials:
    - Charcoal strip solvent
    - Use different solvents (ethyl ether and dichloromethane)
    - Rinse all glass- and lab-ware with acetone and hexane prior use with samples
    - Evaporate solvent under different gaseous conditions (N2, air stream in fume hood)
    - Use different types of lab-ware for extraction (e.g. leave out all plastic components)
- Change dilution factors -> use higher volumes of samples with same extraction conditions to dilute possible contamination

In addition to pure culture medium a parallel set of samples will be spiked with 500pg/ml E2 before extraction to determine extraction efficiency, precision and accuracy.
Extracts produced under the above conditions will be tested for contamination with E2 using commercially available ELISAs. Two different dilutions of each sample will be run per ELISA.

Methods:
Cell culture medium will be prepared as outlined in ATL-SOP (Culturing of the H295R human adrenocortical carcinoma cell line (ATCC CLR-2128)). For all striping experiments an activated charcoal solution of 1% of total test solution volume will be added. Extraction of medium will be conducted following the procedures described in ATL-SOP 253 and as described above. Hormone ELISAs will be performed as outlined by the manufacturers or in ATL-SOP 253.

Statistics:
Descriptive statistics (averages, +/- SD, CV's).

Expected Outcome:
As the supplemented medium contains low amounts of fetal FBS it cannot be excluded that this medium contains measurable residues of T, E2 and progesterone. These hormones should not be detectable in stock medium. Striping with activated charcoal will remove any hormone residues from the test solution.

Hormone spikes can be detected accurately and depending on the concentration spiked into medium, recovery rates will be between 70 and 130% percent of nominal concentrations. The low spike concentrations are likely to be less precise since these will be closer to the MDL and thus will be more variable; higher spike concentrations will be more precise.

4.2 Summary of Results

4.2.1 Evaluation of plastic and nanopure water:

Estradiol (E2) was detected in extracted nanopure water and in analyses conducted with supplemented medium that was extracted without plastic containers and caps (Figure 4.1). A statistical analysis (t-test) of E2 concentrations for the two sample types showed that there were not statistically significant differences between extracted nanopure water and supplemented medium assayed with out plastic ($p = 0.47$). In addition, charcoal stripping of the supplemented medium resulted in approximately a 29-fold reduction in measured E2 concentrations. In total, this result indicates that the source of the background E2 concentrations measured in the supplemented medium is not from contaminated water or from leaching of materials from plastic that interfere with the

ELISA. Rather, it most likely is an artifact of the extraction process. Furthermore, since charcoal stripping the extract removes the interferences, it is also likely that this interference is most like due to some type of organic compound that is not estradiol, but is able to either physically or chemical react with the ELISA. Finally, the average E2 concentration reported in the supplemented medium assayed without plastics was 298 ± 20 pg/ml was within the range of E2 concentrations that have been reported in other tests conducted in Experiment 1A with the SOP253 ELISA protocol. In these tests, the average E2 concentrations ranged from 285 to 581 pg/ml.

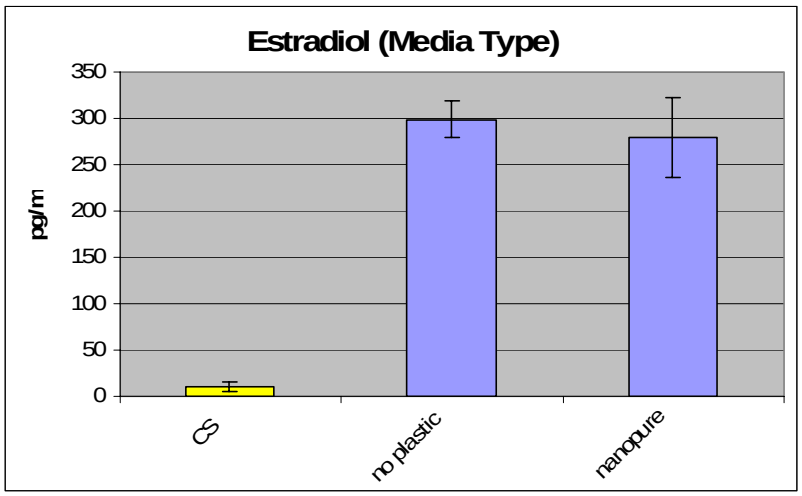

Figure 4.1. Comparison of estradiol (E2) concentrations in water, supplemented medium, and supplemented medium that was charcoal stripped.

4.2.2 Evaluation of Extraction Solvent

Extraction solvent did not have a significant effect on the measured E2 concentrations in either nanopure water or supplemented medium as determined by t-test comparisons (Figure 4.2). When nanopure water E2 concentrations were compared, concentrations in the ether extracts were approximately 6% greater than those extracted with dichloromethane (DCM) but these differences were not significant ($p = 0.34$). For medium, E2 concentrations in the DCM extracts were approximately 2% greater than ether extracts but also were not significantly different ($p = 0.71$). In a separate evaluation, nanopure water extracts were compared to supplemented medium extract E2 concentrations. As was seen for the other comparisons, the difference between nanopure water and medium extracts was not statistically significant for ether (5% difference; p=

0.27) extractions. In contrast, there was a significant difference between medium and nanopure water E2 concentrations (15% difference; p = 0.02) in samples extracted with DCM. However, this difference was not considered significant in that the concentrations observed for medium in both solvent extracts were within the range observed in other studies conducted with the Cayman ELISA kits. Finally, charcoal stripping reduced apparent E2 concentrations from both solvent extracts to levels less than the operating range of the standard curve. In conclusion, solvent type had no effect on the background E2 concentrations measured in either nanopure water or supplemented medium. In addition, the source of the background most likely is not due to estrogen or estradiol contamination in either the solvent or water used in the assay.

