DRAFT

STUDY PLAN FOR PREVALIDATION OF THE SLICED TESTIS ASSAY

EPA Contract Number 68-W-01-023
WA 3-5, Task 16

August 1, 2003

PREPARED FOR

GARY E. TIMM
WORK ASSIGNMENT MANAGER
U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

BATTELLE
505 KING AVENUE
COLUMBUS, OH 43201

TABLE OF CONTENTS

List of Figures

Page

List of Tables

Endocrine Disruptor Screening Program
Contract No. 68-W-01-023
Work Assignment 3-5, Task 16

Study Plan for Prevalidation of the Sliced Testis Assay

INTRODUCTION

In 1996, the Food Quality Protection Act (FQPA) amendments were enacted by Congress to authorize the EPA to implement an Endocrine Disruptor Screening Program (EDSP) on pesticides and other substances found in food or water sources for endocrine effects in humans (FQPA, 1996). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial substances, and pesticides. A two-tiered approach will be utilized. Tier 1 employs a combination of *in vivo* and *in vitro* screens, and Tier 2 involves *in vivo* testing methods using two-generation reproductive studies. A steroidogenesis assay is proposed as one of the Tier 1 screening battery assays.

A detailed review paper (DRP) about steroidogenesis was prepared (EPA 2002). The DRP (1) summarized the state of the science of the *in vivo, ex vivo,* and *in vitro* methodologies available for measuring gonadal steroidogenesis; (2) for each methodology, presented a review of the individual assays and representative data generated by investigators who used the assay to evaluate a substance for steroidogenic-altering activity; (3) provided an evaluation of the various methodologies and the assays as tools for screening substances with suspected steroidogenic activity; (4) recommended a particular screening method and assay as a screening tool; and (5) described the strengths, weaknesses, and implications for further research associated with the recommended screening assay.

Although a promising tool, the sliced testis assay remains to be fully tested as an assay that can meet all the demands of an endocrine disruptor screening tool. Concerns raised by the EPA and EDMVS during discussions on June 11, 2002, and thereafter suggested that experiments be conducted to ensure the optimization of the assay prior to more rigorous prevalidation and validation testing. The most notable concerns were associated with 1) various incubation variables, 2) variables that affect optimal hCG stimulation, 3) characterization of the parenchymal post-slicing equilibration time, 4) parenchymal viability, and 5) detection of Leydig cell toxicity. In addition to these most notable concerns, other factors that could potentially affect the optimal performance of this assay were identified. The factors tested are illustrated in Figure 1. The results of these experiments have been evaluated to a large extent but the evaluation is not completed. Upon completion, sensitivity analysis of the optimization study results will also be performed in order to determine whether a few additional tests are needed. If not, then the optimization study results will be used to proceed with assay prevalidation.

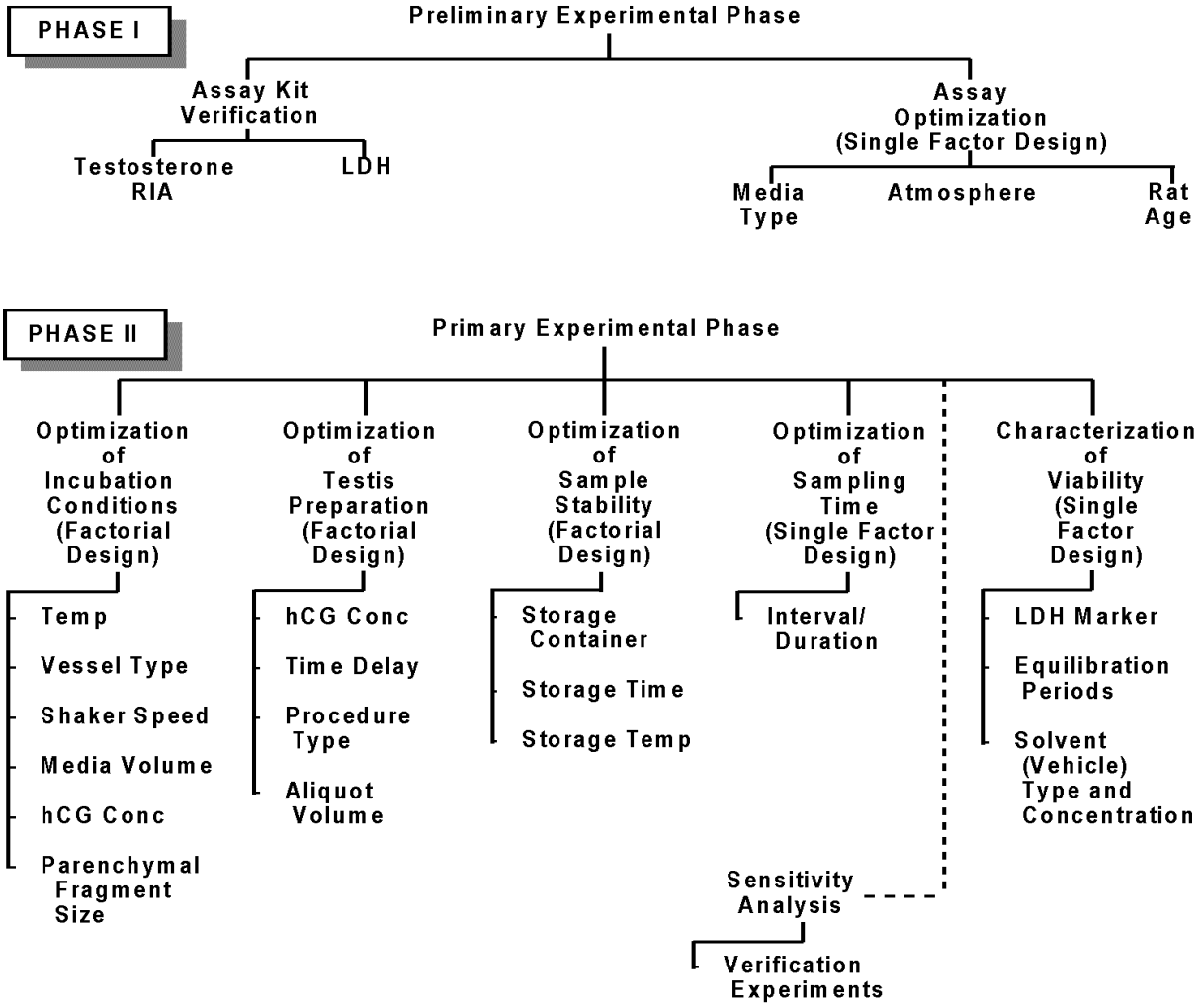


Figure 1. Sliced Testis Assay Experimental Design for Optimization

The planned purpose for the sliced testis assay is to develop a screening tool that will assess the steroidogenic pathway capacity of the Leydig cell so as to identify substances with toxic effects on hormone production. The objective of this study plan is to use the now optimized assay to obtain baseline (media only) information, obtain intra- and inter-laboratory variability estimates, identify a chemical and corresponding concentration that can be used as a positive control, evaluate cytotoxicants for assay responsiveness and selection of a cytotoxicant positive control, and test several chemicals with different modes of action in order to test assay relevance. The study plan includes using multiple laboratories - one lead laboratory and three participating laboratories. Also, the study plan describes the training procedures to be used to transfer the assay conduct method from the lead laboratory to the participating laboratories. The study plan distributes the experiments to the lead laboratory and/or participating laboratories based on the level of experience a given lab has conducting the assay and purpose for conducting

the experiments. If information is needed that will eventually be incorporated into the design of the experiment, then only the lead laboratory was assigned to conduct the study. Obviously, if inter-laboratory variability estimates were a goal, then the participating laboratories were involved. Table 1 below summarizes the prevalidation experiments, order that the studies will be conducted, and laboratory assignments.

Table 1. Prevalidation Study Plan Experiments

Study Number	Study Type	Laboratory Assignment
1	Baseline/Contralateral Testis Fragment	Lead Laboratory
2	Evaluation of AG as Positive Control	Lead Laboratory
3	Selection of Cytotoxicant as Positive Control	Lead Laboratory
4	Training/Hands-On Experience	Lead Laboratory + 3 Participating Laboratories
5	Baseline Experiment	Lead Laboratory + 3 Participating Laboratories
6	Positive Control Experiment	Lead Laboratory + 3 Participating Laboratories
7	Multichemical Experiments	Lead Laboratory

Prevalidation of the sliced testis assay is the main focus of this study plan. This study plan covers the following topics:

- Endpoint measurements/background information
- Protocol issues needing resolution
- Study design to address the protocol issues
- Recommended test substances
- The detailed study protocol
- Statistical methods for comparing the performance of the assay
- Data format.

1.0 ENDPOINT MEASUREMENTS/BACKGROUND

Steroidogenesis is a specific pathway of chemical reactions that result in the production of gonadal intermediary and end-product hormones. The pathway (1) begins with intracellular signal transduction, (2) continues with cholesterol production in the cytoplasm and transport to the mitochodrial inner membrane, and (3) ends with a set of multi-step enzymatic conversions from cholesterol to the end-product hormones.

1.1 STEROIDOGENESIS

1.1.1 Signal Transduction

Signal transduction describes the intracellular biochemical reactions that occur after stimulation of the luteinizing hormone (LH) membrane bound receptor and up to initiation of cholesterol transport to the mitochondria. The LH receptor is coupled with a G-protein and, when stimulated by LH or human chorionic gonadotropin (hCG), interacts with adenylate cyclase to form cyclic adenosine 3',5'-cyclic monophosphate (cAMP). Increased cAMP, the second messenger, stimulates protein kinase A, which initiates cholesterol biosynthesis and cholesterol transport protein synthesis (Cooke, 1996; Stocco, 1999). The receptor-mediated biochemical reactions are illustrated in Figure 2 (Stocco, 1999).

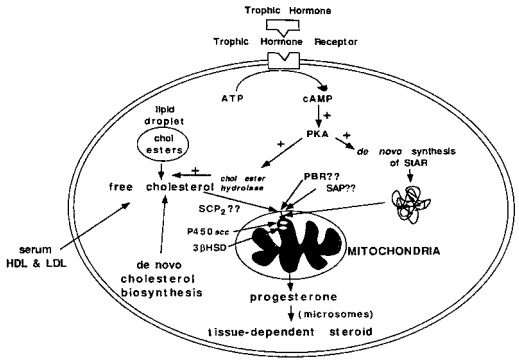


Figure 2. Intracellular Biochemical Steroidogenic Pathway Following Trophic Hormone Stimulation

Calcium (Ca^{2+}) is involved in the signal transduction of the steroidogenic pathway (Janszen et al., 1976). In order for the maximal stimulation of steroidogenesis to occur, intracellular calcium levels must increase following LH binding. The calcium-mediated reactions also involve calmodulin, a calcium binding protein (Hall et al., 1981). Chloride (Cl^-) and arachidonic acid have also been implicated in steroidogenic signal transduction (Choi and Cooke, 1990; Naor, 1991; Cooke, 1996). Arachidonic acid appears to produce a direct inhibitory effect and an indirect stimulatory effect on steroidogenesis. Steroid hormone production is inhibited when arachidonic acid activates protein kinase C. However, metabolism of arachidonic acid to its metabolites, e.g., leukotrienes, stimulates cholesterol transport into the mitochondria, thereby enhancing steroid hormone production. Other intracellular substances shown to affect steroidogenesis include free radicals, i.e., superoxide anion and hydroxyl free radical, as well as hydrogen peroxide and nitric oxide (Clark et al., 1994; Davidoff et al., 1995).

1.1.2 Cholesterol Synthesis and Transport

Cholesterol is the common precursor to the formation of all gonadal steroid hormones. The primary source of cellular cholesterol is the serum. Cholesterol is transported to the cell via serum protein carriers, e.g., high- or low-density lipoprotein (HDL or LDL). Once inside the cell, cholesterol is immediately utilized, or it can be stored, e.g., in lipid droplets. A second, minor source of cholesterol is *de novo* synthesis, which increases following hormone stimulation. Upon LH-induced stimulation, mobilization of newly synthesized and stored cholesterol (enzymatic hydrolysis of cholesterol esters) in lipid droplets occurs. Cholesterol is transported out of the cytoplasm and into the mitochondria. In the mitochondria, cholesterol is transported from the outer to the inner membrane, which is the rate-limiting step in steroidogenesis. The transport of cholesterol from the outer to the inner mitochondrial membrane requires a transport protein. LH stimulation of steroidogenic cells activates *de novo* production of the cholesterol transport protein. This protein is essential for steroidogenesis and, since it mediates the rate-limiting step of steroid hormone production, it is referred to as the steroid acute regulatory (StAR) protein. In the mitochondria, StAR protein transports cholesterol to the inner mitochondrial membrane, where the side-chain cleavage enzyme ($P450_{SCC}$) catalyzes cholesterol into pregnenolone.

1.1.3 Enzymatic Conversions

Enzymatic conversion of cholesterol to pregnenolone constitutes the initial step in a series of biochemical reactions that culminate in end-product hormone production. Figure 3 illustrates the final stage of the steroidogenic biosynthetic pathway, as well as the cell types for males and females and the intracellular location of various enzymatic steps of the steroidogenic pathway.

The first enzyme reaction is the conversion of cholesterol to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme ($P450_{SCC}$). $P450_{SCC}$ activity is also considered to be a rate-limiting step in the production of gonadal steroid hormones (Kagawa & Waterman, 1995).

The second enzymatic reaction results in the conversion of pregnenolone to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase/Δ^5 - Δ^4 isomerase (3β-HSD). At this point, the steroidogenic pathway bifurcates into a Δ^5- hydroxysteroid pathway (starting with pregnenolone) and a Δ^4-ketosteroid pathway (starting with progesterone) and, even though the same enzymes use different substrates along the parallel pathways, both pathways eventually converge, culminating in the production of androstenedione.

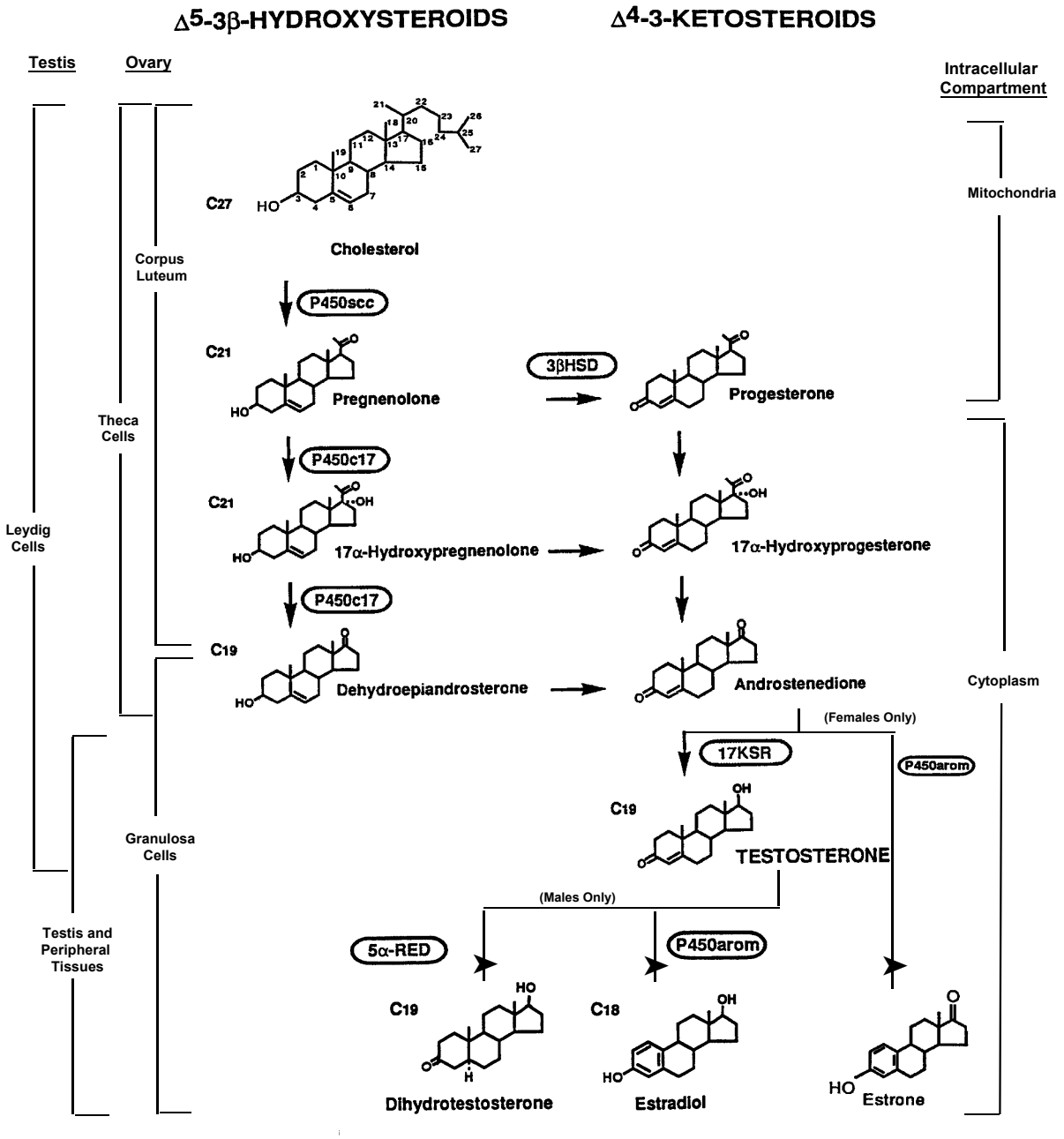


Figure 3. Enzymatic Conversions of Cholesterol and Intermediate/End-Product Hormones

The third enzymatic reaction involves cytochrome P450 17α-hydroxylase/C_{17-20} lyase (P450c17). For the Δ^5-hydroxysteroid pathway, P450c17 initially catalyzes the conversion of pregnenolone to 17α-hydroxypregnenolone, which is then converted to DHEA. As mentioned above, DHEA is converted to androstenedione by 3β-HSD. Likewise for the Δ^4- ketosteroids, P450c17 converts progesterone to 17α-hydroxyprogesterone, which is then converted to androstenedione.

The next enzymatic reaction involves the conversion of androstenedione to testosterone by 17-ketosteroid reductase (17KSR), which is also referred to as 17β-hydroxysteroid dehydrogenase (17β-HSD). The production of testosterone is considered an end-hormone product. A second possible reaction involving androstenedione occurs in the female, whereby androstenedione is converted to estrone by aromatase.

In the male, testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase. DHT is significantly more potent as an androgen than testosterone and is also considered an end-product hormone. DHT is produced primarily in peripheral tissues, although it is also found in the testis.

The last enzyme in the steroidogenic pathway is aromatase. Aromatase converts testosterone into estradiol and, in the female, androstenedione into estrone. In short, aromatase converts androgenic substances into estrogenic substances. As mentioned above for testosterone and DHT, estradiol and estrone are considered end-product hormones of the steroidogenic pathway. Aromatase is found in many different peripheral tissues, as well as male and female gonadal tissue.

In summary, cholesterol is the common precursor for production of steroid hormones. A series of biochemical reactions involving different enzymes results in conversion of cholesterol to end-hormone products: testosterone, DHT, estradiol, and estrone. The steroidogenic pathway is regulated by gonadotropins and end-product hormones. An alteration of the regulatory mechanisms, as well as direct effects on the substrates and enzymes of the steroidogenic pathway, can affect end-hormone product formation, thereby possibly resulting in reproductive system toxicity.

1.2 SLICED TESTIS STEROIDOGENESIS ASSAY

1.2.1 Basis for Selection

Steroid hormones produced by the gonads affect most of the organs in the body including bone, muscle, brain, and reproductive organs. It is for this reason that the EDSTAC recommended that an assay that measures steroidogenic function be considered as a component of the Tier 1 Screening (T1S) battery. An evaluation of steroidogenic assays and the criteria for a screen as presented in the DRP on steroidogenesis resulted in selection of the sliced testis assay. The objective of this assay is to detect disruption of the steroidogenic pathway. It may: (1) be used as one of the protocols recommended by EDSTAC for the Tier 1 screening battery,

(2) serve as a follow-up test for certain substances for which additional data are required or desired, and/or (3) predict the likelihood that steroidogenesis and downstream biologically dependent processes would be affected by the same or similar substances *in vivo*. The endpoint, testosterone, was selected for its potential to detect toxicant-induced alterations of steroidogenesis in testicular tissue.

This *in vitro* assay has been used with fetal, neonatal, and adult testes, and is not limited to mammalian species, having been used to assess steroidogenesis in fish, reptile, avian, and amphibian systems as well. Thus, the steroidogenesis bioassay as a component in the T1S phase should be broadly understood to screen for any disruption of the overall steroid biosynthetic pathway. The sliced testis steroidogenesis assay has the capacity to evaluate simultaneously all of the processes involved with gonadal synthesis of steroid hormones (signal transduction, transcription, translation, synthesis, and cellular secretion of the steroids).

The sliced testis assay has been used to identify substances that alter steroidogenesis. Examples of experimental studies from the literature that used the sliced testis assay for measuring steroidogenesis are summarized in Table 2.

Table 2. Representative Studies Using the Sliced Testis Assay

Animal/Type of Preparation	Treatment & Stimulant	Measured Response	Reference
Crl:CD BR rats/Testes Slices (50 mg)	Various conditions, factors, and chemicals	Various changes in testosterone production	Powlin et al., 1998
Adult male Long-Evans rats/Testes Slices (1/4)	Ethane dimethanesulfonate @ 0, 3, 10, 32, 100, 320, 1000, or 3200 µg/mL media/ ovine LH (100 ng/mL)	↓ testosterone production	Gray et al., 1995
Male Long-Evans Hooded rats (3-25 weeks of age)/ Testes Slices (1/4)	Vinclozolin @ 5 to 100 mg/kg/day, gavage, for 22 weeks/hCG, 50 IU	↑ basal and hCG stimulated testosterone @ 15 and 100 mg/kg/day	Fail et al., 1995
Male Long-Evans Hooded rats (3-14 weeks of age)/ Testes Slices (1/4)	Methoxychlor @ 50 or 200 mg/kg/day, gavage, for 11 weeks/hCG, 50 IU	↓ basal testosterone production no effect on HCG stimulated testosterone production	Fail et al., 1994
Adult male SD rat/Testes Slices (1/4)	Ethane dimethanesulfonate @ 0, 500, or 3000 µM/ 100 mIU/mL hCG	↓ testosterone production	Laskey et al., 1994
Adult male OFA rat/Testis Slices (~1/4)	^{14}C-pregnenolone (50 mCi/mmol; 200 nCi - a tracer amount)	~70 and 15 percent of the ^{14}C-radioactivity was testosterone and androstenedione	Gurtler and Donatsch, 1979

In summary, the most salient features of this assay and the basis for its consideration are that it identifies substances that alter steroid hormone production and can be conducted at a minimal cost, quickly, and simply with standard laboratory equipment and basic laboratory training, which are all important characteristics of an assay to be used as a screen for identifying

substances with steroidogenic-altering activity. The sliced testis assay is stable and the parenchyma remains viable over a sufficient time period to measure changes in end-product hormone production. In addition, the assay is relatively sensitive and specific; uses parenchyma that maintains the cytoarchitecture of the organ; uses a reduced number of animals; will be relatively easy to standardize (by optimization); and has a well-defined endpoint in testosterone, which can be modified to include additional intermediate hormonal endpoints if so desired.

1.2.2 Summary of Optimization Studies

Although the advantages and strengths, as described above, provide a basis for further consideration of this assay as the screening tool for substances that alter steroidogenesis, the sliced testis assay also has some limitations, which prompted performing experiments to optimize the assay. Some limitations of the assay are not able to be resolved. For example, substances that require metabolic activation will not be identified as substances that alter steroid hormone production. Also, substances that are insoluble in the media or cannot be formulated in a soluble vehicle are unable to be tested. Aside from these limitations, there are a number of variables that were tested in order to determine the settings that optimized the assay. An important objective of the optimization experiments was to determine the level of a given factor that would reduce to a minimum the variability of the assay.

The experimental design factors that were tested in an attempt to optimize the sliced testis assay are illustrated in Figure 1. Although the optimization experiments are completed and the results analyzed, a complete analysis (including sensitivity analysis) is in progress. However, an initial evaluation of the optimization experimental findings suggest the following as preliminary optimal conditions:

- incubation temperature - 34 to 37 degrees
- vessel type - scintillation vial
- shaker speed - low (~135 rpm)
- incubation medium volume - 5 mL
- hCG concentration - 0.1 IU/mL (final concentration)
- parenchymal fragment wt - 0.15 mg (range 0.130 - 0.170 mg)
- testis preparation conditions - cold buffered saline
- testis preparation time - less than 1 hour
- media sampling volume - less than 0.5 mL
- cell damage and destruction was sensitive to LDH analysis

The present prevalidation study plan and protocol include these conditions for conducting the sliced testis assay prevalidation experiments. However, pending complete statistical sensitivity analysis, these conditions are subject to modification.

1.2.3 Sliced Testis Steroidogenesis Assay Procedure

As can be deduced by the previous information, the sliced testis assay has been used by many researchers over the past couple of decades. The parameters and settings that are used can, and often do, vary from laboratory to laboratory and researcher to researcher. However, based on the results of the optimization studies, the experimental factors and their levels can now be more standardized. The optimal assay procedure is described and illustrated below (Figure 4).

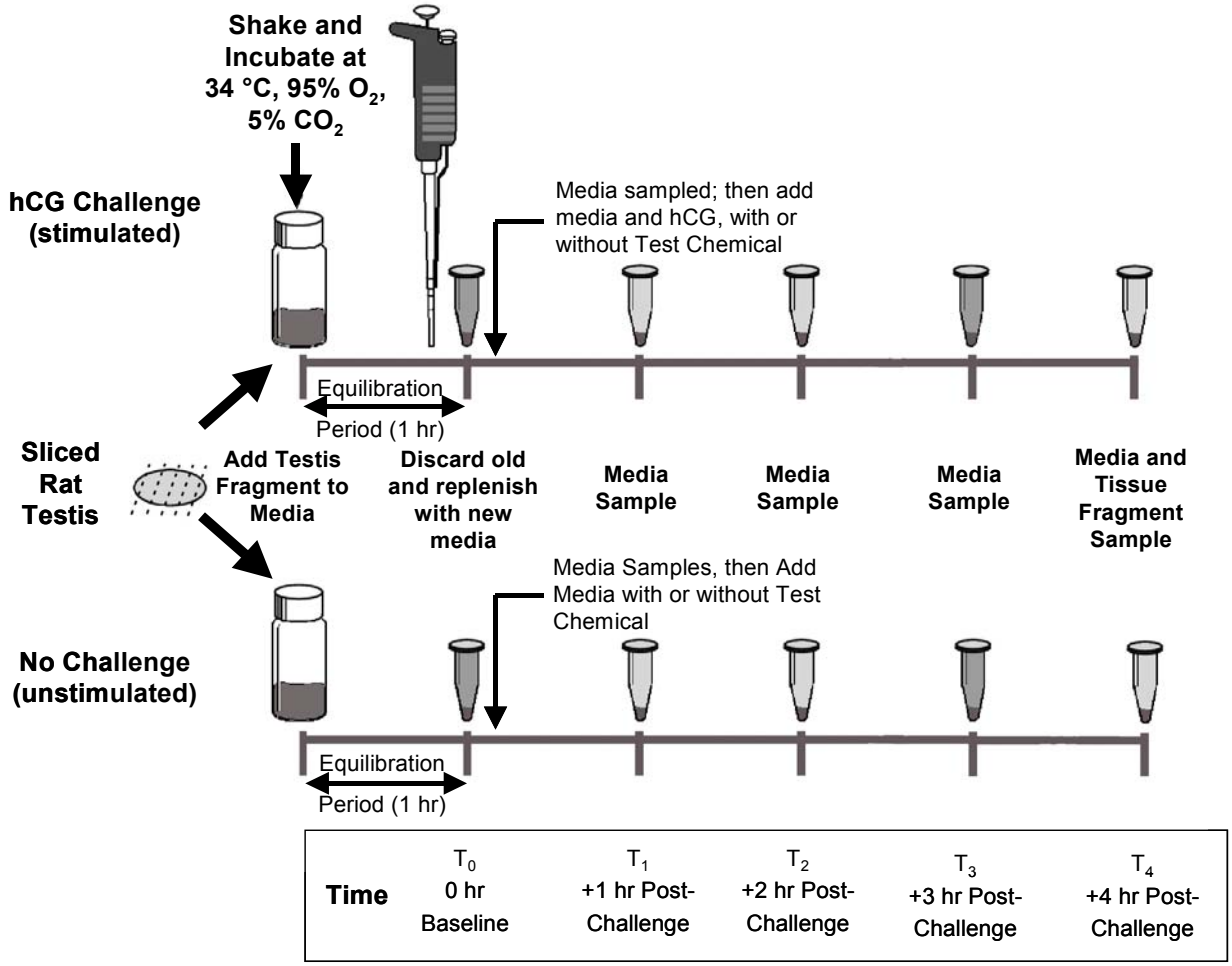


Figure 4. Technical Flow Illustration of the Sliced Testis Steroidogenesis Assay

The assay procedure will begin at a time that will enable the technician to complete all incubations and sample collections before approximately noon on a given day (does not include the analyses). An 11 to 15 week old male Sprague-Dawley rat will be euthanized. The testes will be removed [develop SOP for testis removal and preparation]. The testes will be weighed

[acceptance criterion, i.e., >1000 mg] and placed in cold (4^0 C) Dulbeccos Phosphate Buffered Saline (DPBS; undiluted; pH 7.2 - 7.4). The time from removal of the testis to the time of slicing
will be less than 1 hour. Each testis will have the tunica albicans removed and will be sliced longitudinally to yield pieces weighing within 10% of 0.150 grams (between 0.135 g and 0.165 g). Each slice will be placed into a tightly capped scintillation vial containing 5 mL of 95% O_2 / 5% CO_2 gassed media. [develop SOP for testis slice distribution among test tubes]. The media will be medium-199 without phenol red (Invitrogen), which will have been adjusted to a pH of 7.4.

The vials containing the testicular sections and media will be incubated at 34^0C [range 34 to 37^0C] on a shaker at low speed, i.e., 135 rpm. After the first period of incubation, 1 hour, the contents of the vial will be gently centrifuged. The equilibration media will be discarded without analysis. New media (5.0 mL) will be placed in the original vial. One aliquot (0.5 mL) of media [need further evaluation of aliquot volume] will be collected for analysis (Time 0, Baseline Sample). The sample aliquot will be analyzed for testosterone. [develop SOPs for testosterone RIA] To one-half of the vials, a 0.5 mL volume of media without hCG (unstimulated) will be added, and to the other half of the vials, 0.5 mL of media with hCG (stimulated) will be added to the vials to replace the volume removed for analysis, as well as to initiate testosterone production (hCG stimulated vials only). The final hCG concentration will be 0.1 IU/mL. [Note: For assays using a test chemical, the test chemical will also be formulated in the media so that when the replacement media is added, at time 0, the final target concentration will be achieved.]

At 1, 2, 3 and 4 hours post-challenge, one 0.5 mL sample (or smaller) of media will be collected from each of the hCG-stimulated and non-stimulated vials. After each sampling, the appropriate media with or without hCG will be added to the vial to replace the volume removed for analysis. The sample aliquots removed at each time point will be analyzed for testosterone. Also, at the end of the incubation period, e.g. 4 hours, remove the tissue fragment and collect a sample for microscopic examination.

Media samples collected at the specified time points will be frozen and stored at -70 degrees C and analyzed within one month after collection. Testosterone will be analyzed in duplicate for each sample using an RIA method modified for rat samples. All samples for a given day's set of runs will be analyzed in the same testosterone RIA , if possible. Tissue samples will be thawed, sectioned, stained, and mounted for microscopic examination. The staining procedure is specific for beta-HSD, an enzyme specific to Leydig cells.

2.0 REFERENCE CHEMICAL RESOLUTION ISSUES

There are no sliced testis assay issues that require resolution by experimentation prior to initiation of the protocol included in this study plan. The protocol is believed to be complete and ready for implementation. However, prior to implementation of the multichemical study, there are chemistry issues that must be resolved. Reference chemical use issues include:

- chemical characterization
- formulation development
- formulation analysis
- stability determination

In addition, there are some unanswered questions that are believed necessary to address during prevalidation in order to design the validation study plan. The experiments needed to obtain this additional information were built into the design of the prevalidation study plan. The issues yet to be resolved include:

- contralateral fragment variability
- evaluation of aminoglutethimide as a positive control
- evaluation of cytotoxicants

The experimental design for these studies are described in Section 4.0.

Prevalidation will use a variety of reference chemicals to test how the sliced testis assay responds. Some chemicals will be evaluated for their use as a positive control and others will be used to determine the relevance of the assay. These latter reference chemicals will be selected to have different modes of action. All such chemical usage will require that the reference chemicals be characterized prior to use, as well as prepared into a formulation that can be added to the incubation mixture as a solution. In addition, confirmation of the formulation concentration requires development of an analysis procedure. Finally, the stability of each formulation needs to be determined. Each of these issues are addressed in more detail in the following subsections.

2.1 CHEMICAL CHARACTERIZATION

A single lot of each reference chemical will be procurred so that all prevalidation experiments are performed using the same lot. The lot obtained will have a purity >95 - 98 percent. Upon receipt of each reference chemical, the identity will be confirmed by running an IR spectrum and comparing it to a reference spectrum from the manufacturer or from the literature. Within 30 days of using a reference chemical in the assay, the purity will be determined using one chromatographic method and one additional or complementary method.

2.2 FORMULATION DEVELOPMENT

Prior to testing, the solubility of the reference chemical in the media at a concentration that will result in the desired final concentration in the incubation mixture will need to be determined. Formulation development will be based on the highest concentration needed for a given reference chemical. In addition, a formulation will be developed using a) media only or b) a vehicle that can be used to dissolve the reference chemical prior to mixing it with the media.

Only select solvents will be used as vehicles. The vehicles that will be used and their concentrations were determined during the optimization studies. Based on these results, selected concentrations of ethanol, DMSO, or Tween 20 will be used as vehicles if the chemical is not soluble in the media.

It will be important to confirm that the formulation is a solution. This will be done by analyzing the formulation before and after filtering since the results of the two analyses will be similar if the formulation is a solution. In addition, since the media is gassed in the assay, the solubility of gassed formulation will be tested.

Formulation development will seek to prepare a solution of the reference chemical in media, with and without hCG, so that a given aliquot of the formulation, when added to the incubation mixture, will result in the desired final concentration of reference chemical to be tested, as well as the final total incubation volume. Also, if a vehicle is needed to make the formulation, then the vehicle concentration would be formulated at an acceptable level.

When formulations for multiple laboratories are required, a formulation will be prepared in a sufficient batch size that will allow the same formulation to be dispensed to all laboratories. If different concentrations are to be tested for a given reference chemical, then the central laboratory will prepare a stock solution, which will be shipped to the laboratories. Each laboratory will then use the stock solution to prepare the various concentrations to be tested.

Formulations, if solutions, will be prepared to within 5 percent of target.

2.3 FORMULATION ANALYSIS

For each chemical to be tested, a method for analysis will be developed for the reference chemical in media/vehicle over the concentration range to be tested.