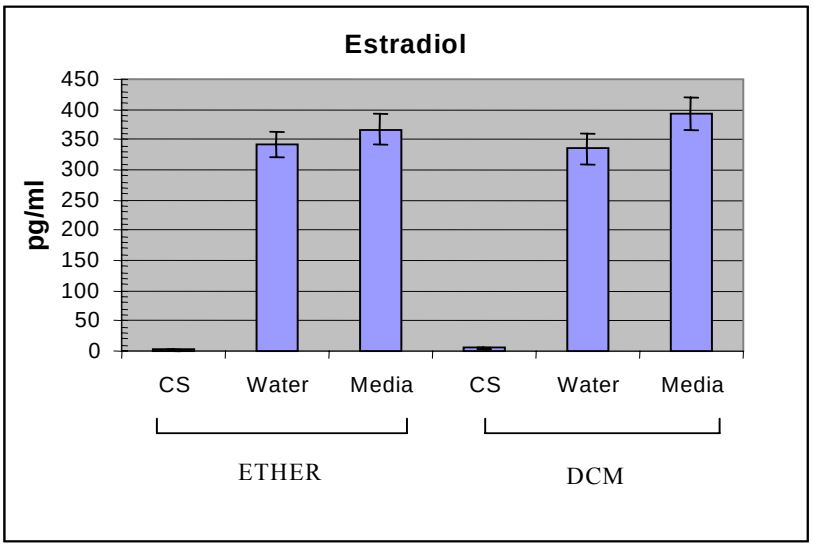

Figure 4.2. Measured estradiol (E2) concentrations in nanopure water and supplemented medium extracted with either diethyl ether (ether) or dichloromethane (DCM). CS indicates charcoal stripping of supplemented medium extract (CAY 9-15-2004).

4.2.3 Evaluation of Glass Treatment

Glassware as a potential source of apparent E2 contamination was examined by comparing new glassware used as outlined in standard protocols (SOP 253) with new glassware that was rinsed with acetone and hexane prior to use in the assay. When measured E2 concentrations from normal and solvent rinsed glassware were compared, no significant differences E2 concentrations were observed between the two glassware

treatments (Figure 4.3). A comparison between nanopure water, supplemented medium and supplemented medium spiked with 500 pg/ml E2 showed that the treatment of glassware did not have a significant ($p > 0.05$) effect on the measured E2 concentrations. Furthermore, a comparison between extracted nanopure water and medium E2 concentrations were not significantly different ($p > 0.05$). This result indicates that the background levels of the apparent E2 were not a result of contamination but most likely due to some other interaction with the solvent. As a result, glassware was determined not to be a source of the background E2 concentrations measured in nanopure water or the medium used in the culture of H295R cells.

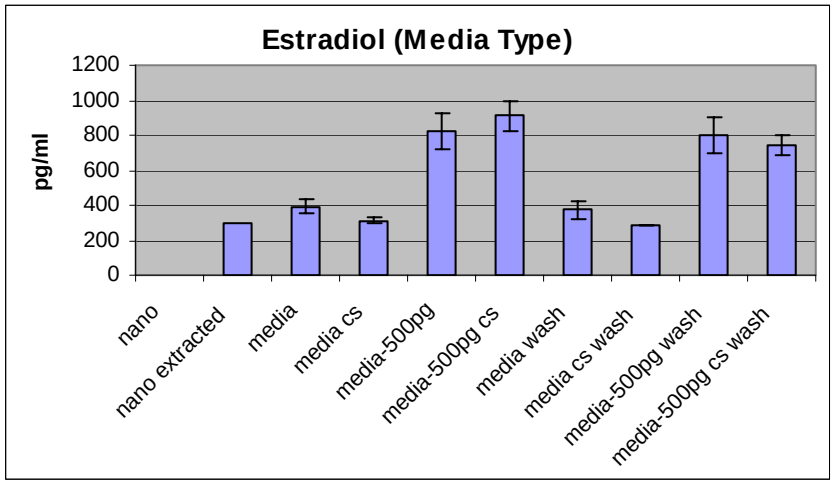

Figure 4.3. Comparison of E2 concentrations measured in nanopure water, supplemented medium and medium spiked with 500 pg/ml in washed and unwashed glassware. All glassware was new. Washed indicates that the glassware was rinsed with acetone and hexane prior to use in the assay (Cay 9-21-200).

4.2.4 Effect of Evaporation on E2 concentrations

Nitrogen gas was evaluated as a potential source of the E2 background in samples extracted with ether were either evaporated under nitrogen or air-dried prior to reconstitution with EIA buffer (Figure 4.4). For extracted nanopure water and medium, E2 concentrations in the air-dried samples were approximately 1.2-fold greater than those measured in the nitrogen evaporated samples. While these differences between the two treatments were significant ($p < 0.05$), it is important to note that the overall variability in

measured E2 concentrations for each sample and treatment was relatively low (CVs < 5%). As a result, small changes in E2 concentrations between treatments were found to be significantly different. This result is unlike that observed in other assays where the average CVs for E2 analysis have ranged from 10 to 25%. To further evaluate the effect of nitrogen evaporation of E2 concentrations, nanopure water was compared to supplement medium. For the nitrogen evaporated samples, supplemented medium E2 concentrations were approximately 1.1-fold greater than those measured in nanopure water. Again, while this difference was statistically significant ($p = 0.08$), the significance of this difference was due to very low variability (average CV =0.36%) for each sample. In the air-dried samples, there was 1.1-fold greater E2 concentration in the supplemented medium than nanopure water that was not statistically significant ($p > 0.05$). Overall, the results from this test indicate that nitrogen evaporation was not a source of the E2 background measured in extracted samples of nanopure water or cell medium.