Prior to shipment of a formulation to the laboratory(ies), a sample of the stock solution formulation to be shipped will be taken and analyzed. Samples will be analyzed in duplicate. If the determined concentration is not within 5 percent of the target concentration, then the formulation will be re-prepared.

After the laboratory(ies) have used the diluted formulations, a sample will be shipped back to the central laboratory and archived for possible analysis at a later date if there is some question about the results of the assay.

2.4 STABILITY DETERMINATION

Prior to being used in the assay, the formulations will be tested for stability. Stability testing will be performed at the concentration of the stock solution formulation. Stability will be determined at time 0 (day of preparation) and at weekly intervals for a period of at least 6 weeks and, thereafter, at 9 and 12 weeks. Stability will be tested at room temperature and at

refrigerated temperatures. Stability will be tested using amber glass bottles with Teflon-lined lids.

3.0 STUDY DESIGN (PREVALIDATION OF THE SLICED TESTIS ASSAY)

Each of the studies planned for prevalidation is summarized in the table below and are described in more detail in the following subsections.

Study Number	Study Type	Laboratory Assignment
1	Baseline/Contralateral Testis Fragment	Lead Laboratory
2	Evaluation of AG as Positive Control	Lead Laboratory
3	Selection of Cytotoxicant as Positive Control	Lead Laboratory
4	Training/Hands-On Experience	Lead Laboratory + 3 Participating Laboratories
5	Baseline Experiment	Lead Laboratory + 3 Participating Laboratories
6	Positive Control Experiment	Lead Laboratory + 3 Participating Laboratories
7	Multichemical Experiments	Lead Laboratory

3.1 BASELINE AND CONTRALATERAL TESTIS FRAGMENT STUDY

The lead laboratory will conduct this experiment using the optimized assay conditions. The objectives of this experiment are two-fold. First, to have an experienced laboratory conduct the assay under conditions considered optimal for minimizing the assay variability in testosterone concentrations measured over time. Second, to evaluate the variability in contralateral testes fragments for the purposes of deciding whether future experimental designs can use randomized fragment assignments or if a blocking design is essential and, if the latter, what blocking design would be most favorable.

The purposes of this experiment are:

- to demonstrate competence of lead lab using optimized assay conditions
- to estimate response trends in T production in the absence of inhibiting chemicals
- to estimate Leydig cell density after four hours of incubation, in the absence of inhibiting chemicals
- to determine the variability in assay response associated with animals, contralateral testes within animals, testis fragments within testes

The experimental design is summarized in the following table:

Time (Hours from Equilibration)	hCG	Animal	Testis	Fragment #	Incubation (Run) #
0	no	1 - 3	A	1	1 -3
	yes			2	4 - 6
1	no	1 - 3	A	1	1 -3
	yes			2	4 - 6
2	no	1 - 3	A	1	1 - 3
	yes	1 - 3	A	2	4 - 6
		1	B	1, 2	7, 8
		2	A	3, 4	9, 10
			B	1, 2	11, 12
		3	A	3, 4	13, 14
			B	1, 2	15, 16
		4	A	1, 2	17, 18
			B	1, 2	19, 20
		5	A	1, 2	21, 22
			B	1, 2	23, 24
3	no	1 - 3	A	1	1 - 3
	yes			2	4 - 6
4	no	1 - 3	A	1	1 - 3
	yes	1 -3	A	2	4 - 6
		1	B	1, 2	7, 8
		2	A	3, 4	9, 10
			B	1, 2	11, 12
		3	A	3, 4	13, 14
			B	1, 2	15, 16
		4	A	1, 2	17, 18
			B	1, 2	19, 20
		5	A	1, 2	21, 22
			B	1, 2	23, 24

The experiment summarized in the above table represents one replicate study. A replicate study is an independently conducted experiment; separated by day and using freshly prepared reagents. Two full replicates will be conducted for runs 1 to 24 and a third partial replicate will be carried out for runs 1 to 6. Runs 1 to 6 within each replicate will use two fragments taken from a single testis from each of three animals. Runs 7 to 24 within each of the two full replicate will use five animals, two testes within each animal, and two fragments from each testis. Each replicate will use different animals. The total number of individual fragments and incubations used to conduct this experiment is 24.

The sampling time points from the media range from 2 to 5 depending on the sample type. If two samples, then the time points are at 2 and 4 hours post-equilbration. If five, then the sampling time points are 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. Thus, the overall total number of testosterone samples for analysis is 324 samples [Runs 1 to 6 has 5 time points with duplicate analyses for 60 samples and runs 7 to 24 has 2 time points with duplicate analyses for 72 samples, which gives a total of 132 samples/replicate.

The principal endpoint is testosterone (T) concentration. T analyses will be carried out in duplicate. Results of the duplicate T analyses will be averaged. Statistical analysis will be carried out on the averages the duplicates.

The statistical analysis will be carried out by the lead laboratory. Mixed effects repeated measures models will be fitted to the data to describe trends in concentration vs. time and to estimate animal-to-animal, testis-to-testis within animal, and fragment-to-fragment within testis components of variation. For the T concentration analyses fixed effects terms in the models will describe linear and non-linear trends in T for hCG stimulated testis fragments and for non-stimulated testis fragments. Random effects terms in the model will estimate variance components that account for variation among fragments within testes, testes within animals, animals within replicates, and variation among replicates, primarily for the hCG stimulated testis fragments. A correlation structure will be fitted to describe the relation among the responses based on the same vial (i.e., the same fragment) at the various times. The testis-to-testis variation within animals and the fragment-to-fragment variation within testes will be determined only for the hCG stimulated testis fragments.

The results of the model fit will be parametric functions that describe the time trends and variance components that describe the within and among replicate variation about the time trends.

Residuals from the model fits will be examined to determine goodness-of-fit to the model assumptions, to assess the nature of the random variation about the model, and to search for outlying observations. Outliers may be individual responses that deviate from the trend within their vial, individual vials that deviate from the average across vials within their replicate, and individual replicates that deviate from the average across replicates. Each of these types of residuals will be examined.

Based on the model fits multiple endpoints will be reported. Endpoints include

- T concentrations at 1, 2, 3, 4 hours following equilibration, with and without hCG stimulation
- Ratios of T concentrations with and without hCG stimulation, at 1, 2, 3, 4 hours following equilibration
- Response trends, with and without hCG stimulation
- Ratios of trends with and without hCG stimulation
- Average Leydig cell densities after two hours of incubation and after four hours, with and without hCG stimulation.

For each response the average value across runs, the standard error of estimate, and confidence intervals about the average will be reported. Estimates of run-to-run variation within replicates and replicate-to-replicate variation will be reported. Separate variance components will be estimated for responses with hCG stimulation and for responses without hCG stimulation.

Random effects terms in the model will describe variance components that account for animal-to-animal variation, testis-to-testis variation within animal, and fragment-to-fragment variation within testis. These components of variation will be estimated for hCG stimulated testis fragments at two hours and at four hours following equilibration. A correlation structure will be fitted to describe the relation among the responses at the various times based on the same vial (i.e. the same fragment). The results of the model fit will be parametric functions that describe the time trends and estimates of the variance components.

For each response estimates of variance components will be reported.

The results of this experiment would require technical review and approval to determine whether the assay is performing satisfactorily before proceeding to the next study.

3.2 EVALUATION OF AMINOGLUTETHIMIDE (AG) AS A POSITIVE CONTROL

The lead laboratory will conduct this positive control experiment with AG using the optimized assay conditions. Although AG is anticipated to be the positive control for the assay, verification is warranted. In order to evaluate the responsiveness of the assay on a day-to-day basis, this study plan proposes to use a positive control with every set of samples. Along with the testis fragments used for the media-vehicle control and test chemicals (multichemical testing), the tissue fragment response to a known gonadal steroidogenic inhibitor is proposed for use as a positive control. One concentration of the inhibitor would be used once the inhibitor and concentration are finalized. Now that the optimized assay is available, the use of aminoglutethimide (AG) as a potential positive control substance can be evaluated. In addition, this experiment attempts to determine a single concentration of AG that could be used when included in the multichemical tests. Furthermore, once the positive control substance and the concentration are determined, then the response from this treatment will serve to build historical control data.

The Lead Laboratory alone will evaluate AG for its potential use as a positive control. This experiment will be conducted immediately following an evaluation of the results from the contralateral fragment experiment and approval has been given to proceed. The experimental design for this experiment is described as follows:

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment ID Number
Media-Vehicle control	no	3	1 - 3
Media-Vehicle control	yes	3	4 - 6
Media + AG (low)	yes	3	7 - 9
Media + AG (mid)	yes	3	10 - 12
Media + AG (high)	yes	3	13 - 15

The experiment summarized in the above table represents one replicate study. A replicate study is an independently conducted experiment; separated by day and using freshly prepared reagents. Two replicates would be conducted. The overall experiment would use three rats/replicate study; three testes total/replicate study (based on using 5 fragments/testis). Within each replicate, the five fragments from a single testis would be distributed among the five test conditions. Thus, each repetition of the test within a replicate will involve testes fragments from the same testes. The overall total number of individual fragments and incubations used to conduct this experiment is 30.

The sampling time points (5) from the media are 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. In addition, at the end of the incubation period, a piece of the tissue fragment will be collected and snap-frozen for beta-HSD staining and microscopic analysis. Thus, the overall total number of testosterone samples for analysis is 300 samples [30 runs x 5 sampling time points x 2 (duplicate) analyses] and the overall total number of tissue samples is 30 [30 runs x 1 time point].

The data analysis and statistical evaluation will be similar to the intra-laboratory analysis that described later in this study plan when this positive control experiment is conducted by the Lead Laboratory and the three participating laboratories. While the purpose of this initial experiment is to determine whether AG will be useful as a positive control and, if so, determine a single concentration to use in subsequent studies, the second conduct of this experiment using multiple laboratories has as its primary purpose to begin to estimate inter- and intra-laboratory variability.

The results of this experiment would require technical review and approval to determine whether the assay is performing satisfactorily, AG is a satisfactory positive control, and a single concentration is selected before proceeding to the next study.

3.3 SELECTION OF A CYTOTOXICANT AS A POSITIVE CONTROL

The lead laboratory will conduct this positive control experiment with various cytoxicant substances to be tested using the optimized assay conditions. It is of interest to determine whether a given test chemical is cytotoxic to the cellular components of the testes. Chemicals with cytotoxic activity are classified as (for the purposes of the present study plan) a) general cellular toxicants, which refers to those chemicals that are non-specific cytotoxicants, or b) specific cytoxicants, which refers to chemicals that are specifically toxic to the Leydig cell. The purpose of this set of experiments is to test different known general cytoxicants for their effect on testosterone production (RIA analysis) and on the Leydig cell (beta-HSD staining). From these results, a chemical that can be used as a cytotoxicant positive control will be selected in order to provide information about the tissue fragment response when cellular destruction is the cause of the decrease in testosterone concentrations. As was described above for AG, the cytotoxicant positive control will be used at one concentration along with the media-vehicle control when performing multichemical testing.

The four general chemical cytotoxicants presently being considered for testing are SDS; 2, 4-dinitrophenol; methylnitrosourea; sodium azide. Three concentrations of each will be tested. Prior to being tested in the sliced testes assay, it is necessary to determine whether these agents interfere with the testosterone RIA. Consequently, a mid concentration testosterone standard will be spiked with the cytotoxicant and compared to the results obtained from a vehicle spiked control (same final volumes). If there is no interference, then the cytotoxicant will be tested using the sliced testis assay.

A Leydig cell specific staining method and microscopic evaluation will be used to evaluate the viability of these cells. At the end of the incubation period, a piece will be taken from each fragment for staining. The media-vehicle control fragment will be used to evaluate the result of incubation with the test chemical treatment. The staining procedure is specific for cells containing 3β-HSD (see Section 1.0), a steroidogenic enzyme specific to the Leydig cells when examining testis tissue. The general procedure is described as follows:

- after the last sample collection, snap freeze the tissue, section it (~15 um), and mount it on slides. Stain the slide using the following solutions and procedure: a) etiocholanolone stock solution (1 mg/ml in DMSO), b) 2 mg Nitroblue Tetrazolium in 0.6 ml Etiocholanolone stock, c) 10 mg NAD+ dissolved in 9.5 ml warm Dulbeccos Phosphate buffered saline (DPBS), and d) 10 mg NAD+ dissolved in 9.5 ml warm Dulbeccos Phosphate buffered saline (DPBS). Mix solutions b and c. Cover section tissue on slide with staining solution for 1 - 2 hours. Rinse in deionized water. Fix in 10% formalin in DPBS with 5% sucrose. Coverslip with glycerol:DPBS (1:1) and seal with nail polish. [Ref.: Payne et al., (1980). Endocrinology 106:1424; Klinefelter et al., (1993). In: Methods in Toxicology, Vol. 3, pp. 166-181.

In order to increase efficiency and conserve animal usage, only the stimulated (hCG-with) incubations will be performed. This seems reasonable since the cytotoxicant response will decrease the testosterone concentration and it will be difficult to assess a chemical-induced decrease in testosterone concentration in a non-stimulated tissue fragment. The experimental design for this experiment is described as follows:

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment(s)
Media-Vehicle control	no	3	1 - 3
Media-Vehicle control	yes	3	4 - 6
Positive control	yes	3	7 - 9
Media + Cytotoxicant A (low)	yes	3	10 - 12
Media + Cytotoxicant A (mid)	yes	3	13 - 15
Media + Cytotoxicant A (high)	yes	3	16 - 18
Media + Cytotoxicant B (low)	yes	3	19 - 21
Media + Cytotoxicant B (mid)	yes	3	22 - 24
Media + Cytotoxicant B (high)	yes	3	25 - 27

The experiments listed in the above table represent one replicate study. Note that two of the four cytotoxicants are tested per replicate study. A replicate study is an independently conducted experiment; separated by day and using freshly prepared reagents. Two replicates would be conducted. The overall experiment would use three rats/replicate study; three testes total/replicate study (based on obtaining 8 to10 fragments/testis). Testes will be at least 1000 mg so that at least nine fragments can be obtained from a testis. These nine fragments will be divided among the nine test conditions. The overall total number of individual fragments and incubations used to conduct this experiment is 54. The two replicate experimental designs will be repeated with the other two cytotoxicants.

The sampling time points (5) from the media are 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. In addition, at the end of the incubation period, a piece of the tissue fragment will be collected and snap-frozen for beta-HSD staining and microscopic analysis. Thus, the overall total number of testosterone samples for analysis is 540 samples [54 runs x 5 sampling time points x 2 (duplicate) analyses] and the overall total number of tissue samples is 54 [54 runs x 1 time point].

Regarding data analysis and the statistical evaluation, the principal endpoints are testosterone (T) concentration and Leydig cell counts. T analyses will be carried out in duplicate. Results of the duplicate testosterone analyses will be averaged. Statistical analysis will be carried out on the averages of the duplicates. Mixed effects repeated measures models

will be fitted to the data to describe trends in T concentration vs. time and trends in the cytotoxicant concentration. Fixed effects terms in the models will describe linear and non-linear T time trends and cytotoxicant concentration trends for hCG stimulated testis and differences relative to the hCG stimulated media-vehicle control. Random effects terms in the model will describe variance components that account for variation among the repeat vials within replicates and variation among replicates. A correlation structure will be fitted to describe the relation among the responses at the various times based on the same vial (i.e. the same fragment) and at the various cytotoxicant concentrations, based on fragments taken from the same testis. The results of the model fit will be parametric functions that describe the time trends and concentration trends and variance components that describe the within and among replicate variation about the time trends and concentration trends.

Residuals from the model fits will be examined to determine goodness-of-fit to the model assumptions, to assess the nature of the random variation about the model, and to search for outlying observations. Outliers may be individual responses that deviate from the time trend within their vial or from the concentration trend across vials, individual vials that deviate from the average across vials with the same conditions within their replicate, and individual replicates that deviate from the average across replicates. Each of these types of residuals will be examined.

Based on the model fits multiple endpoints will be reported. Possible endpoints include T concentrations at 1, 2, 3, 4 hours following equilibration for the media-vehicle control and graded cytotoxicant concentrations, differences or ratios of T concentrations from the same groups with hCG stimulation to the media-vehicle control with hCG stimulation at 1, 2, 3, 4 hours following equilibration, response trends in time or in cytotoxicant concentration with hCG stimulation, and differences or ratios of trends to the media-vehicle control with hCG stimulation. For each response the average value across runs, the standard error of estimate, and confidence intervals about the average will be reported. Estimates of run-to-run variation within replicates and replicate-to-replicate variation will be reported. Differences between the T concentrations in the presence of the cytotoxicant and the T concentrations in the media-vehicle control group will also be analyzed.

The results of this experiment would require technical review and approval to determine whether the assay is performing satisfactorily, a single chemical is selected as a positive control, and a single concentration is selected before proceeding to the next study.

3.4 TRAINING AND HANDS-ON EXPERIENCE

In addition to the lead laboratory, additional laboratories will be required to participate in order for inter-laboratory assay variability to be estimated. These participating laboratories were selected following a review of their responses to a sliced testis assay prevalidation proposal. Three laboratories were selected from those solicited for participation. It is believed important to provide training and give the participating laboratories experience prior to using their results from conducting the assay for estimating inter-laboratory variability. In this way, the assay

variability that is generated by laboratories judged to be competent in the conduct of the assay can be evaluated. The procedures planned for training the participating laboratories and providing hands-on experience are described in the following paragraphs.