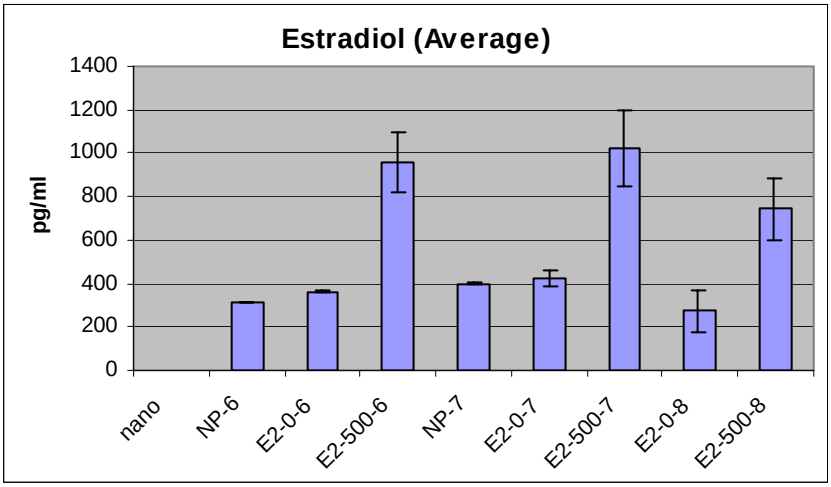

Figure 4.4. Concentration of estradiol in samples extracted with ether. NP indicates extracted nanopure water, E2-0 is supplemented medium and E2-500 is supplemented medium spiked with 500 pg/ml E2. Samples labeled with 6 indicate nitrogen evaporation while 7 indicates air-dried samples. (CAY 9-23-2004).

4.2.5 E2 Spiked Sample Recoveries

E2 concentrations were measured in nanopure water, supplemented medium, and supplemented medium spiked with E2 to characterize the accuracy and precision of the

SOP253 ELISA. Concentrations of E2 in extracted nanopure water and supplemented medium were 388 ± 46 and 475 ± 36 pg/ml, respectively indicating that E2 was absent or at very low concentrations in the H295R cell medium (Figure 4.5). In samples spiked with 250 or 500 pg/ml E2, concentrations measured in the supplemented medium were 907 ± 165 and 1188 ± 392 pg/ml, respectively. Concentrations of E2 measured in the 250 and 500 pg spiked samples represented E2 recoveries of 173% and 142%, respectively. These results indicate that recoveries tend to overestimate the spiked nominal concentrations for SOP253 (Figure 4.6).

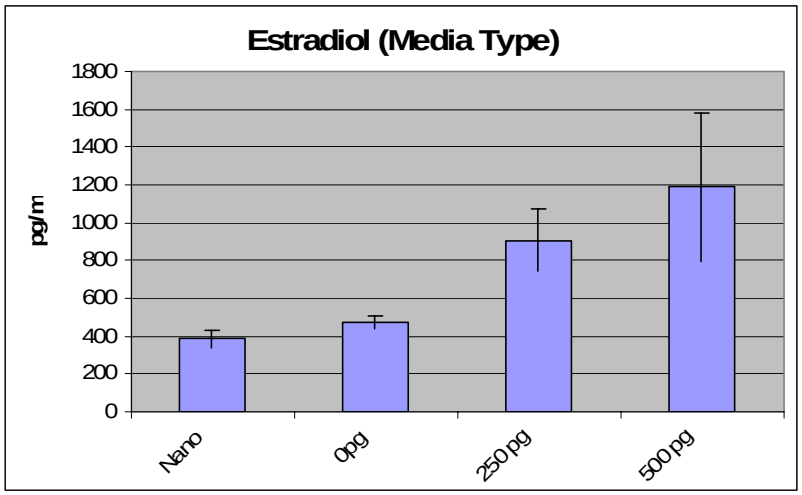

Figure 4.5. Concentration of estradiol (E2) in nanopure water, supplemented medium, and supplemented medium spiked with E2 (SOP253 10-1-2004).

Figure 4.6. Linear regression of measured estradiol (E2) concentrations in supplemented cell culture medium vs nominal E2 concentrations (SOP253 10-1-2004).

4.3 Further Approach:

Results for these tests indicate that the background E2 concentrations that have been measured in extracted samples is not the result of contamination, rather seems to be due to the extraction procedure itself that interferes with the ELISA assays. The use of plastic, glassware treatment or nitrogen evaporation had little or no effect on the measured E2 background. Furthermore, while charcoal striping of nanopure water or medium prior to extraction does not affect the background signal measured by the ELISA kits, charcoal striping after extraction removes up to 90% of the background values. As a result, additional studies would be needed to evaluate and characterize each ELISA kit to ascertain potential sources of this interference relative to the measurement of E2 in cell culture medium.

Based on the results obtained with the Cayman and SOP253 ELISAs, it was determined that further experiments will be required to optimize the assays as background media E2 concentrations could not be lowered by applying the above measures. However, future experiments will focus primarily on the OB and Cayman ELISA kits as both tests were highly precise at the 1000 pg/ml spike range and are commercially available and do not require laborious optimizations when set up de novo in a laboratory.

5.0 EXPERIMENT 2

5.1 Protocol

Experiment #: 2 **Date:** 7/6/04 **Expt. Leader:** LB

Title: Preparation of H295R medium for development of GC/MS and ELISA methods.

Questions: How are cells cultured and medium collected to provide sufficient material for subsequent method development?

Experimental Design:

Cells were cultured in 75 x 100 mm flasks according to cell culture ATL-SOP (Culturing of the H295R human adrenocortical carcinoma cell line (ATCC CLR-2128)). Medium was removed from the cells after 72 hours and frozen at -80°C.

Methods:

Prior to the start of the experiment, cells were removed from liquid nitrogen, placed in culture flasks, and kept in culture for 2-3 d until confluency was reached (see ATL-SOP Culturing of the H295R human adrenocortical carcinoma cell line (ATCC CLR-2128)). Once confluency was reached, cells were washed 3X with sterile PBS and new medium was added to the flasks. After 72 h, the medium was removed from the cells, placed into a 50 ml centrifuge tube, and frozen at –80 °C. The medium was subsequently thawed and placed in 1 ml aliquots in Eppendorf tubes and frozen at –80 °C until use in subsequent experiments.

Statistics:

N/A

Expected Outcome:

N/A

5.2 Summary of Results

Cells were successfully cultured and medium was collected and stored according to standard protocols. This medium will be used in the evaluation of ELISA test kits, GC/MS/MS method development.

6.0 EXPERIMENT 3

6.1 Protocol

Experiment #: 3 **Date**: 6/14-15/04 **Expt. Leader:** MM/AT

Title: Determination of testosterone and estradiol in H295R medium from cells exposed to aminoglutethimide and progesterone using commercially available ELISA kits from Cayman (preliminary testing). (Medium from EPA #GS-10F-0041L steroidogenesis project)

Questions:

1. Is testosterone and estradiol production by the H295R cells detectable in the medium using Cayman ELISA kits?

2. What are dilutions of extracts that contain measurable concentrations of E2 and T?

3. What is the intra-assay variation?

4. Are there any differences in hormone concentrations between culture medium from exposed and non-exposed cells?

Experimental Design: Medium from experiment of project H295R steroidogenesis (EPA #GS-10F-0041L) is extracted using the ethyl ether extraction procedure described in MSU ATL SOP 253. The extracts are serially diluted and run in the ELISA kits using the protocols provided by the manufacturers. Appropriate statistical methods are applied to determine intra- and inter-assay variability.

Methods:

Reconstitute 1 ml of ether extracted medium in either 250 µl Phosgel buffer or EIA buffer (MSU-ATL SOP 253). Make dilutions in the assay buffer provided by the manufacturer. For initial kit screening, samples are diluted 1:10, 1:50 and 1:100 and run on the kit plate in quadruplicate. Follow kit instructions with regards to blank, non-specific binding and total binding wells.

Statistics:

Descriptive statistics (means +/- SD, CV's); compare the average concentrations measured by the different test kits using basic parametric tests.

Expected Outcome:

Testable hypotheses:

- Hormone concentrations are in the linear range of the E2 and T immunoassays.
- There are no differences in absolute hormone concentrations between the different dilutions run in the assay.
- Exposure to aminoglutethimide and progesterone (each 100 μM in DMSO) do cause significant suppression or elevation of hormone concentrations in culture medium when compared to solvent controls.

6.2 Summary of results

Concentrations of testosterone and estradiol were determined in the medium of H295R cells to evaluate the effect of two chemicals, aminoglutethimide and progesterone, on hormone production. Cell medium used in these analyses was taken from previous exposures that were conducted to evaluate steroidogenic gene expression. The results obtained from both the solvent controls and treatments are given.

6.2.1 Testosterone

Testosterone was detected in the H295R medium for all treatments examined in the experiment (Table 6.1). However, samples measured in the 1:10 dilution were outside the operating range of the standard curve and were not used in any data analyses. Concentrations of T measured in the 1:50 and 1:100 dilutions were within the linear range of the standard curve and were used to evaluate the effect of chemicals on testosterone production.

Table 6.1: Concentration of testosterone in H295R cell medium exposed to aminoglutethimide (AMG) and progesterone (Prog). [a]

		Dilution		
Treatment	Sample ID	1:10	1:50	1:100
Control	P276	3280 ± 249 [b]	10,500 ± 1460	17,800 ± 797
100μ AMG	P271	2340 ± 279 [b]	7380 ± 1460 [c]	11,100 ± 2360 [c]
Control	P266	3340 ± 340 [b]	11,000 ± 2090	20,000 ± 3520
100μ Prog	P261	2790 ± 250 [b]	7790 ± 966 [c]	11,200 ± 2800 [c]

[a] Testosterone concentration in pg/ml and are reported as means and standard deviations.
[b] Samples not included in data analyses due to concentrations being outside standard curve operating range
[c] Treated sample significantly different from same control ($p < 0.05$).

Except for one sample, the concentrations of testosterone measured in the 1:100 dilutions were significantly greater than those measured in the 1:50 dilution ($p < 0.05$). The only exception to this trend was observed for sample P261, a sample collected from cell treated with progesterone. In this sample, testosterone concentrations in the 1:100 dilution were greater than that in the 1:50 dilution (1.4-fold) but this difference was not statistically significant ($p = 0.09$). When testosterone concentrations of the same dilution were compared, cells exposed to either aminoglutethimide or progesterone had testosterone concentrations that were significantly less ($p < 0.05$) than that observed in medium collected from the solvent controls. However, when the testosterone concentrations for each dilution were combined, no significant differences were observed between the solvent controls and the treated cells (Figure 6.1).