Standardized study documents will be produced by the lead laboratory. These documents will include a study protocol and standard operating procedures (SOPs). While the standard protocol and study specific SOPs may be customized according to each laboratory format, the content of the documents will be the same. The study specific SOPs to be developed by the lead laboratory will focus on aspects of the assay that, if varied, will impact on the assay variability and performance. The procedures to be addressed by study specific SOPs include:

- Testis removal/Fragment production
- Reagent preparation
- Fragment assignment, incubation and media sampling
- Testosterone RIA
- Fragment processing for staining and slide preparation

In addition, forms for data collection will be standardized. In this way, the data deemed necessary for collection is more likely to occur. In addition, if collected on the same form, then the data will be readily amenable to efficient review by outside laboratory monitors, e.g. lead laboratory, Data Coordination Center (DCC).

Training of the participating laboratories' technical staff will take place at the lead laboratory. The senior investigator/head technician for each participating laboratory will be trained in the conduct of the assay at the lead laboratory. The training will include a review of the relevant study documents described above and any necessary demonstrations regarding various aspects of the assay (starting at testis removal through to data analysis). Furthermore, the lead laboratory will be set-up to allow the participating laboratory staff to demonstrate proficiency in conducting the assay by supervising them as they conduct a baseline (media only) experiment as described in subsection 3.5 and a positive control experiment as described in subsection 3.6, except only one replicate per laboratory would be conducted.

The results of these proficiency experiments will be reviewed by the lead laboratory and the EPA prior to proceeding to the next studies.

3.5 BASELINE EXPERIMENT

This study will be conducted by the lead laboratory and three participating laboratories. The purposes of this study are:

- to estimate baseline inter- and intra-laboratory variability (which will also provide information to design the validation experimental design)
- to evaluate the knowledge transfer of assay procedures to participating laboratories
- to begin collection of historical control and variability data.

The experimental design of this study is summarized in the following table.

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment ID Number
Media	no	3	1 - 3
Media	yes	3	4 - 6

The information presented in the table is one replicate of the experiment. A total of three independent replicates would be conducted by each laboratory. The overall study would use one rat/replicate study and one testis total/replicate study. The overall total number of individual fragments or incubations used is 18 for each laboratory [(3 runs with + 3 runs without hCG) x 3 replicate studies].

The sampling time points (5) from the media are at time 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. No fragments will be collected for 3β-HSD staining and microscopic examination. Thus, the overall total number of testosterone samples for analysis is 180 samples/ laboratory [18 runs x 5 time points x 2 (duplicate) analyses].

Regarding the data analysis and statistical evaluation, each laboratory will carry out three complete study replicates. The results for each analysis will be reported individually, with sufficient identifying information to determine which results correspond to duplicate analyses, to different time points within one vial, to different vials within the same replicate, and to different replicates. Each laboratory will maintain databases to include all data generated during the study. The databases will have uniform structure, formatting, and variable naming across laboratories. Data will be reported for each individual sample generated. Test conditions, background environmental conditions, and results for each analysis for each sample at each time point will be reported. Analysis results to be reported are testosterone (T) concentration (ng/mg testis/hr). Detection limits and indications of inability to detect will be reported, as will confirmation of the acceptability or non-acceptability of each individual value.

The statistical analysis will be divided into intra-laboratory and inter-laboratory components. The intra-laboratory analyses will be carried out by each laboratory individually, based on a common analysis plan. Mixed effects repeated measures models will be fitted to the data to describe trends in concentration vs. time. Fixed effects terms in the models will describe linear and non-linear trends in T time trends for hCG stimulated testis fragments and for non-stimulated testis fragments. Random effects terms in the model will describe variance components that account for variation among the repeat vials within replicates and variation among replicates. A correlation structure will be fitted to describe the relation among the responses at the various times based on the same vial (i.e. the same fragment and among fragments from the same testis). The results of the model fit will be parametric functions that

describe the time trends and variance components that describe the within and among replicate variation about the time trends.

Residuals from the model fits will be examined to determine goodness-of-fit to the model assumptions, to assess the nature of the random variation about the model, and to search for outlying observations. Outliers may be individual responses that deviate from the trend within their vial, individual vials that deviate from the average across vials within their replicate, and individual replicates that deviate from the average across replicates. Each of these types of residuals will be examined.

Based on the model fits, multiple endpoints will be reported. Possible endpoints include T concentrations at 1, 2, 3, 4 hours following equilibration with and without hCG stimulation, ratios of T concentrations with and without hCG stimulation at 1, 2, 3, 4 hours following equilibration, response trends with and without hCG stimulation, and ratios of trends. For each response the average value across runs, the standard error of estimate, and confidence intervals about the average will be reported. Estimates of run-to-run variation within replicates and replicate-to-replicate variation will be reported.

The inter-laboratory analysis will be carried out by the DCC. The objective of the inter-laboratory analysis is to assess the extent of variation across laboratories with respect to average response and variability of response. For each endpoint the average response and associated standard error, the variation among runs within replicates, and the variation among replicates will be determined for each laboratory. Comparisons of within laboratory variance components will be made among the participating laboratories based on control charts to assess homogeneity of variance. The reference for comparisons will be either the lead laboratory results or the average results across all laboratories. If there is no evidence of lab-to-lab variation the within laboratory variance components will be pooled among laboratories. Otherwise separate within laboratory variance components will be specified for each laboratory.

The average responses will be compared across laboratories based on random effects one-way analysis of variance. The among laboratories variance in average response will be added to the within laboratory variance, resulting in the total assay variability. Estimates and confidence intervals will be reported for each laboratory based on the total assay variability. Estimates and confidence intervals will be reported on the differences between the participating laboratory results and the lead laboratory results and the differences between each laboratory's results and the average of the laboratory results based on the total assay variability.

3.6 POSITIVE CONTROL EXPERIMENT

This study will be conducted by the lead laboratory and three participating laboratories. The purposes of this study are:

- to evaluate aminoglutethimide (AG) as a positive control for the assay
- to determine AG (positive control) inter- and intra-laboratory variability

- to determine time effect on the assay by comparing Baseline to Pilot experiments using the media control data
- to begin collection of historical positive control and variability data.

The experimental design of this study is summarized in the following table.

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment(s)
Media-Vehicle control	no	3	1 - 3
Media-Vehicle control	yes	3	4 - 6
Cytotoxicant control	yes	3	7 - 9
Media + AG (low)	yes	3	10 - 12
Media + AG (mid)	yes	3	13 - 15
Media + AG (high)	yes	3	16 - 18

The information presented in the table is one replicate of the experiment. Three replicates of the study will be run. The non-stimulated incubations (non hCG) were excluded from the study since the affect of AG on the non-stimulated tissue fragments would not be expected to reduce the baseline levels to any great extent. The overall study would use three rats/replicate study and three testes total/replicate study. Six fragments will be taken from one testis from each rat and allocated to the six test conditions. The overall total number of individual fragments or incubations used is 54 for each laboratory.

The sampling time points (5) from the media are at time 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. In addition, at the end of the incubation period, a piece of the tissue fragment will be collected and snap-frozen for 3β-HSD staining and microscopic examination. Thus, the overall total number of testosterone samples for analysis is 540 samples/ laboratory [54 runs x 5 time points x 2 (duplicate) analyses]. The overall total number of tissue samples for microscopic examination are 54/laboratory [54 runs x 1 time point].

Regarding data analysis and statistical evaluation, the statistical analysis will be divided into intra-laboratory and inter-laboratory components. The intra-laboratory analyses will be carried out by each laboratory individually, based on a common analysis plan. Mixed effects repeated measures models will be fitted to the data to describe trends in T concentration vs. time and trends in AG concentration. Fixed effects terms in the models will describe linear and non-linear T time trends and AG concentration trends for hCG stimulated testis and differences relative to the media-vehicle control. Random effects terms in the model will describe variance components that account for variation among the repeat vials within replicates and variation among replicates. A correlation structure will be fitted to describe the relation among the

responses at the various times based on the same vial (i.e. the same fragment and fragments taken from the same testis) and at the various AG concentrations. The results of the model fit will be parametric functions that describe the time trends and concentration trends and variance components that describe the within and among replicate variation about the time trends and concentration trends.

Residuals from the model fits will be examined to determine goodness-of-fit to the model assumptions, to assess the nature of the random variation about the model, and to search for outlying observations. Outliers may be individual responses that deviate from the time trend within their vial or from the concentration trend across vials, individual vials that deviate from the average across vials with the same conditions within their replicate, and individual replicates that deviate from the average across replicates. Each of these types of residuals will be examined.

Based on the model fits multiple endpoints will be reported. Possible endpoints will be include T concentrations at 1, 2, 3, 4 hours following equilibration for the media-vehicle control and graded AG concentrations, with hCG stimulation, or in AG concentration with hCG stimulation. For each response the average value across runs, the standard error of estimate, and confidence intervals about the average will be reported. Estimates of run-to-run variation within replicates and replicate-to-replicate variation will be reported. Differences or ratio between the T concentrations in the presence of AG and the T concentrations in the media-vehicle control group will also be analyzed.

The inter-laboratory analysis will be carried out by the DCC. The objective of the inter-laboratory analysis is to assess the extent of variation across laboratories with respect to average response and variability of response. For each endpoint developed in the intra-laboratory analysis the average response and associated standard error, the variation among repetitions within replicates, and the variation among replicates will be calculated for each laboratory. Comparisons of within laboratory variance components will be made among the participating laboratories based on control charts to assess homogeneity of variance. The reference for comparisons will be either the lead laboratory results or the average results across all laboratories. If there is no evidence of lab-to-lab variation the within laboratory variance components will be pooled among laboratories. Otherwise separate within laboratory variance components will be specified for each laboratory.

The average responses will be compared across laboratories based on random effects one-way analysis of variance. The among laboratories variance in average response will be added to the within laboratory variance, resulting in the total assay variability. Estimates and confidence intervals will be reported for each laboratory based on the total assay variability. Estimates and confidence intervals will be reported on the differences between the participating laboratory results and the lead laboratory results and between each laboratory's results and the average of the laboratory results based on the total assay variability.

The responses included in the intra-laboratory and inter-laboratory analyses will be categorized as primary or secondary endpoints. The primary endpoints will be restricted to a small number (two to five) of the most important responses for comparisons. Power calculations to assess inference sensitivity versus sample size will be based on the primary endpoints. The studies will be powered to attain desired sensitivity for comparisons of the primary endpoints within or among laboratories. The secondary endpoints are the (relatively large number of) remaining responses that are included in the statistical analyses. The studies will not be powered for comparisons among the secondary endpoints.

For purposes of assessing sample size versus inference sensitivity for the validation tests, two responses have been selected as primary endpoints. These are:

- differences between T concentrations associated with the high aminoglutethimide concentration and the media-vehicle control at three hours past equilibration, with hCG stimulation.

- Sum of absolute differences between T concentrations associated with the high aminoglutethimide concentration and the media-vehicle control at 1, 2, 3, and 4 hours past equilibration, with hCG stimulation.

The second response approximates the area between the T concentration versus time curves for these two conditions.

Sensitivity analyses will be carried out based on response variability estimates obtained from the analyses. Numbers of replicates per laboratory necessary to detect heterogeneity of replicate-to-replicate variability within laboratories will be assessed. Numbers of laboratories and numbers of replicates per laboratory necessary to detect various levels of coefficient of variation across laboratories with high power or to detect various levels of ratio of between laboratory standard deviation to within laboratory standard deviation with high power will be determined.

3.7 MULTICHEMICAL EXPERIMENTS

This study will be conducted by the lead laboratory only. The purposes of this study are:

- to determine the response of the assay when challenged with putative positive test chemicals. The test chemicals will differ in their mode(s) of action, which will include altering signal transduction, cholesterol transport into the mitochondria, and the series of enzymatic reactions that lead to the production of testosterone in the gonadal steroidogenic pathway.
- to determine the response of the assay when challenged with putative negative test chemicals.
- to select test chemicals and concentrations that would be used for validation of the assay.

- to estimate inter-laboratory variability based on the results from the assay using the selected test chemicals.

The experimental design of this study for any two test chemicals is summarized in the following table:

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment(s)
Media-Vehicle control	no	3	1 - 3
Media-Vehicle control	yes	3	4 - 6
Positive control - Aminogluthethimide @ 1 conc	yes	3	7 - 9
Cytotoxicant control - TBD @ 1 conc	yes	3	10 - 12
Media + Test Chemical A (low)	yes	3	13 - 15
Media + Test Chemical A (mid)	yes	3	16 - 18
Media + Test Chemical A (high)	yes	3	19 - 21
Media + Test Chemical A (high)	no	3	22 - 24
Media + Test Chemical B (low)	yes	3	25 - 27
Media + Test Chemical B (mid)	yes	3	28 - 30
Media + Test Chemical B (high)	yes	3	31 - 33
Media + Test Chemical B (high)	no	3	34 - 36

The information presented in the table is one replicate of the experiment. The non-stimulated incubations (non hCG) were excluded from the study since the affect of a test chemical on the non-stimulated tissue fragments would not be expected to reduce the baseline levels to any great extent. However, a high concentration test chemical with non-stimulated tissue fragment was retained in the experimental design to confirm this response. In addition, in the case when the assay would be used to evaluate a chemical with an unknown effect on the testosterone concentration, it would be important to test the non-stimulated fragment at the high concentration to determine whether the test chemical had a stimulatory effect on the testosterone concentration. A total of two independent replicates would be conducted. The overall study would use two rats/replicate study and four testes total/replicate study. The overall total number of individual fragments or incubations used is 72.

The sampling time points (5) from the media are at time 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. In addition, at the end of the incubation period, a piece of the tissue fragment will be collected and snap-frozen for 3β-HSD staining and microscopic examination. Thus, the overall

total number of testosterone samples for analysis is 720 samples [72 runs x 5 time points x 2 (duplicate) analyses]. The overall total number of tissue samples for microscopic examination are 72 [72 runs x 1 time point].

Separate statistical analyses will be carried out for each test chemical. Consideration will be given to pooling the media-vehicle control groups across those chemical tests that use the same vehicle. The statistical analysis considerations are the same as those described above in the previous section, except that there are two replicates per chemical rather than three. Extent of replicate-to-replicate variation can be determined from these tests only in a qualitative manner.

4.0 RECOMMENDED REFERENCE CHEMICALS FOR MULTICHEMICAL TESTING

The purpose of this study plan is to design experiments that will provide information to evaluate the relevance of the sliced testis assay. For this reason, reference chemicals are recommended for testing in this study plan. In this prevalidation study plan, the optimized sliced testis assay will be evaluated for its capacity to detect an effect of a substance on gonadal steroidogenesis. It is predicted that the assay will detect interference with key steps in the steroidogenic pathway because interference with any of these steps will result in a statistically significant change in measured testosterone production relative to the controls. The key processes include a) decreases in signal transduction, b) interference with the transport of cholesterol from the cytoplasm to the mitochondria by the steroid acute regulatory protein and c) inhibition of various enzymes in the steroidogenic pathway (see Section 1.0).

Selection of reference chemicals were primarily based on their reported mechanism of action as it relates to the gonadal steroidogenic pathway. The most desired reference chemicals for testing are those with a single site of action (high specificity) regardless of the exposure concentration. However, most reference chemicals are inhibitory at more than one site of the steroidogenic pathway. In addition, in those instances when a reference chemical is active at one site, the effect is usually only at the low concentration and, as the concentration increases, the effect may include more than a single site (decreased specificity). While mostly positive reference chemicals were selected, there was also a negative reference chemical included in order to evaluate whether a false positive effect would be seen.

The proposed reference chemicals for testing are summarized below:

Test Chemical	Mode of Action[a]	Test Concentrations[b]	Reference
Aminoglutethimide (positive control)	$P450_{SCC}$, aromatase	10, 100, and 1000 µM	Powlin et al., 1998; Johnston, 1997; Uzgiris et al., 1977
Diethylumbelliferyl phosphate	cAMP-stimulated accumulation of StAR	TBD	Choi et al., 1995
Dimethoate	StAR protein	TBD	Walsh et al., 2000

Test Chemical	Mode of Action[a]	Test Concentrations[b]	Reference
Ketoconazole	$P450_{SCC}$, P450c17	0.1, 1, and 10 µM	Powlin et al., 1998; Kan et al., 1985; Albertson et al., 1988; DeCoster et al., 1989; Chaudhary/Stocco, 1989; Malozowski et al., 1985, 1986
Trilostane[c]	3β-HSD	TBD	Takahashi et al., 1990
Genistein or epostane	3β-HSD	TBD	Ohno et al., 2002; Tanaka et al., 1992
Flutamide	P450c17	10, 100, and 1000 µM	Powlin et al., 1998; Ayub and Levelll, 1987
Finasteride[d] or MK-434	5α-reductase	10, 100, and 1000 µM (MK-434 - TBD)	Morris, 1996
Fenarimol	aromatase	TBD	Vinggaard et al., 2000
Vinclozolin (negative chemical)	antiandrogen	TBD	--
Bisphenol A	c AMP	TBD	--
Lindane	c AMP	TBD	--

a. Site of action that leads to a decrease in Testosterone concentration for those chemicals with inhibitory activity. Negative reference chemicals would have no effect on the testosterone concentration.
b. Final concentrations in the incubation mixture.
c. Requires synthesis by commercial laboratory. Availability may be difficult due to proprietary claim.
d. Commercial source may limit availability.

5.0 STUDY PROTOCOL

The study protocol is included with this study plan as an attachment.

6.0 DATA FORMAT

A uniform data format will be developed for the laboratories to record and present data generated by conducting the sliced testis assay. Data forms will be developed for the testosterone concentrations measured at each time point for a given fragment of testis and the fragments used for staining in order to determine the number of viable Leydig cells. Regarding the data forms for collecting data, the results for each analysis will be reported individually, with sufficient identifying information to determine which results correspond to duplicate analyses, to different time points within one vial, to different vials within the same replicate, and to different replicates. These raw data collection forms will be used to process the data and report it in a summarized format as ng T/mg testis/hr for the testosterone analysis and cell number/cm^2 for the

Leydig cell analysis. The forms and format to be used are under development.

The lead laboratory will maintain a database to include all data generated during the study. The databases will have uniform structure, formatting, and variable naming across laboratories. Test conditions, background environmental conditions, and results for each analysis for each sample at each time point will be reported. These data bases are under development.

Additional details regarding data review and distribution, as well as reporting format and submission are included in Appendix B.

7.0 GLP MONITORING-QUALITY ASSURANCE UNIT

Prevalidation will be conducted in accordance with Good Laboratory Practice (GLP) regulations. A GLP-compliant protocol will direct the work of the laboratory(ies). The overall prevalidation work will be monitored by Battelle's Quality Assurance Officer and the laboratory work at each laboratory will be monitored by the individual laboratories' Quality Assurance Unit.

A QAPP for prevalidation will be prepared by the lead laboratory and this QAPP will apply to all participating laboratories as well. Battelle's QA Officer will visit the participating laboratories prior to initiation of testing to ensure they are GLP compliant.

8.0 TECHINICAL MONITORING/COORDINATION - ADMINISTRATIVE SUPPORT

Administrative support, in the way of technical monitoring and coordination of activities, will be performed by an individual from Battelle or the lead-laboratory with relevant experience with the overall program, purposes for conducting individual experiments in the prevalidation, set-up and conduct of assay, interpretation of results, and timely submission of deliverables.

This individual will make visits to the participating labs. Visits to each laboratory will occur prior to initiating laboratory work and during the conduct of the baseline experiment. In addition, the focus of this individual will be to:

- to establish milestones for lead/participating laboratories based on EPA schedule
- to coordination of knowledge transfer between lead laboratory and participating laboratories. Includes working with lead laboratory to develop/transfer protocol and study specific SOPs to participating laboratories and the implementation of such SOPs at the participating laboratories
- to coordinate the development and production of a particular report format with the lead laboratory and EPA, which can then be transferred and used by the participating laboratories in order to report results in a consistent format
- to coordinate the schedule for chemical procurement, formulation preparation and analysis, and stability determinations with the Chemical Repository and lead/participating laboratories based on EPA milestone schedule.

9.0 REFERENCES

Albertson, B.D., Frederick, K.L., Maronian, N.C., Feuillan, P., Schorer, S., Dunn, J.F., Lariaux, D.L. (1988). The effect of ketoconazole on steroidogenesis: I. Leydig cell enzyme activity in vitro. *Res. Commun. Chem. Pathol. Pharmacol.*, Jul;**61**:17-26.

Ayub, M., and Levell, M. J. (1987). Inhibition of rat testicular 17β-hydroxylase and 17,20-lyase activities by anti-androgens (flutamide, hydroxyflutamide, RU23908, cytoproterone acetate (*in vitro*). *J. Steroid Biochem.*, **28**:43-47.

Chaudhary, L. R. and Stocco, D. M. (1989). Inhibition of hCG- and cAMP-stimulated progesterone production in MA-10 mouse Leydig tumor cells by ketoconazole. *Biochem. Inter.* **18**, 251-262.

Choi, M.S.K. and Cooke, B. A. (1990). Evidence for two independent pathways in the stimulation of steroidogenesis by luteinizing hormone involving chloride channels and cyclic AMP. *FEBS Letters*, **261**:402-404.

Choi, Y. S., Stocco, D. M., and Freeman, D. A. (1995). Diethylumbelliferyl phosphate inhibits steroidogenesis by interfering with a long-lived factor acting between protein kinase A activation and induction of the steroidogenic acute regulatory protein (StAR). *Eur. J. Biochem.*, **234**(2):680-685.

Clark, B. J., Wells, J., King, S. R., and Stocco, D. M. (1994). The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells: Characterization of a steroidogenic regulatory protein (STAR). *J. Biol. Chem.* **269(45)**, 28314-28322.

Cooke, B. A. (1996). Leydig cell structure and function during aging. In: *The Leydig Cell* (eds. Payne, A. H., Hardy, M. P., and Russel, L. D.), Cache River Press, Vienna, IL.

Davidoff, M. S., Middendorff, R., Mayer, B., and Holstein, A. F. (1995). Nitric oxide synthase (NOS-i) in Leydig cells of the human testis. *Arch. Histol. Cytol.*, **58**:17-30.

Fail, P. A., Pearce, S. W., Anderson, S. A., Tyl, R. W., and Gray, L. E., Jr. (1995). Endocrine and reproductive toxicity of vinclozolin (VIN) in male Long-Evans hooded rats. *The Toxicologist* **15**, 293 (Abstract 1570).

Fail, P. A., Pearce, S. W., Anderson, S. A., and Gray, L. E., Jr. (1994). Methoxychlor alters testosterone and LH response to human chorionic gonadotropin (hCG) or gonadotropin-releasing hormone (GNRH) in male Long-Evans hooded rats. *Biology of Reproduction* **50(1)**, 106 (Abstract 206).

Gray, L. E., Klinefelter, G., Kelce, W., Laskey, J., Ostby, J., and Ewing, L. (1995). Hamster

Leydig cells are less sensitive to ethane dimethanesulfonate when compared to rat Leydig cells both *in vivo* and *in vitro*. *Toxicol. Appl. Pharmacol.* **130**, 248-256.

Gürtler, J. and Donatsch, P. (1979). Effects of two structurally different antispermatogenic compounds on the synthesis of steroids in rat testes. *Arch. Toxicol.* **41(2)**, 381-385.

Janszen, F.H.A., Cooke, B. A., van Driel, M.J.A., and Van der Molen, H. J. (1976). The effect of calcium ions on testosterone production in Leydig cells from rat testis. *Biochem J.*, **160**:433-437.

Johnston, J. O. (1997). "Aromatase Inhibitors." In: *Biochemistry and Function of Sterols*. Eds. Parish, E. J. and Nes, W. C. CRC Press, pp 23-53.

APPENDIX A

Draft Protocol for the Prevalidation of the Sliced Testis Assay

EPA Contract No.: 68-W-01-023 (Battelle Prime Contractor)
Contract No.:
Study Code:
Master Protocol No.:

TITLE: Draft Prevalidation of the Sliced Testis Assay

SPONSOR: Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2693

TESTING FACILITY: [Name and address of lab]

PROPOSED EXPERIMENTAL START DATE:

PROPOSED EXPERIMENTAL TERMINATION DATE:

AMENDMENTS:

Number	Date	Section(s)	Page(s)
1			
2			
3			
4			

APPROVED BY:

[Name, title, lab affiliation] Date
Study Director

[Name, title, lab affiliation] Date
Management

Gary E. Timm Date
Work Assignment Manager
Endocrine Disruptor Screening Program
U.S. EPA

David P. Houchens, Ph.D. Date
Program Manager
Endocrine Disruptor Screening Program
Battelle Memorial Institute

L. Greg Schweer Date
Project Officer
Endocrine Disruptor Screening Program
U.S. EPA

REVIEWED BY:

J. Thomas McClintock, Ph.D. Date
EPA Quality Assurance Manager

Terri L. Pollock, B.A. Date
Quality Assurance Manager
Battelle Memorial Institute

[Name, title, and lab affiliation] Date
Quality Assurance Specialist

TABLE OF CONTENTS

1.0 BACKGROUND AND OBJECTIVES

1.1 BACKGROUND

In 1996, the Food Quality Protection Act (FQPA) amendments were enacted by Congress to authorize the EPA to implement an Endocrine Disruptor Screening Program (EDSP) on pesticides and other substances found in food or water sources for endocrine effects in humans (FQPA, 1996). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial substances, and pesticides. A two-tiered approach will be utilized. Tier 1 employs a combination of *in vivo* and *in vitro* screens, and Tier 2 involves *in vivo* testing methods using two-generation reproductive studies. A steroidogenesis assay is proposed as one of the Tier 1 screening battery assays.

A detailed review paper (DRP) about steroidogenesis was prepared. The DRP (1) summarized the state of the science of the *in vivo, ex vivo,* and *in vitro* methodologies available for measuring gonadal steroidogenesis; (2) for each methodology, presented a review of the individual assays and representative data generated by investigators who used the assay to evaluate a substance for steroidogenic-altering activity; (3) provided an evaluation of the various methodologies and the assays as tools for screening substances with suspected steroidogenic activity; (4) recommended a particular screening method and assay as a screening tool; and (5) described the strengths, weaknesses, and implications for further research associated with the recommended screening assay.

The *in vitro* sliced testis steroidogenesis assay was selected as the most promising screening tool for identifying substances with steroidogenic-altering activity. The sliced testis assay was recommended because it can be conducted at a minimal cost, quickly, and simply with standard laboratory equipment and basic laboratory training; the preparation is stable and the parenchyma remains viable over a sufficient time period to measure changes in end-product hormone production; the assay is relatively sensitive and specific; the assay uses parenchyma that maintains the cytoarchitecture of the organ; the assay uses a reduced number of animals (up to quartered testis slices); the assay will be relatively easy to standardize (by optimization); and the assay has a well-defined endpoint in testosterone and, if desired, can be modified to include additional intermediate hormonal endpoints. The experiments necessary to identify the factors and conditions that optimize the assay have been completed and much of the data analysis has been finished. While there may be some minor changes to the procedure, the assay as described in the present protocol describes what is presently believed to be the optimized assay procedure. As such, this procedure will be used to proceed with the experiments believed necessary to pre-validate the assay.

1.2 OBJECTIVES

The objectives of the prevalidation studies will be to assess the relevance of the sliced testes assay for detecting compounds that inhibit steroidogenesis. Relevance will be assessed by demonstrating that the assay can detect inhibition of steroid hormone synthesis by determining the change in the production of testosterone.

These prevalidation studies will be performed by a lead laboratory and three additional participating laboratories. The table below summarizes the different studies to be conducted and the laboratories that will be involved in the conduct of each study.

Study Phase	Study Type	Laboratory Assignment
1	Baseline/Contralateral Testis Fragment	Lead Laboratory
2	Evaluation of AG as Positive Control	Lead Laboratory
3	Selection of Cytotoxicant as Positive Control	Lead Laboratory
4	Training/Hands-On Experience	Lead Laboratory + 3 Participating Laboratories
5	Baseline Experiment	Lead Laboratory + 3 Participating Laboratories
6	Positive Control Experiment	Lead Laboratory + 3 Participating Laboratories
7	Multichemical Experiments	Lead Laboratory

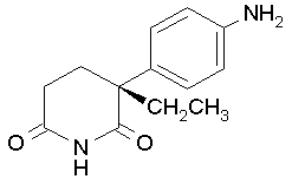
2.0 CHEMICALS

Below are a list of reagents to be used and test chemicals to be tested using the assay. Information provided for each test chemical includes identification and physical-chemical properties.

2.1 TEST CHEMICALS

2.1.1 Aminoglutethimide (Positive Control)

CAS Number: 55511-44-9
Synonyms:
Lot Number:
Purity:
Appearance: Solid
Molecular Formula: $C_{13}H_{16}N_2O_2$
Molecular Weight: 232.3
Storage, Bulk Chemical:
Storage, Test Solution:



2.1.2 Dimethoate

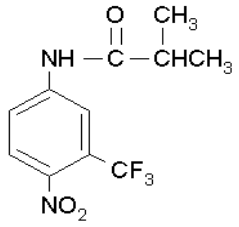
CAS Number: 60-51-5
Synonyms: MDL# MFCD00053676
Lot Number:
Purity:
Supplier:
Appearance:
Molecular Formula: $C_6H_{12}NO_3PS_2$
Molecular Weight: 229.3
Storage, Bulk: 2-8^{0}C
Storage, Test Solution:

2.1.3 Fenarimol

CAS Number: 60168-88-9
Synonyms: MDL# MFCD00055325
Lot Number:
Purity:
Supplier:
Appearance:
Molecular Formula: $C_{17}H_{12}Cl_2NO$
Molecular Weight: 331.2
Storage, Bulk:
Storage, Test Solution:

2.1.4 Flutamide

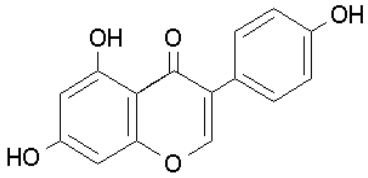
CAS Number: 13311-84-7
Synonyms: 2 Methyl-–(4 nitro-3-[trifluoromethyl]phenyl propanamide, MDL# MFCD00072009
Lot Number:
Purity:
Supplier:
Molecular Formula: $C_{11}H_{11}F_3N_2O_3$
Molecular Weight: 276.2
Storage, Bulk: Room Temp.
Storage, Test Solution:



2.1.5 Genistein

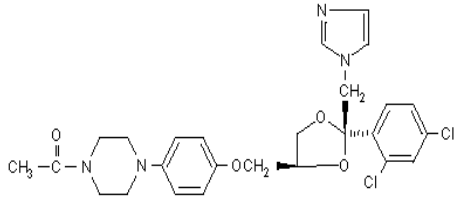
CAS Number: 446-72-0
Synonyms: 5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one
4′,5,7-Trihydroxyisoflavone, MLD # MFCD00016952
Lot Number:
Purity:

Supplier:
Appearance: off-white solid
Molecular Formula: $C_{15}H_{10}O_5$
Molecular Weight: 270.2
Storage, Bulk: -0^0C
Storage, Test Solution:



2.1.6 Ketoconazole

CAS Number: 65277-42-1
Synonyms:(±)-cis-1-Acetyl-4-(4-[(2-[2,4-dichlorophenyl]-2-[1H-imidazol-1-ylmethyl]-1,3-dioxolan-4-yl)-methoxy]phenyl)piperazine, MLD # MFCD00058579
Lot Number:
Purity:
Supplier:
Appearance:
Molecular Formula: $C_{26}H_{28}Cl_2N_4O_4$
Molecular Weight: 531.4
Storage, Bulk: 2-8^0C
Storage, Test Solution:



2.1.7 Diethylumbelliferyl Phosphate

CAS Number: 65277-42-1
Synonyms:(±)-cis-1-Acetyl-4-(4-[(2-[2,4-dichlorophenyl]-2-[1H-imidazol-1-ylmethyl]-1,3-dioxolan-4-yl)-methoxy]phenyl)piperazine, MLD # MFCD00058579
Lot Number:
Purity:
Supplier:
Appearance:
Molecular Formula: $C_{26}H_{28}Cl_2N_4O_4$

Molecular Weight: 531.4
Storage, Bulk: 2-8^{0}C
Storage, Test Solution:

2.1.8 MK-434

CAS Number: 65277-42-1
Synonyms:(±)-cis-1-Acetyl-4-(4-[(2-[2,4-dichlorophenyl]-2-[1H-imidazol-1-ylmethyl]-1,3-dioxolan-4-yl)-methoxy]phenyl)piperazine, MLD # MFCD00058579
Lot Number:
Purity:
Supplier:
Appearance:
Molecular Formula: $C_{26}H_{28}Cl_2N_4O_4$
Molecular Weight: 531.4
Storage, Bulk: 2-8^{0}C
Storage, Test Solution:

2.1.9 Trilostane

CAS Number: 65277-42-1
Synonyms:(±)-cis-1-Acetyl-4-(4-[(2-[2,4-dichlorophenyl]-2-[1H-imidazol-1-ylmethyl]-1,3-dioxolan-4-yl)-methoxy]phenyl)piperazine, MLD # MFCD00058579
Lot Number:
Purity:
Supplier:
Appearance:
Molecular Formula: $C_{26}H_{28}Cl_2N_4O_4$
Molecular Weight: 531.4
Storage, Bulk: 2-8^{0}C
Storage, Test Solution:

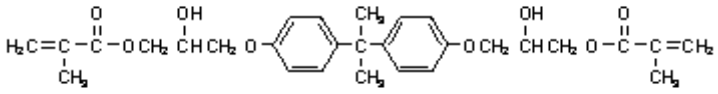
2.1.10 Vinclozolin (Negative Test Chemical)

Chemical Name: 3-(3,5-Dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione
CAS Number: 5-0471-44-8
Supplier: BASF
Manufacturer's Batch No.: To be added to the protocol by amendment
Appearance: To be added to the protocol by amendment
Molecular Formula: $C_{12}H_9Cl_2NO_3$
Molecular Weight: 286.114
Storage Conditions of Bulk Chemical:To be added to the protocol by amendment

Storage Conditions of Dosing Suspensions:To be added to the protocol by amendment

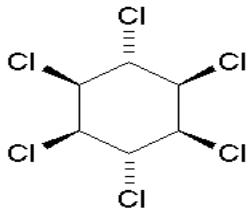
2.1.11 Bisphenol A

CAS Number: 1565-94-2
Synonyms: MDL # MFCD01317012
Lot Number:
Purity:
Appearance: liquid
Molecular Formula: $C_{29}H_{36}O_8$
Molecular Weight: 512.6
Storage, Bulk:
Storage, Test Solution:



2.1.12 Lindane

CAS Number: 58-89-9
Synonyms:1α,2α,3β,4α,5α,6β-Hexachlorocyclohexane, γ-1,2,3,4,5,6-Hexachlorocyclohexane,
MDL number: MFCD00135947
Lot Number:
Purity:
Supplier:
Appearance:
Molecular Formula: $C_6H_6Cl_6$
Molecular Weight: 290.8
Storage, Bulk: Room Temp
Storage, Test Solution:



2.2 STANDARDS

Standardization of the analytical assays will require testosterone for the RIA method. In addition, hCG will be used as a stimulant of the sliced testis bioassay. These substances will be considered standards and will be handled and documented according to the laboratory's Standard Operating Procedures for standards.