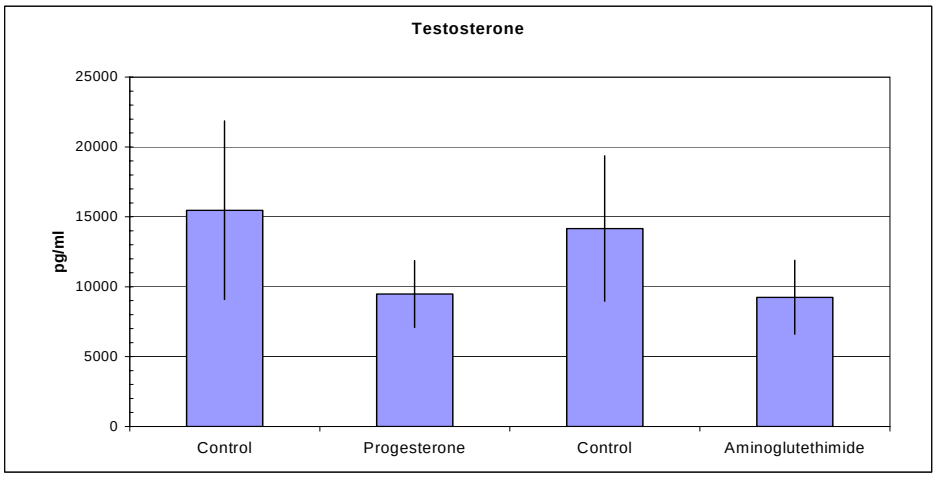

Figure 6.1. Testosterone concentrations in cell medium of H295R cell exposed to progesterone or aminoglutethimide. Controls are solvent control (0.1% DMSO) and data represent all dilutions for each sample included in the data analysis.

6.2.2 Estradiol

Estradiol H295R cell medium hormone concentrations measured in the 1:10 and 1:50 dilutions were within the linear range of the standard curve for both aminoglutethimide and progesterone treatments and their respective controls (Table 6.2). However, with one exception, estradiol concentrations measured at the greatest dilution (1:100) were less than the detection limit of the assay. The exception was sample P266 where progesterone exposure significantly increased ($p = 0.0034$) the production of E2 such that it was

detectable at the 1:100 dilution. As a result, the 1:100 dilution data for both progesterone and aminoglutethimide treatment were not used in any statistical analysis. For all other samples, a dilution effect was observed in that the concentrations of estradiol measured in the 1:50 dilution were significantly greater ($p < 0.05$) than those measured at a dilution of 1:10.

Table 6.2: Concentration of estradiol in H295R cell medium exposed to aminoglutethimide (AMG) and progesterone (Prog). [a]

		Dilution		
Treatment	Sample ID	1:10	1:50	1:100
Control	P276	263 ± 66	534 ± 40	424 ± 130 [b]
100µ AMG	P271	116 ± 17.4 [c]	398 ± 32.7 [c]	559 ± 123 [b]
Control	P266	322 ± 25.4	475 ± 162	835 ± 249 [b]
100µ Prog	P261	2570 ± 115 [c]	1980 ± 314 [c]	2240 ± 409 [b]

[a] Estradiol concentration in pg/ml and are reported as means and standard deviations.
[b] Samples not included in data analyses due to concentrations being outside standard curve operating range
[c] Treated sample significantly different from same dilution control ($p < 0.05$).

A comparison of E2 concentrations by dilution showed that exposure to aminoglutethimide significantly reduced ($p < 0.05$) E2 concentration as compared to solvent controls (Figure 6.2). In the progesterone exposure, E2 concentrations in medium from exposed cells exposed had E2 concentrations that were significantly greater ($p < 0.05$) than the E2 concentrations measured in medium from control cells.

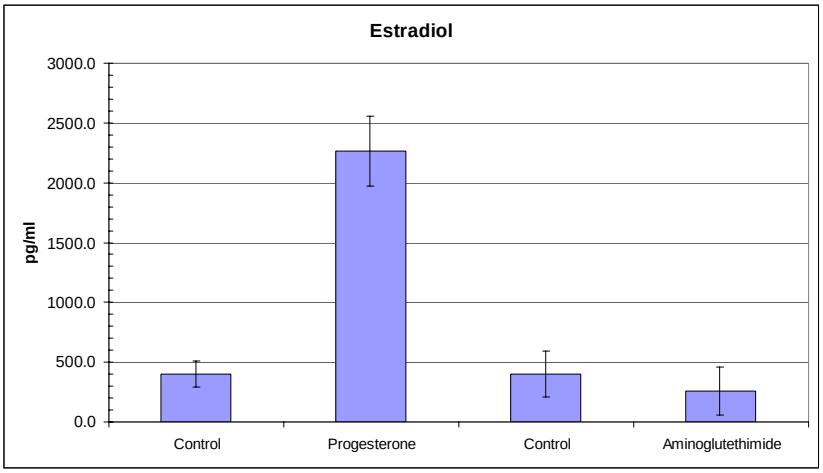

Figure 6.2. Estradiol concentrations in cell medium of H295R cell exposed to progesterone or aminoglutethimide. Controls are solvent control (0.1% DMSO) and data represent all dilutions for each sample included in the data analysis.