2.2.1 Testosterone

Chemical Name: Testosterone
CAS No.:58220
Molecular Formula/Weight:288.4
Solubility: Clear colorless to very faint yellow solution at 100 mg/mL in chloroform
Supplier: Sigma-Aldrich Chemical Company
Lot No.: TBD
Purity:NLT 98%
Storage Conditions: 2 Year shelf life
A safety protocol exists for the use of the radioactive form of testosterone.

2.2.2 Human Chorionic Gonadotropin (hCG)

Chemical Name: hCG
CAS No.: 9002-61-3
Molecular Formula/Weight: 36,700
Solubility: H2O
Supplier: Calbiochem
Lot No.: TBD
Purity: TBD
Storage Conditions: Freezer (-20 degrees C). Following reconstitution, aliquot and freeze (-20 degrees C). Stable for 2 years as supplied.

2.3 CHEMICAL SAFETY AND HANDLING

Once the chemicals to be tested and used according to the final protocol are determined, then an MSDS for each chemical will be included as an attachment to the protocol.

3.0 ANIMALS

3.1 TEST ANIMALS

An IACUC protocol will be submitted and approved before the beginning of the prevalidation experiments. Animals will be sequentially ordered on an as needed basis for each of the experiments.

3.1.1 Species

Sprague Dawley Derived Outbred Albino Rat [Crl:CD® (SD)IGSBR], known as the Charles River CD® Rat.

3.1.2 Supplier

Charles River Laboratories, Inc., Raleigh, NC.

3.1.3 Rationale

The use of live animals is necessary to provide fresh testicular tissue for the assay optimization. Alternative test systems are not available for the assessment of chemical effects on the cultured testicular cells. The albino rat is the species of choice and the Sprague-Dawley rat has been employed in previous reproductive toxicity studies.

3.1.4 Number and Sex

Male rats will be used to conduct the experiments in this protocol. Regarding the number of animals that will be used, an approximation can be made based on the design of each experiment. Approximately 8 - 10 fragments can be obtained from each testis. The numbers below are nominal numbers and actual numbers used may vary based individual laboratory procedures, e.g.sentinel animals for health monitoring.

Study Phase	Study Type	Number of Male Rats
1	Baseline/Contralateral Testis Fragment	10
2	Evaluation of AG as Positive Control	2
3	Selection of Cytotoxicant as Positive Control	4
4	Training/Hands-On Experience	8
5	Baseline Experiment	12

Study Phase	Study Type	Number of Male Rats
6	Positive Control Experiment	36
7	Multichemical Experiments	~40

3.1.5 Age and Weight

The animals will be approximately 10 weeks of age on the scheduled animal receipt date for the experiments. Testes removal will be when the animals are between 11 and 15 weeks of age. Males should be approximately 275 to 400 grams. Testis that weight 1000 ± 200 mg will be used for testing.

3.1.6 Body Weight

All animals will be weighed at least once during acclimation and quarantine.

3.1.7 Identification

Animals shall be uniquely identified by eartag within three days of arrival. The method of identification and animal numbers will be documented in the study records.

3.1.8 Limitation of Discomfort

There should be no discomfort or injury to animals; if any animal becomes severely debilitated or moribund, it will be humanely terminated by CO_2 asphyxiation. All necropsies will be performed after terminal anesthesia with CO_2. Animals will not be subjected to undue pain or distress.

3.1.9 Culled Animals

Animals received with the shipment, but not used in the study, will be removed from the study room. Records will be kept documenting the fate of all animals received for the study.

3.1.10 Quality Control and Sentinels

Sentinel Animals will not be necessary since the animals will not be in house for more than 1 month. The animals will be ordered as close as possible to the time of use. If any quality control is necessary, it will be conducted on the test animals during the quarantine period.

3.2 HUSBANDRY

3.2.1 Conditions

The animal portions of this protocol will be carried out under standard laboratory conditions. The animals will be housed 1 per cage upon arrival, during the acclimation period, and until used for testing in solid-bottom polycarbonate cages with stainless steel wire lids (Laboratory Products, Rochelle Park, NJ), with Sani-Chip® cage litter (P.J. Murphy Forest Products Corp., Montville, NJ). The cage dimensions will be 8"x19"x10.5" (height) for all phases of this study.

All animals will be housed in approved Animal Facilities following arrival and until needed. The animal rooms should be air-conditioned, and temperature and relative humidity are continuously monitored, controlled, and recorded using an automatic system. The target environmental ranges will be 64 to 79˚F (18-26˚C) for temperature and 30 to 70% relative humidity with a 12 hours light:12 hours dark cycle per day (NRC Guide, 1996; see below). Temperature and/or relative humidity excursions outside the target ranges will be documented in the study records and in the final report. The specific animal rooms used will be documented in the study records and in the final report. At all times, the animals will be handled, cared for, and used in compliance with the NRC Guide for the Care and Use of Laboratory Animals (1996).

3.2.2 Diet

Purina Certified pelleted Rodent Diet® (No. 5002, PMI Feeds, Inc., St. Louis, MO) will be available ad libitum. The analysis of each feed batch for nutrient levels and possible contaminants will be performed by the supplier, examined by the Study Director, and maintained in the study records. It is anticipated that contaminant levels will be below certified levels and will not affect the design, conduct, or conclusions of this study. The feed will be stored at approximately 60-70˚F, and the period of use will not exceed six months from the milling date.

3.2.3 Water

Animals will receive tap water (at RTI this will be the city municipal supply. Water will be available ad libitum by plastic water bottles with butyl rubber stoppers and stainless steel sipper tubes. The supplier per EPA specifications should measure contaminant levels of the city water at regular intervals. Documentation of these analyses will be inspected by the Study Director and maintained in the study records. It is anticipated that contaminant levels will be below the maximal levels established for potable water and will not affect the design, conduct, or conclusions of these studies.

4.0 SLICED TESTIS ASSAY PROCEDURE

[Note: The procedure described below is the preliminary optimized assay pending completion of the sensitivity analysis of the optimization study results.]

The assay procedure will begin at a time that will enable the technician to complete all incubations and sample collections before approximately noon on a given day (does not include the analyses). An 11-week old male Sprague-Dawley rat will be euthanized. The testes will be removed [develop SOP for testis removal and preparation]. The testes will be weighed [acceptance criterion] and placed in cold (4^0 C) Dulbeccos Phosphate Buffered Saline (DPBS; undiluted; pH 7.2 - 7.4). The time from removal of the testis to the time of slicing will be less than 1 hour. Each testis will have the tunica albicans removed and will be sliced longitudinally to yield pieces weighing within 10% of 0.175 grams (between 0.157 g and 0.192 g). [actual slice weight to be determined based on optimization results]. Each slice will be placed into a tightly capped scintillation vial containing 5 mL of 95% O_2 / 5% CO_2 gassed media. [develop SOP for testis slice distribution among test tubes]. The media will be medium-199 without phenol red (Invitrogen), which will have been adjusted to a pH of 7.4. [check on media components].

The vials containing the testicular sections and media will be incubated at 36^0C [temperature to be determined based on optimization results] on a shaker at 135 rpm [actual RPMs will be based on optimization study results]. After the first period of incubation, 1 hour [actual equilibration period will be based on optimization study results], the contents of the vial will be filtered using filter paper [check on use of gentle centrifugation]. The equilibration media will be discarded without analysis. If microscopic examination of the fragment is to be performed, then remove a piece of the fragment, transfer it to a screw-cap cryovial, snap freeze the sample in liquid nitrogen, and store it in a -70 degrees freezer until removed for further processing. New media (5.0 mL) will be placed in the original vial and the testis tissue will be returned to the vial. One aliquot (0.5 mL) of media [need further evaluation of aliquot volume] will be collected for analysis (Time 0, Baseline Sample). The sample aliquot will be analyzed for testosterone. [develop SOPs for testosterone RIA] To one-half of the vials, a 0.5 mL volume of media without hCG (unstimulated) will be added, and to the other half of the vials, 0.5 mL of media with hCG (stimulated; final concentration to be determined based on optimization study results) will be added to the vials to replace the volume removed for analysis, as well as to initiate testosterone production (hCG stimulated vials only). [Note: For assays using a test chemical, the test chemical will also be formulated in the media so that when the replacement media is added, at time 0, the final target concentration will be achieved.]

At 1, 2, 3 and 4 hours post-challenge, one 0.5 mL sample of media will be collected from each of the hCG-stimulated and non-stimulated vials. After each sampling, the appropriate media with or without hCG will be added to the vial to replace the volume removed for analysis. The sample aliquots removed at each time point will be analyzed for testosterone. Also, at the end of the incubation period, e.g. 4 hours, remove the tissue fragment and collect a sample for

microscopic examination. Transfer the tissue piece to a cryovial, snap freeze it in liquid nitrogen, and store at -70 degrees C until removed for processing.

Media samples collected at the specified time points will be frozen and stored at -70 degrees C and analyzed within one month after collection. Testosterone will be analyzed in duplicate for each sample using an RIA method modified for rat samples. All samples for a given day's set of runs will be analyzed in the same testosterone RIA , if possible. Tissue samples will be thawed, sectioned, stained, and mounted for microscopic examination. The staining procedure is specific for beta-HSD, an enzyme specific to Leydig cells.

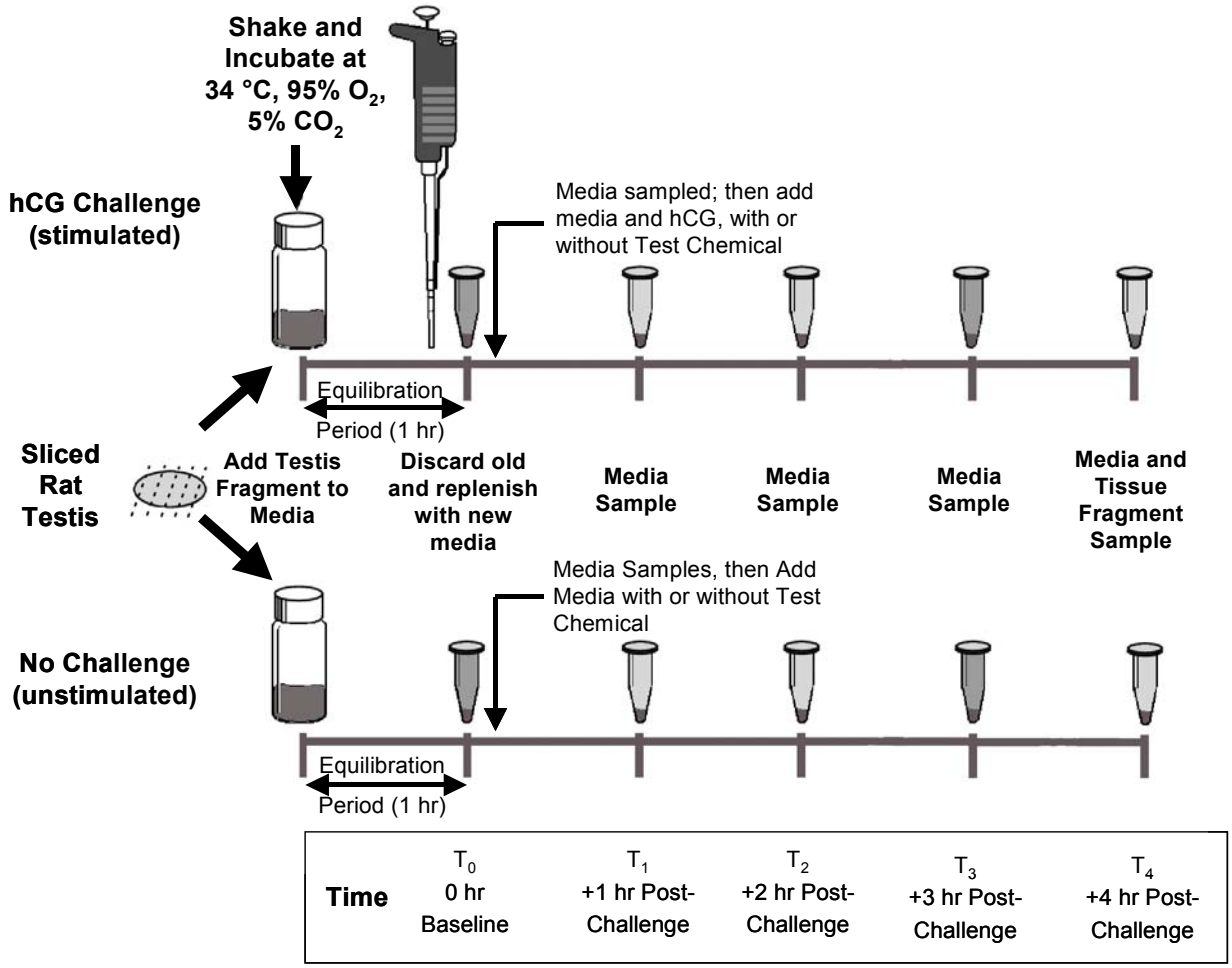


Figure 1. Technical Flow Illustration of the Sliced Testis Assay

5.0 Prevalidation STUDY DESIGN

5.1 BASELINE/CONTRALATERAL TESTIS FRAGMENT EXPERIMENT

This experiment will be conducted by the lead laboratory only.

The purposes of this experiment are:

- to demonstrate competence of lead lab using optimized assay conditions
- to estimate response trends in T production in the absence of inhibiting chemicals
- to estimate Leydig cell density after four hours of incubation, in the absence of inhibiting chemicals
- to determine the variability in assay response associated with animals, contralateral testes within animals, testis fragments within testes

The experimental design is summarized in the following table:

Time (Hours from Equilibration)	hCG	Animal	Testis	Fragment #	Incubation (Run) #
0	no	1 - 3	A	1	1 -3
	yes			2	4 - 6
1	no	1 - 3	A	1	1 -3
	yes			2	4 - 6
2	no	1 - 3	A	1	1 - 3
	yes	1 - 3	A	2	4 - 6
		1	B	1, 2	7, 8
		2	A	3, 4	9, 10
			B	1, 2	11, 12
		3	A	3, 4	13, 14
			B	1, 2	15, 16
		4	A	1, 2	17, 18
			B	1, 2	19, 20
		5	A	1, 2	21, 22
			B	1, 2	23, 24

3	no	1 - 3	A	1	1 - 3
	yes			2	4 - 6
4	no	1 - 3	A	1	1 - 3
	yes	1 -3	A	2	4 - 6
		1	B	1, 2	7, 8
		2	A	3, 4	9, 10
			B	1, 2	11, 12
		3	A	3, 4	13, 14
			B	1, 2	15, 16
		4	A	1, 2	17, 18
			B	1, 2	19, 20
		5	A	1, 2	21, 22
			B	1, 2	23, 24

The experiment summarized in the above table represents one replicate study. A replicate study is an independently conducted experiment; separated by day and using freshly prepared reagents. Two full replicates will be conducted for runs 1 - 24 and a third partial replicate will be carried out for runs 1 - 6. Runs 1-6 within each replicate will use two fragments taken from a single testis from each of three animals. Runs 7-24 within each of the two full replicate will use five animals, two testes within each animal, and two fragments from each testis. Each replicate will use different animals. The total number of individual fragments and incubations used to conduct this experiment is 24.

The sampling time points from the media range from 2 to 5 depending on the sample type. If two samples, then the time points are at 2 and 4 hours post-equilbration. If five, then the sampling time points are 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. Thus, the overall total number of testosterone samples for analysis is 324 samples [Runs 1 - 6 has 5 time points with duplicate analyses for 60 samples and runs 7 - 24 has 2 time points with duplicate analyses for 72 samples, which gives a total of 132 samples/replicate.

5.2 EVALUATION OF AMINOGLUTETHIMIDE (AG) AS A POSITIVE CONTROL

This experiment will be conducted by the lead laboratory only.

The purpose of this experiment is to determine the optimized assay response to AG when tested at three concentrations. From this information, AG may be selected as a positive control when the sliced testis assay is used to evaluate the effect of a test chemical. The experimental design is summarized in the following table:

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment ID Number
Media-Vehicle control	no	3	1 - 3
Media-Vehicle control	yes	3	4 - 6
Media + AG (low)	yes	3	7 - 9
Media + AG (mid)	yes	3	10 - 12
Media + AG (high)	yes	3	13 - 15

The experiment summarized in the above table represents one replicate study. A replicate study is an independently conducted experiment; separated by day and using freshly prepared reagents. Two replicates will be conducted. The overall experiment will use three rats/replicate study; three testes total/replicate study (based on using 5 fragments/testis). Within each replicate, the 5 fragments from a single testis will be distributed among the 5 test conditions. The overall total number of individual fragments and incubations used to conduct this experiment is 30.

The sampling time points (5) from the media are 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. In addition, at the end of the incubation period, a piece of the tissue fragment will be collected and snap-frozen for beta-HSD staining and microscopic analysis. Thus, the overall total number of testosterone samples for analysis is 300 samples [30 runs x 5 sampling time points x 2 (duplicate) analyses] and the overall total number of tissue samples is 30 [30 runs x 1 time point].

5.3 CYTOTOXICITY STUDY

This experiment will be conducted by the lead laboratory only.

The purpose of this experiment is to determine the optimized assay response to various cytotoxicants. Each cytotoxicant will be tested at three concentrations. From this information, one cytotoxicant may be selected as a positive control and included in the experimental design when the sliced testis assay is used to evaluate the effect of a test chemical.

The cytotoxicants to be tested are: SDS; 2,4-dinitrophenol; methylnitrosourea; sodium azide. Three concentrations of each cytotoxicant will be tested.

Prior to using a given cytotoxicant in the sliced testis assay, the highest concentration of the cytotoxicant to be tested will be evaluated for interference with the testosterone RIA. Briefly, a mid concentration testosterone standard will be spiked with the cytotoxicant and compared to the results obtained from a vehicle spiked control (same final volumes). If there is no interference, then the cytotoxicant will be tested using the sliced testis assay.