However, when the all sample dilutions are analyzed together, the differences between the solvent control and the aminoglutethimide exposed cells was not statistically significant ($p > 0.05$) (Figure 6.2). In contrast, when the dilutions for each sample were analyzed together, the difference between the solvent controls and progesterone-exposed cells was significantly different ($p < 0.05$).

6.3 Further considerations

These initial experiments demonstrated that there were measurable concentration testosterone and estradiol in the medium of H295R cells. However, dilution seemed to affect the concentration of both testosterone and estradiol as measured by the Cayman ELISA kit. There are four possibilities that could account for this effect including:

1. Pipetting error while preparing serial dilutions.
2. Matrix dilutions do not parallel the standard curve.
3. Preparation of test kit standards might have affected the slope of the standard curve (For some of the kits the dried standard is directly diluted in buffer. As steroid hormones are not very soluble in water this might have resulted in adhesion of the hormone to the wall of the stock solution vial and subsequent resulted in a serial dilution error).
4. Test kits were expired what might have affected the slope of the standard curve.

In order to identify the cause for the above problem we first addressed the 4th possibility and will use newly ordered test kits. In addition, concentration ranges to be tested will be extended to 4 different dilutions (1:10, 1:50, 1:100 and 1:200) to more accurately and precisely characterized the linear ranges of the ELISA kit to measure hormone concentrations in the matrices to be tested.

7.0 EXPERIMENT 4

7.1 PROTOCOL

Experiment #: 4 **Date**: 7/13-8/2/04 **Expt. Leader:** MM/EH/AT

Title: Determination of testosterone and estradiol in H295R medium using commercially available ELISA kits from Cayman, Oxford Biomedical, and Assay Designs.

Questions:

1. Is testosterone and estradiol production by the H295R cells detectable in the medium using the commercial ELISA kits?

2. How do the results from the different test kits compare to one another?

3. What is the intra-assay and inter-assay variation?

Experimental Design:

Medium collected from Experiment 2 is extracted using the ethyl ether extraction procedure as described in MSU ATL SOP 253. The extracts are serially diluted and run in the ELISA kits using the protocols provided by the manufacturers. Appropriate statistical methods are applied to determine intra- and inter-assay variability.

Methods:

Reconstitute 1 ml of extracted medium in either 250 µl Phosgel buffer or EIA buffer (MSU-ATL SOP 253). If the commercial kit includes an extraction buffer, use that buffer rather than Phosgel to reconstitute the extract. Make dilutions in the assay buffer provided by the manufacturer. For initial kit screening, samples are diluted 1:10, 1:25, 1:500 and 1:100 and run on the kit plate in quadruplicate. Follow kit instructions with regards to blank, non-specific binding and total binding wells.

Statistics:

Descriptive stats (means +/- SD, CV's); compare the average concentrations measured by the different test kits using basic parametric tests.

Expected Outcome:

Testable hypotheses:

- T and E2 concentrations are detectable in cell culture medium after 72hrs using the above commercial ELISA kits.
- The average T and E2 concentrations measured by the different commercial ELISA kits are not significantly different.
- Serial dilutions of culture medium extracts are parallel to the linear range of the standard curve.

7.2 Summary of Results

7.2.1 Cayman

Testosterone was quantified in the H295R cell medium at all dilutions evaluated in the study (Table 7.1). Concentrations of T from the 1:50 and 1:100 dilutions were within the linear range of the standard curve while the testosterone concentrations measured in the 1:10 and 1:25 dilutions were greater the standard curve and were not considered for any further evaluations. Although not statistically significant, concentrations from 1:100 dilutions were approximately 1.2-fold greater than those measured in 1:50 dilutions. Intra-assay variability as measured in different extracts from the same sample at the same dilution was low with an average CV of 6.8%. The variability T concentration as measured for all dilutions was acceptable with an average CV of 24%. The reconstitution solvent had an affect on the quantification of testosterone. At the 1:50 dilution, the concentration of T measured in phosgel buffer was approximately 1.3-fold greater than those measured in EIA buffer and this difference was statistically significant ($p=0.01$). For the 1:100 dilution, concentrations of T measured in phosgel buffer were approximately 1.6-fold greater than those measured in EIA buffer and this difference was also statistically significant ($p < 0.05$). In addition, the differences in measured concentration between the 1:50 and 1:100 dilutions were greater in the phosgel buffer (1.24-fold) than was observed in the EIA buffer (1.1-fold). As a result, it was determined that samples should not be reconstituted in phosgel, rather that EIA buffer would be used in future analyses.

Concentrations of E2 from the 1:10, 1:25 and 1:50 extract dilutions were within the linear range of the standard curve of the Cayman assay (Table 7.2). However, concentrations from the 1:100 dilution were less than the detection limit of the assay and were not used in any calculations. There were slight but no statistically significant effects of dilution on the absolute concentrations of E2 measured in the assay. The concentrations of E2 in the 1:10 dilution were approximately 1.37-fold greater than the concentrations measured in the 1:50 dilution but due to the variability, these differences were not significant. Overall, the inter-assay variability between different extracts of the same sample at the same dilution was relatively great with an average CV of 24%. Reconstitution buffer had a slight effect on the measured E2 concentrations. For instance, the E2 concentrations in the phosgel buffer were approximately 1.37-fold greater than those measured in the EIA buffer. While none of these differences were statistically significant, it was decided to conduct all future assays with the EIA reconstitution buffer. The average concentration of E2 in the H295R cell medium was 826 pg/ml.

Table 7.1: Determination of Testosterone (T) in H295R cell medium by several commercial ELISA test kits. [a]

		Dilutions [b]			
Test	Date	1:10	1:25	1:50	1:100
Cayman	7-13-04	3100 [c] (149)	4850 (662)	6740 (318)	7950 (330)
AD	7-13-04	4870 [c] (753)	4830 (915)	5520 (335)	4520 (1840)
OB	7-15-04	1490 [c] (33)	2770 [c] (80)	3800 (66)	4260 (121)

[a] Test kits evaluated were from Cayman, Assay Designs (AD), and Oxford Biomedical (OB)
[b] Testosterone concentration (pg/ml) are reported as means and standard deviations (in brackets).
[c] Concentrations were reported outside the standard curve range, as a result these values were not used in calculations.

7.2.2 Assay Designs

In the alkaline phosphatase ELISA for testosterone, only the 1:10 dilution was outside the standard curve range and could not be used in the analyses (Table 7.1). While there were some differences in T concentrations noted at different dilutions, no trend was apparent

between dilution and T concentrations in this ELISA. The reconstitution buffer had a significant impact on the quantification of T in cell medium. On average, samples reconstituted with phosgel had T concentrations that were approximately 1.8-fold greater than samples reconstituted in EIA. In addition, the differences between the two buffers were statistically significant at the 1:50 and 1:100 dilutions. The basis for these differences was not determined in the study. As a result, for consistency, only the results obtained with samples reconstituted in EIA buffer were used to evaluate the assays. The average concentration testosterone in the H295R cell medium for all dilutions was 4870 ± 753 pg/ml with a CV of 15%.

Table 7.2: Concentration of 17β-Estradiol (E2) in H295R cell medium by several commercial ELISA test kits. [a]

		Dilutions [b]			
Test	Date	1:10	1:25	1:50	1:100
Cayman	7-13-04	925 (237)	813 (255)	750 (70)	1070 [c] (307)
AD	7-13-04 (P)	763 (226)	934 (335)	918 (251)	1390 (337)
	7-27-04 (C)	927 (175)	1118 (132)	1470 (218)	2160 (205)
OB	7-15-04	581 (114)	706 (184)	795 (63.7)	1350 (141)

[a] Test kits evaluated were from Cayman, Oxford Biomedical (OB) and two kits from Assay Designs (AD) with a phosphatase (P) and chemoluminescence (C) detections systems.
[b] Estradiol concentration (pg/ml) are reported as means and standard deviations.
[c] Concentrations were reported outside the standard curve range, as a result these values were not used in calculations.

Two ELISA kits, one alkaline phosphatase (P) and one chemoluminescence (C), were used to measure E2 in the H295R cell medium (Table 7.2). In the alkaline phosphatase ELISA test, sample C4 had exceptionally high E2 concentrations in comparison to the other samples high and was assumed to be in error. Potential sources of the error may been introduced during either the extraction procedure or while preparing serial dilutions. As a result, this sample was not included in any data evaluation or statistical analyses. When E2 concentrations at each dilution were compared in the ELISA (P) assay, there was approximately a 1.8-fold increase in E2 concentrations between the 1:10 to 1:100 dilutions. In addition, the differences in E2 concentrations

between the 1:50 and 1:100 dilution were statistically significant ($p < 0.05$). The causes for this dilution effect have not yet been fully investigated. When reconstitution buffer was evaluated, the E2 concentrations measured in samples reconstituted in phosgel were approximately 1.5-fold greater than those reconstituted in EIA buffer. While these differences were not statistically significant, the trend was consistent across all dilutions. Again for consistency, only the samples reconstituted in EIA buffer were used for data analysis. The average E2 concentrations measured in H295R cell medium for all dilutions with the ELISA (P) assay was 1079 ± 265 pg/ml with a CV of 25%.

In the E2 chemoluminescence ELISA, there was approximately a 2.3-fold increase in E2 concentrations for samples diluted from 1:10 to 1:100 (Table 7.2). A closer comparison of the data showed that many of the differences between dilutions were statistically significant (Table 7.3). This effect of dilution is of particular concern in that E2 concentrations determined in samples at all dilutions were within the operating range of the standard curve and that no other sources of potential contamination or error could be ascertained from the data. An evaluation of the effect of reconstitution buffer was conducted but due to the relatively great variability in the data, no definitive conclusions could be drawn. As a result, only the EIA reconstituted samples were used in data analyses. The average E2 concentration in the H295R cell medium determined for all samples at all dilutions was 1580 ± 527 pg/ml with a CV of 33%.

Table 7.3: P-values for comparison of the differences in E2 concentrations for differentially diluted samples. [a]

Dilutions [b]	1:10	1:25	1:50	1:100
1:10	-	0.2	0.03	0.002
1:25			0.09	0.003
1:50				0.02

[a] E2 concentration determined in H295R medium by AD alkaline phosphatase ELISA
[b] All diluted samples were within the operating range of the standard curve.

A comparison of the result from the two E2 ELISA kits show that there were differences relative to variability and absolute E2 concentrations determined in H295R cell medium. On average, the chemoluminescence ELISA had measured E2 concentrations that were approximately 1.4-fold greater than those measured with the alkaline phosphatase ELISA. In addition, the variability associated with the

chemoluminescence ELISA was greater than that associated with alkaline phosphatase ELISA with CVs of 33% and 25%, respectively. As a result, the characteristics of the chemoluminescence ELISA were not as acceptable as those obtained with the alkaline phosphatase assay would not be an acceptable test for further development for the H295R protocols.