The experimental design is summarized in the following table:

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment(s)
Media-Vehicle control	no	3	1 - 3
Media-Vehicle control	yes	3	4 - 6
Positive control	yes	3	7 - 9
Media + Cytotoxicant A (low)	yes	3	10 - 12
Media + Cytotoxicant A (mid)	yes	3	13 - 15
Media + Cytotoxicant A (high)	yes	3	16 - 18
Media + Cytotoxicant B (low)	yes	3	19 - 21
Media + Cytotoxicant B (mid)	yes	3	22 - 24
Media + Cytotoxicant B (high)	yes	3	25 - 27

The experiment summarized in the above table represents one replicate study. Note that two cytotoxicants are tested per replicate study. A replicate study is an independently conducted experiment; separated by day and using freshly prepared reagents. Two replicates will be conducted. The overall experiment will use three rats/replicate study; three testes total/replicate study (based on obtaining 8 - 10 fragments/testis). Testis will be at least 1000 mg so that at least 9 fragments are obtained per testis. The overall total number of individual fragments and incubations used to conduct this experiment is 54.

The sampling time points (5) from the media are 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. In addition, at the end of the incubation period, a piece of the tissue fragment will be collected and snap-frozen for beta-HSD staining and microscopic analysis. Thus, the overall total number of testosterone samples for analysis is 540 samples [54 runs x 5 sampling time points x 2 (duplicate) analyses] and the overall total number of tissue samples is 54 [54 runs x 1 time point].

5.4 TRAINING/HANDS-ON EXPERIENCE

The lead and participating (3) laboratories will be involved in the conduct of this phase of prevalidation.

Training of the participating laboratories' technical staff will take place at the lead laboratory. The senior investigator/head technician for each participating laboratory will be trained in the conduct of the assay at the lead laboratory. The training will include a review of the relevant study documents and any necessary demonstrations regarding various aspects of the assay (starting at testis removal through to data analysis). Furthermore, the lead laboratory will be set-up to allow the participating laboratory staff to demonstrate proficiency in conducting the assay by supervising them as they conduct a baseline (media only) experiment and a positive control experiment - both of which are described in further detail in the following subsections. However, only one replicate of each experiment will be conducted by each participating laboratory.

5.5 BASELINE EXPERIMENT

The lead and participating (3) laboratories will be involved in the conduct of this phase of prevalidation.

The purposes of this study are:

- to estimate baseline inter- and intra-laboratory variability (which will also provide information to design the validation experimental design)
- to evaluate the knowledge transfer of assay procedures to participating laboratories.
- to begin collection of historical control and variability data.

The experimental design of this study is summarized in the following table:

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment ID Number
Media	no	3	1 - 3
Media	yes	3	4 - 6

The information summarized in the table is one replicate of the experiment. A total of three independent replicates will be conducted by each laboratory. The overall study will use one rat/replicate study and one testis total/replicate study. The overall total number of individual fragments or incubations used is 18 for each laboratory [(3 runs with + 3 runs without hCG) x 3 replicate studies].

The sampling time points (5) from the media are at time 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. In addition, at the end of the incubation period, a piece of the tissue fragment will be collected and snap-frozen for 3β-HSD staining and microscopic examination. Thus, the overall total number of testosterone samples for analysis is 180 samples/ laboratory [18 runs x 5 time points x 2 (duplicate) analyses]. The overall total number of tissue samples for microscopic examination are 18/laboratory [18 runs x 1 time point].

5.6 POSITIVE CONTROL EXPERIMENT

The lead and participating (3) laboratories will be involved in the conduct of this phase of prevalidation.

The purposes of this study are:

- to evaluate aminoglutethimide (AG) as a positive control for the assay
- to determine AG (positive control) inter- and intra-laboratory variability
- to determine time effect on the assay by comparing Baseline to Pilot experiments using the media control data
- to begin collection of historical positive control and variability data

The experimental design of this study is summarized in the following table.

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment(s)
Media-Vehicle control	no	3	1 - 3
Media-Vehicle control	yes	3	4 - 6
Cytotoxicant control	yes	3	7 - 9
Media + AG (low)	yes	3	10 - 12
Media + AG (mid)	yes	3	13 - 15
Media + AG (high)	yes	3	16 - 18

The information summarized in the table is one replicate of the experiment. The non-stimulated incubations (non hCG) were excluded from the study since the affect of AG on the non-stimulated tissue fragments would not be expected to reduce the baseline levels to any great extent. A total of three independent replicates will be conducted by each laboratory. The overall study will use three rats/replicate study and three testes total/replicate study. Six fragments will be taken from one testis from each rat and allocated to the six test conditions. The overall total number of individual fragments or incubations used is 54 for each laboratory.

The sampling time points (5) from the media are at time 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. In addition, at the end of the incubation period, a piece of the tissue fragment will be collected and snap-frozen for 3β-HSD staining and microscopic examination. Thus, the overall total number of testosterone samples for analysis is 540 samples/ laboratory [54 runs x 5 time points x 2 (duplicate) analyses]. The overall total number of tissue samples for microscopic examination are 54/laboratory [54 runs x 1 time point].

5.7 MULTICHEMICAL EXPERIMENTS

This experiment will be conducted by the lead laboratory only.

The purposes of this study are:

- to determine the response of the assay when challenged with putative positive test chemicals. The test chemicals will differ in their mode(s) of action, which will include altering signal transduction, cholesterol transport into the mitochondria, and the series of enzymatic reactions that lead to the production of testosterone in the gonadal steroidogenic pathway.
- to determine the response of the assay when challenged with putative negative test chemicals.

- to select test chemicals and concentrations that would be used for validation of the assay.
- to estimate interlaboratory variability based on the results from the assay using the selected test chemicals.

The test chemicals and their concentrations that will be tested are summarized in the following table:

Test Chemical	Mode of Action[a]	Test Concentrations[b]	Reference
Aminoglutethimide (positive control)	$P450_{SCC}$, aromatase	10, 100, and 1000 µM	Powlin et al., 1998; Johnston, 1997; Uzgiris et al., 1977
Diethylumbelliferyl phosphate	cAMP-stimulated accumulation of StAR	TBD	Choi et al., 1995
Dimethoate	StAR protein	TBD	Walsh et al., 2000
Ketoconazole	$P450_{SCC}$, P450c17	0.1, 1, and 10 µM	Powlin et al., 1998; Kan et al., 1985; Albertson et al., 1988; DeCoster et al., 1989; Chaudhary/Stocco, 1989; Malozowski et al., 1985, 1986
Trilostane[c]	3β-HSD	TBD	Takahashi et al., 1990
Genistein or epostane	3β-HSD	TBD	Ohno et al., 2002; Tanaka et al., 1992
Flutamide	P450c17	10, 100, and 1000 µM	Powlin et al., 1998; Ayub and Levelll, 1987
Finasteride[d] or MK-434	5α-reductase	10, 100, and 1000 µM (MK-434 - TBD)	Morris, 1996
Fenarimol	aromatase	TBD	Vinggaard et al., 2000
Vinclozolin (negative chemical)	antiandrogen	TBD	--
Bisphenol A	cAMP	TBD	--
Lindane	cAMP	TBD	--

a. Site of action that leads to a decrease in Testosterone concentration for those chemicals with inhibitory activity. Negative test chemicals would have no effect on the testosterone concentration.
b. Final concentrations in the incubation mixture.
c. Requires synthesis by commercial laboratory. Availability may be difficult due to proprietary claim.
d. Commercial source may limit availability.

The experimental design of this study for any two test chemicals is summarized in the following table:

Sample Type	hCG	Number of Incubations (Runs)	Testis Fragment(s)
Media-Vehicle control	no	3	1 - 3
Media-Vehicle control	yes	3	4 - 6
Positive control - Aminogluthethimide @ 1 conc	yes	3	7 - 9
Cytotoxicant control - TBD @ 1 conc	yes	3	10 - 12
Media + Test Chemical A (low)	yes	3	13 - 15
Media + Test Chemical A (mid)	yes	3	16 - 18
Media + Test Chemical A (high)	yes	3	19 - 21
Media + Test Chemical A (high)	no	3	22 - 24
Media + Test Chemical B (low)	yes	3	25 - 27
Media + Test Chemical B (mid)	yes	3	28 - 30
Media + Test Chemical B (high)	yes	3	31 - 33
Media + Test Chemical B (high)	no	3	34 - 36

The information summarized in the table is one replicate of the experiment. The non-stimulated incubations (non hCG) were excluded from the study since the affect of AG on the non-stimulated tissue fragments would not be expected to reduce the baseline levels to any great extent. However, a high concentration test chemical with non-stimulated tissue fragment was retained in the experimental design to confirm this response. In addition, in the case when the assay would be used to evaluate a chemical with an unknown effect on the testosterone concentration, it would be important to test the non-stimulated fragment at the high concentration to determine whether the test chemical had a stimulatory effect on the testosterone concentration. A total of three independent replicates will be conducted. The overall study will use two rats/replicate study and four testes total/replicate study. The overall total number of individual fragments or incubations used is 72.

The sampling time points (5) from the media are at time 0 (after a 1 hour equilibration) and 1, 2, 3, and 4 hours post-equilibration. Each sample will be analyzed for testosterone in duplicate. In addition, at the end of the incubation period, a piece of the tissue fragment will be

collected and snap-frozen for 3β-HSD staining and microscopic examination. Thus, the overall total number of testosterone samples for analysis is 720 samples [72 runs x 5 time points x 2 (duplicate) analyses]. The overall total number of tissue samples for microscopic examination are 72 [72 runs x 1 time point].

6.0 CHEMISTRY

The test chemicals and AG (positive control) will be procurred, characterized, formulated, and analyzed as described below. A central chemistry laboratory will perform these activities. However, the formulations prepared by the central chemistry laboratory will be limited to the stock solution, which will be shipped to the assay testing laboratories, where the appropriate dilutions will be made.

6.1 CHEMICAL PROCUREMENT AND CHARACTERIZATION

A single lot of each test chemical will be procurred so that all prevalidation experiments are performed using the same lot. The lot obtained will have a purity >95 - 98 percent. Upon receipt of each test chemical, the identity will be confirmed by running an IR spectrum and comparing it to a reference spectrum from the manufacturer or from the literature. Within 30 days of using a test chemical in the assay, the purity will be determined using one chromatographic method and one additional or complementary method.

6.2 FORMULATION PREPARATIONS

Prior to testing, the solubility of the test chemical in the media at a concentration that will result in the desired final concentration in the incubation mixture will need to be determined. Formulation development will be based on the highest concentration needed for a given test chemical. In addition, a formulation will be developed using a) media only or b) a vehicle that can be used to dissolve the test chemical prior to mixing it with the media.

Only select solvents will be used as vehicles. The vehicles that will be used and their concentrations were determined during the optimization studies. Based on these results, selected concentrations of ethanol, DMSO, or Tween 20 will be used as vehicles if the chemical is not soluble in the media.

It will be important to confirm that the formulation is a solution. This will be done by analyzing the formulation before and after filtering since the results of the two analyses will be similar if the formulation is a solution. In addition, since the media is gassed in the assay, the solubility of gassed formulation will be tested.

Formulation development will seek to prepare a solution of the test chemical in media, with and without hCG, so that a given aliquot of the formulation, when added to the incubation mixture, will result in the desired final concentration of test chemical to be tested, as well as the final total incubation volume. Also, if a vehicle is needed to make the formulation, then the vehicle concentration would be formulated at an acceptable level.

When formulations for multiple laboratories are required, a formulation will be prepared in a sufficient batch size that will allow the same formulation to be dispensed to all laboratories. If different concentrations are to be tested for a given test chemical, then the central laboratory will prepare a stock solution, which will be shipped to the laboratories. Each laboratory will then use the stock solution to prepare the various concentrations to be tested.

Formulations, if solutions, will be prepared to within 5 percent of target.

6.3 FORMULATION ANALYSIS

For each chemical to be tested, a method for analysis will be developed for the test chemical in media/vehicle over the concentration range to be tested.

Prior to shipment of a formulation to the laboratory(ies), a sample of the stock solution formulation to be shipped will be taken and analyzed. Samples will be analyzed in duplicate. If the determined concentration is not within 5 percent of the target concentration, then the formulation will be re-prepared.

After the laboratory(ies) have used the diluted formulations, a sample will be shipped back to the central laboratory and archived for possible analysis at a later date if there is some question about the results of the assay.

6.4 FORMULATION STABILITY

Prior to being used in the assay, the formulations will be tested for stability. Stability testing will be performed at the concentration of the stock solution formulation. Stability will be determined at time 0 (day of preparation) and at weekly intervals for a period of at least 6 weeks and, thereafter, at 9 and 12 weeks. Stability will be tested at room temperature and at refrigerated temperatures. Stability will be tested using amber glass bottles with Teflon-lined lids.

7.0 ENDPOINT MEASUREMENTS

The sliced testis assay incubation media will be sampled at selected time points and the media analyzed for testosterone concentration using an RIA method. In addition, at the conclusion of the incubation period, the testis fragment will be sampled and processed for

Leydig cell identification using a staining procedure specific for 3β-HSD.

7.1 TESTOSTERONE RIA PROCEDURE

A radioimmunoassay (RIA) commercial kit (Diagnostic Products Corporation, Los Angeles, CA), that utilizes ^{125}I-testosterone and a testosterone-specific antibody affixed to polypropylene culture tubes, will be used to measure testosterone.

Testosterone (Sigma, St. Louis, MO; T-1500) to be used for preparing the standard curve will be stored desiccated at room temperature. A standard will be prepared in ethanol (0.1 mg/ml). Up to an 8-point standard curve but not less than a 4-point standard curve will be prepared using standards with concentrations of 0.07, 0.16, 0.41, 1.02 , 2.56, 6.4, 16, and 40 ng/ml in PBS-Gel Buffer (0.1 M phosphate buffered saline with 0.1% (w/v) sodium azide and 0.1% (w/v) gelatin, pH 7.4). In addition, procedural controls will be included in each run. These procedural controls will include low or high serum testosterone samples and two reagent blanks. The standard curve points and the procedural controls will be prepared in quadruplicate; the bioassay unknowns and the internal standards (see below) will be prepared in duplicate. The volume of all standards and controls (including bioassay unknowns) will be adjusted to 50 µL by adding the PBS-Gel Buffer. Next, 1 ml ^{125}I-testosterone will be added to each antibody-coated tube and mixed (Vortex). The tubes will be incubated in a 37˚C water bath for three hours, during which time testosterone, whether labeled or unlabeled, will compete for testosterone specific antibody binding sites. At the end of the incubation period, the free (unbound) testosterone, in the supernatant fluid of all tubes will be aspirated and wiped clean of fluid. The bound testosterone will be counted in a gamma counter for 1 minute. The concentration of testosterone will be estimated against the standard curve. Values will be reported as a mean concentration (ng/mL) of duplicate analyses.

Verification of the testosterone assay will involve preparation of internal standards (no less than three) using spiked media with concentrations ranging from 12.5 to 500 ng/mL. Each concentration will be run at each of three volumes - 10, 25, and 50 µL, to check for parallellism, and each sample will be adjusted to 50 µL by adding the PBS-Gel Buffer. The low and high standards will be analyzed in at least duplicate. Verification will be based on results determined for accuracy, precision, specificity, and linearity. Accuracy will be expressed as the relative error, which will be determined by comparing the measured to the target concentration. Relative errors within 10 percent will be acceptable. Precision will be expressed as the relative standard deviate (RSD) or coefficient of variance (CV), which will be determined by calculating the mean and standard deviation (sd) of the low and high standards. A RSD or CV within 10 percent will be acceptable. The sensitivity will be acceptable if the means of the blanks and low standards are significantly different at the 5 percent significance level. Linear determinations of the standard curve line will be made and a correlation coefficient (r) calculated. An r of 0.90 or greater will be considered acceptable.

Inter- and intra-assay variability will be determined. The intra-assay variability will be determined from the precision results calculated from the results obtained by measuring the low and high standards in triplicate. The inter-assay variability will be determined by repeating analysis of the standards by generating a standard curve on three different days.

7.2 3β-HSD LEYDIG CELL STAINING PROCEDURE

Analysis results will be reported as Leydig cell density (cells/cm^2).

A Leydig cell specific staining method and microscopic evaluation will be used to evaluate the viability of these cells. At the end of the incubation period, a piece will be taken from each fragment for staining. The media-vehicle control fragment will be used to evaluate the result of incubation with the test chemical treatment. The staining procedure is specific for cells containing 3β-HSD, a steroidogenic enzyme specific to the Leydig cells when examining testis tissue. The general staining procedure [Ref.: Payne et al., (1980). Endocrinology 106:1424; Klinefelter et al., (1993). In: Methods in Toxicology, Vol. 3, pp. 166-181.] is described as follows:

- after the last sample collection, snap freeze the tissue, section it (~15 um), and mount it on slides.
- stain the slide using the following solutions and procedure:

  a) etiocholanolone stock solution (1 mg/ml in DMSO),
  b) 2 mg Nitroblue Tetrazolium in 0.6 ml Etiocholanolone stock,
  c) 10 mg NAD+ dissolved in 9.5 ml warm Dulbeccos Phosphate buffered saline (DPBS), and
  d) 10 mg NAD+ dissolved in 9.5 ml warm Dulbeccos Phosphate buffered saline (DPBS).
     - Mix solutions b and c.
     - Cover section tissue on slide with staining solution for 1 - 2 hours.
     - Rinse in deionized water.
     - Fix in 10% formalin in DPBS with 5% sucrose.
     - Coverslip with glycerol:DPBS (1:1) and seal with nail polish.