7.2.3 Oxford Biomedical

In the testosterone assays, the 1:10 and 1:25 dilutions of the T medium extracts were above the linear range of the standard curve of the assay and were not considered for further evaluations (Table 7.1). As a result, only the 1:50 and 1:100 dilutions were used in the data analysis. Analysis of the data from the two greater dilutions showed that there was a slight increase in absolute T concentrations with increasing dilution but that this increase was not statistically significant ($p = 0.12$). The average T concentration for the two greater dilutions was 4030 ± 511 pg/ml with a CV of 8%. There was no effect of reconstitution buffer on the quantification of T in cell medium for any dilution evaluated in the experiment.

All dilutions of H295R cell medium extracts had E2 concentrations that were within the linear range of the standard curve of the ELISA. Analysis of the data at each dilution showed that there was an increase in E2 concentrations with increasing dilution with maximum 2.3-fold increase between the 1:10 and 1:100 dilution. However, these differences were not statistically significant ($p > 0.05$). There were no consistent or statistically significant difference in absolute E2 concentrations of sample reconstituted with either phosgel or EIA buffers. The average E2 concentration measured in the H295R cell medium was 857 ± 337 pg/ml with a CV of 39%.

7.2.4 Comparison of test kits

Differences in measured testosterone concentrations were noted between the three ELISA test kits evaluated in this experiment. The greatest concentrations of testosterone were measured with the Cayman kit while the least were measured with the OB kit. The average concentrations of T measured by the Cayman, AD and OB kits were 6510, 4960

and 4030 pg/ml, respectively. The differences between measured T concentrations for the three ELISA kits were not statistically significant ($p > 0.05$). The greatest variability in measured concentrations was associated with the Cayman kit while the least was with the OB kit. However, it is important to note that most of the variability in these measures was associated with the dilution effect on concentration noted with all three kits. Thus, if one compares the variability of each kit taking into account dilution, the CVs associated with the Cayman and OB kits were 7.5% and 2.3%, respectively. Thus, based on the performance of each kit relative to precision, both the Cayman and OB kits performed equally well. In contrast, the AD alkaline phosphatase ELISA had greater variability within each dilution with an average CV of 22%. The average concentration of T in medium as measured by all three-test kits was 5166 ± 1254 pg/ml medium with a CV of 24%.

For E2, the results from the different kits varied both in the quantification of hormone and in terms of the variability associated with each ELISA. Of the kits tested, the greatest concentration of E2 was associated with the AD chemoluminescence ELISA while the Cayman ELISA had the least. The average concentration of E2 for the Cayman, AD (P), AD (C), and OB ELISAs were 828, 1000, 1420, and 857 pg/ml, respectively. As was observed for testosterone, dilution had an effect on measured E2 concentrations and much of the variability associated with each ELISA was associated with this phenomena. Overall, the greatest intra-assay and interassay variability was observed with AD ELISAs while the least was associated with the Cayman kit. The results using all ELISA kits had an overall average E2 concentration of 1026 ± 272 pg/ml with a CV of 26.5%. However, if the results from the AD (C) ELISA are excluded from the analysis, the average E2 concentration was 895 ± 20 pg/ml with a CV of 2.2%.

7.3 Further Considerations

As was observed in Experiment 3, measurable concentrations of T and E2 were observed in the medium from H295R cells. In addition, there was an apparent dilution effect where the concentration in the medium depended on the dilution used in the assay. This only occurred for several test kits including the Cayman T ELISA and the Assay Designs

alkaline phosphatase E2 ELISA. Based on these results it can be concluded that the age of the test kits (Possibility 3 from Experiment 3, Further consideration section) was not responsible for the dilution effect. There are three possibilities remaining that could account for this:

- Pipetting error while preparing serial dilutions.
- Matrix dilutions do not behave parallel to the standard curve.
- Preparation of test kit standards might have affected the slope of the standard curve (For some of the kits the dried standard is directly diluted in buffer. As steroid hormones are not very soluble in water this might have resulted in adhesion of the hormone to the wall of the stock solution vial and subsequent resulted in a serial dilution error).

Furthermore, the variability among replicates of a sample was relatively great for several of the kits. These kits are:

- Oxford Biomedical E2 ELISA
- All Assay Designs kits
- Cayman E2 ELISA

Finally, the reconstitution solvent also had an effect in some of the ELISAs relative to the background and concentration of T detected in the medium (Cayman T ELISA and Assay Designs T ELISA).

As a result, in future experiments extra care will be taken relative to the preparation of the serial dilutions and two different ways of preparing standard stock solutions will be evaluated. This evaluation will compare the methods outlined in the manufacturers protocols and the method described in ATL-SOP #253. In addition, hormone spikes experiments will be conducted to evaluate the precision of the different ELISAs. These tests will also help identify the reasons for the effect on measured concentrations observed with the different reconstitution buffers. Finally, the Cayman and the OBR assays will be re-run at optimized dilutions to verify the results of this experiment. To further confirm the results of this ELISA kits, testosterone concentrations will also be determined using an ELISA established and validated in our laboratory

(ATL-SOP #253). Due to the numerous problems that were associated with the Assay Designs kits, these ELISAs will not be used in any future tests.

8.0 APPENDICES

Appendix A- Experiment 1A:Raw Data and Results

Appendix B- Experiment 3:Raw Data and Results

Appendix C- Experiment 4:Raw Data and Results

Appendix D- Experiment 1B:Raw Data and Results