8.0 DATA ANALYSIS

The results for each analysis will be reported individually, with sufficient identifying information to determine which results correspond to duplicate analyses, to different time points within one vial, to different vials within the same replicate, and to different replicates. The lead and participating laboratories will maintain a database to include all data generated during the study. The databases will have uniform structure, formatting, and variable naming across

laboratories. Test conditions, background environmental conditions, and results for each analysis for each sample at each time point will be reported.

Analysis results to be reported are testosterone (T) concentration (ng T/mg tissue/hour) and Leydig cell density (cells/cm^2). Detection limits and indications of inability to detect will be reported, as will confirmation of the acceptability or non-acceptability of each individual value.

9.0 STATISTICAL ANALYSIS

9.1 CONTRALATERAL TESTIS FRAGMENT EXPERIMENT

The principal endpoint is testosterone (T) concentration. T analyses will be carried out in duplicate. Results of the duplicate T analyses will be averaged. Statistical analysis will be carried out on the averages the duplicates.

The statistical analysis will be carried out by the lead laboratory. Mixed effects repeated measures models will be fitted to the data to describe trends in concentration vs. time and to estimate animal-to-animal, testis-to-testis within animal, and fragment-to-fragment within testis components of variation. For the T concentration analyses fixed effects terms in the models will describe linear and non-linear trends in T for hCG stimulated testis fragments and for non-stimulated testis fragments. Random effects terms in the model will estimate variance components that account for variation among fragments within testes, testes within animals, animals within replicates, and variation among replicates, primarily for the hCG stimulated testis fragments. A correlation structure will be fitted to describe the relation among the responses based on the same vial (i.e. the same fragment) at the various times. The testis-to-testis variation within animals and the fragment-to-fragment variation within testes will be determined only for the hCG stimulated testis fragments.

The results of the model fit will be parametric functions that describe the time trends and variance components that describe the within and among replicate variation about the time trends.

Residuals from the model fits will be examined to determine goodness-of-fit to the model assumptions, to assess the nature of the random variation about the model, and to search for outlying observations. Outliers may be individual responses that deviate from the trend within their vial, individual vials that deviate from the average across vials within their replicate, and individual replicates that deviate from the average across replicates. Each of these types of residuals will be examined.

Based on the model fits multiple endpoints will be reported. Endpoints include

- T concentrations at 1, 2, 3, 4 hours following equilibration, with and without hCG stimulation
- Ratios of T concentrations with and without hCG stimulation, at 1, 2, 3, 4 hours following equilibration
- Response trends, with and without hCG stimulation
- Ratios of trends with and without hCG stimulation.
- Average Leydig cell densities after two hours of incubation and after four hours, with and without hCG stimulation

For each response the average value across runs, the standard error of estimate, and confidence intervals about the average will be reported. Estimates of run-to-run variation within replicates and replicate-to-replicate variation will be reported. Separate variance components will be estimated for responses with hCG stimulation and for responses without hCG stimulation.

Random effects terms in the model will describe variance components that account for animal-to-animal variation, testis-to-testis variation within animal, and fragment-to-fragment variation within testis. These components of variation will be estimated for hCG stimulated testis fragments at two hours and at four hours following equilibration. A correlation structure will be fitted to describe the relation among the responses at the various times based on the same vial (i.e. the same fragment). The results of the model fit will be parametric functions that describe the time trends and estimates of the variance components.

For each response estimates of variance components will be reported.

9.2 BASELINE EXPERIMENT

The statistical analysis will be divided into intra-laboratory and inter-laboratory components. The intra-laboratory analyses will be carried out by each laboratory individually, based on a common analysis plan. Mixed effects repeated measures models will be fitted to the data to describe trends in concentration vs. time. Fixed effects terms in the models will describe linear and non-linear trends in T time trends for hCG stimulated testis fragments and for non-stimulated testis fragments. Random effects terms in the model will describe variance components that account for variation among the repeat vials within replicates and variation among replicates. A correlation structure will be fitted to describe the relation among the responses at the various times based on the same vial (i.e. the same fragment). The results of the model fit will be parametric functions that describe the time trends and variance components that describe the within and among replicate variation about the time trends.

Residuals from the model fits will be examined to determine goodness-of-fit to the model assumptions, to assess the nature of the random variation about the model, and to search for

outlying observations. Outliers may be individual responses that deviate from the trend within their vial, individual vials that deviate from the average across vials within their replicate, and individual replicates that deviate from the average across replicates. Each of these types of residuals will be examined.

Based on the model fits, multiple endpoints will be reported. Possible endpoints include T concentrations at 1, 2, 3, 4 hours following equilibration with and without hCG stimulation, ratios of T concentrations with and without hCG stimulation at 1, 2, 3, 4 hours following equilibration, response trends with and without hCG stimulation, and ratios of trends. For each response the average value across runs, the standard error of estimate, and confidence intervals about the average will be reported. Estimates of run-to-run variation within replicates and replicate-to-replicate variation will be reported.

The inter-laboratory analysis will be carried out by the DCC. The objective of the inter-laboratory analysis is to assess the extent of variation across laboratories with respect to average response and variability of response. For each endpoint the average response and associated standard error, the variation among runs within replicates, and the variation among replicates will be determined for each laboratory. Comparisons of within laboratory variance components will be made among the participating laboratories based on control charts to assess homogeneity of variance. The reference for comparisons will be either the lead laboratory results or the average results across all laboratories. If there is no evidence of lab-to-lab variation the within laboratory variance components will be pooled among laboratories. Otherwise separate within laboratory variance components will be specified for each laboratory.

The average responses will be compared across laboratories based on random effects one-way analysis of variance. The among laboratories variance in average response will be added to the within laboratory variance, resulting in the total assay variability. Estimates and confidence intervals will be reported for each laboratory based on the total assay variability. Estimates and confidence intervals will be reported on the differences between the participating laboratory results and the lead laboratory results and the differences between each laboratory's results and the average of the laboratory results based on the total assay variability.

9.3 POSITIVE CONTROL EXPERIMENT

Regarding data analysis and statistical evaluation, the statistical analysis will be divided into intra-laboratory and inter-laboratory components. The intra-laboratory analyses will be carried out by each laboratory individually, based on a common analysis plan. Mixed effects repeated measures models will be fitted to the data to describe trends in T concentration vs. time and trends in AG concentration. Fixed effects terms in the models will describe linear and non-linear T time trends and AG concentration trends for hCG stimulated testis and differences relative to the media-vehicle control. Random effects terms in the model will describe variance

components that account for variation among the repeat vials within replicates and variation among replicates. A correlation structure will be fitted to describe the relation among the responses at the various times based on the same vial (i.e. the same fragment) and at the various AG concentrations, based on fragments taken from the same testis. The results of the model fit will be parametric functions that describe the time trends and concentration trends and variance components that describe the within and among replicate variation about the time trends and concentration trends.

Residuals from the model fits will be examined to determine goodness-of-fit to the model assumptions, to assess the nature of the random variation about the model, and to search for outlying observations. Outliers may be individual responses that deviate from the time trend within their vial or from the concentration trend across vials, individual vials that deviate from the average across vials with the same conditions within their replicate, and individual replicates that deviate from the average across replicates. Each of these types of residuals will be examined.

Based on the model fits multiple endpoints will be reported. Possible endpoints will be include T concentrations at 1, 2, 3, 4 hours following equilibration for the media-vehicle control and graded AG concentrations, with hCG stimulation, ratios of T concentrations from the same groups with hCG stimulation at 1, 2, 3, 4 hours following equilibration, response trends in time or in AG concentration with hCG stimulation, and ratios of trends. For each response the average value across runs, the standard error of estimate, and confidence intervals about the average will be reported. Estimates of run-to-run variation within replicates and replicate-to-replicate variation will be reported. Differences between the T concentrations in the presence of AG and the T concentrations in the media-vehicle control group will also be analyzed.

The inter-laboratory analysis will be carried out by the DCC. The objective of the inter-laboratory analysis is to assess the extent of variation across laboratories with respect to average response and variability of response. For each endpoint developed in the intra-laboratory analysis the average response and associated standard error, the variation among repetitions within replicates, and the variation among replicates will be calculated for each laboratory. Comparisons of within laboratory variance components will be made among the participating laboratories based on control charts to assess homogeneity of variance. The reference for comparisons will be either the lead laboratory results or the average results across all laboratories. If there is no evidence of lab-to-lab variation the within laboratory variance components will be pooled among laboratories. Otherwise separate within laboratory variance components will be specified for each laboratory.

The average responses will be compared across laboratories based on random effects one-way analysis of variance. The among laboratories variance in average response will be added to the within laboratory variance, resulting in the total assay variability. Estimates and

confidence intervals will be reported for each laboratory based on the total assay variability. Estimates and confidence intervals will be reported on the differences between the participating laboratory results and the lead laboratory results and between each laboratory's results and the average of the laboratory results based on the total assay variability.

The responses included in the intra-laboratory and inter-laboratory analyses will be categorized as primary or secondary endpoints. The primary endpoints will be restricted to a small number (two to five) of the most important responses for comparisons. Power calculations to assess inference sensitivity versus sample size will be based on the primary endpoints. The studies will be powered to attain desired sensitivity for comparisons of the primary endpoints within or among laboratories. The secondary endpoints are the (relatively large number of) remaining responses that are included in the statistical analyses. The studies will not be powered for comparisons among the secondary endpoints.

For purposes of assessing sample size versus inference sensitivity for the validation tests, two responses have been selected as primary endpoints. These are:

- differences between T concentrations associated with the high aminoglutethimide concentration and the media-vehicle control at three hours past equilibration, with hCG stimulation.

- Sum of absolute differences between T concentrations associated with the high aminoglutethimide concentration and the media-vehicle control at 1, 2, 3, and 4 hours past equilibration, with hCG stimulation.

The second response approximates the area between the T concentration versus time curves for these two conditions.

Sensitivity analyses will be carried out based on response variability estimates obtained from the analyses. Numbers of replicates per laboratory necessary to detect heterogeneity of replicate-to-replicate variability within laboratories will be assessed. Numbers of laboratories and numbers of replicates per laboratory necessary to detect various levels of coefficient of variation across laboratories with high power or to detect various levels of ratio of between laboratory standard deviation to within laboratory standard deviation with high power will be determined.

9.4 MULTICHEMICAL EXPERIMENT

Separate statistical analyses will be carried out for each test chemical. Consideration will be given to pooling the media-vehicle control groups across those chemical tests that use the same vehicle. The statistical analysis considerations are the same as those described above in the

previous section, except that there are two replicates per chemical rather than three. Extent of replicate-to-replicate variation can be determined from these tests only in a qualitative manner.

10.0 RETENTION OF SPECIMENS AND RECORDS

All specimens and records that remain the responsibility of the testing laboratory will be retained in the testing laboratory's archives for the life of the contract.

11.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

This study will be conducted according to Good Laboratory Practice Guidelines. Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) that will be prepared for this study.

12.0 REPORTING

Interim data sets will be submitted from the lead laboratory to the EPA and Battelle upon summarization of the raw data and completion of a technical review for the experiments listed below:

- baseline/contralateral tissue fragment and positive control
- cytotoxicity
- training and hands-on results of participating laboratories

This interim data submission will not be audited by the Quality Assurance Unit and will be stamped as "Preliminary Data". The data sets will be reviewed promptly by the EPA so that a decision can be reached to determine whether to proceed to the next experiment.

Each participating laboratory will prepare a draft final report upon completion of the baseline and positive control experiments. A single draft final report that includes both experiments will be prepared. The draft final reports will be submitted to the lead laboratory, Battelle, and the EPA. Review comments from the lead laboratory and the EPA will be collected by Battelle and submitted to the respective participating laboratory for inclusion of the changes prior to their submission of a final report.

The lead laboratory will prepare a draft final report that will include the results of all experiments, including the final reports submitted by the participating laboratories. This overall draft final report will include the statistical analyses that evaluates inter- and intra-laboratory variability. Upon review of the draft final report by the EPA, the lead laboratory will submit a final report that incorporates any comments from the EPA's review of the draft final report.

The report format used by the laboratories will have a standardized format. This format will be provided to the laboratories prior to initiating report preparation. All reports will include introduction, methods and materials, results, discussion, and conclusion sections. Any difficulties in executing the studies should be noted and there should be data summaries as well as the raw data tables.

13.0 PERSONNEL

Personnel:

Study Director:
Principal Investigator:
Animal Research Facility Vet.:
Animal Research Facility Manager:
LORET Laboratory Supervisor:
Research Toxicologist:
Research Biologists:
Biologists:
Biological Laboratory Assistants:
Statistical Analyses:
Quality Assurance Unit (QAU):

14.0 STUDY RECORDS TO BE MAINTAINED

- Chemical Receipt, Storage and Use Records
- Animal Receipt Records
- Quarantine Animal Health Surveillance Records
- Balance Check Sheets
- Temperature and Relative Humidity Records
- Room Log Sheets
- Feed and Water Analyses
- All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and chemicals used
- Protocol and any Amendments
- List of any Protocol Deviations
- List of Standard Operating Procedures
- Computer records: all records of data sets and statistical analyses

APPENDIX B

Data Coordination and Distribution/Report Submission

DATA COORDINATION AND DISTRIBUTION/REPORT SUBMISSION

The Data Coordination Center (DCC) is the central coordinator of all information being generated under the EDSP. For the sliced testis assay prevalidation, the DCC will foster movement of data from the laboratories to the EPA. Critical data coordination and distribution points are anticipated to occur in between the completion of various experiments. To ensure accurate and timely coordination and analysis of interim data, as well as compilation of data for final report submission, the following procedures will be followed and serve as guidelines to the laboratories.

In general, there are two levels of deliverables. The first level consists of data that are generated upon completion of a given individual experiment and are used to make a decision to proceed to the next experiment. Such data sets will be referred to as interim data. The second level consists of all the data from all the prevalidation experiments. This data set is designated as final data and will be used for submission of the final report. Further descriptions of how each of these data sets will be handled and distributed are described in the following subsections.

INTERIM DATA REVIEW

There are several different decision points that will require submission of interim data for review. The interim data sets to be submitted for review will be generated after completion of the following experiments:

- Lead Laboratory's conduct of the baseline/contralateral tissue fragment and positive control experiments
- Lead Laboratory's conduct of the cytotoxicity experiments
- Training and Hands-on experience results from the participating laboratories conduct of the baseline and positive control experiments using the assay

The Lead laboratory will generate data upon completion of these experiments that will be used to make decisions to proceed to the next phase of prevalidation. In order for the decision to proceed to be made promptly, the lead laboratory will need to process its data rapidly. It is proposed that the laboratory will provide only a technical review of the data for accuracy before making the data available for outside review. This interim data review would proceed without the data going through a formal quality assurance audit. As such, the interim data sets would be stamped as unaudited interim data. In this way, the data generated after each phase could be more quickly transferred to the DCC for collation and statistical analysis of all data sets, thereby providing the EPA and Lead Laboratory with complete data sets and summarized data analysis for them to make decisions to proceed. The flow of interim data that is critical for making a decision to proceed with the next phase is illustrated in Figure B-1.

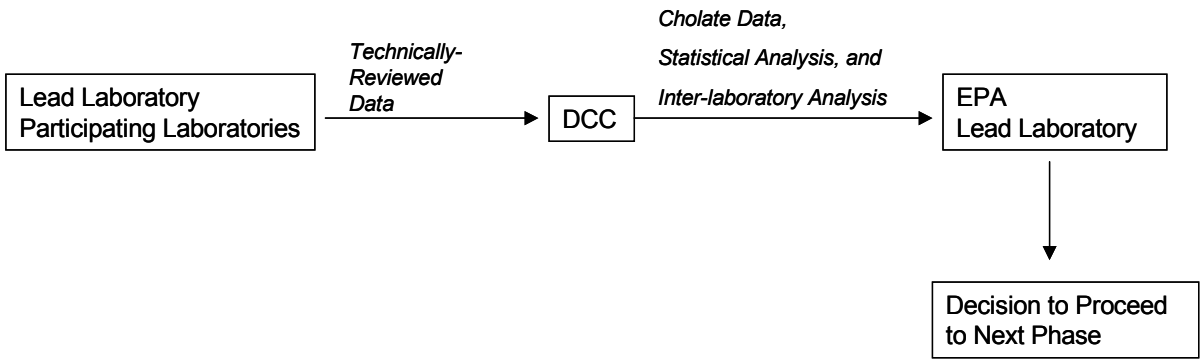


Figure B-1. Interim Data Flow Diagram

FINAL REPORT SUBMISSION

Final Reports will be submitted by all laboratories. The participating laboratories will submit a final report for the baseline and positive control experimental results. Their reports will be written after the positive control experiment is completed since the participating laboratories are not involved in the remaining prevalidation experiment (multi-chemical testing). By contrast, the lead laboratory is assigned the task of putting together the overall study report. It is expected that the lead laboratory will need the participating laboratories' reports, as well as the DCC's summary of the inter-laboratory analysis to complete the report. These reports will need to be prepared and sent to the lead laboratory, such that the overall study report can be completed immediately upon the completion of the multi-chemical test experiment. In general, the flow of information during the course of this study should proceed as illustrated in Figure B-2.

A unified format for data and report submission will be implemented. Prior to the start the training phase, the DCC staff will meet with the lead laboratory staff to discuss the format of the results and statistical analysis files for the data. Based on this discussion, the DCC will put together a draft template and provide it to the lead laboratory and the EPA for review. Comments will be collected via a conference call with the EPA and the lead laboratory staff. Following the conference call, a final template will be developed. The lead laboratory will distribute this information to the participating laboratories at the training session. These steps are recommended to ensure that data is presented in the same format from all laboratories in order to foster the collating process and the inter-laboratory analysis. By being proactive at the beginning of the study, this should minimize time for completing the inter-laboratory analysis, as well as problems with interpretation of data from each laboratory.

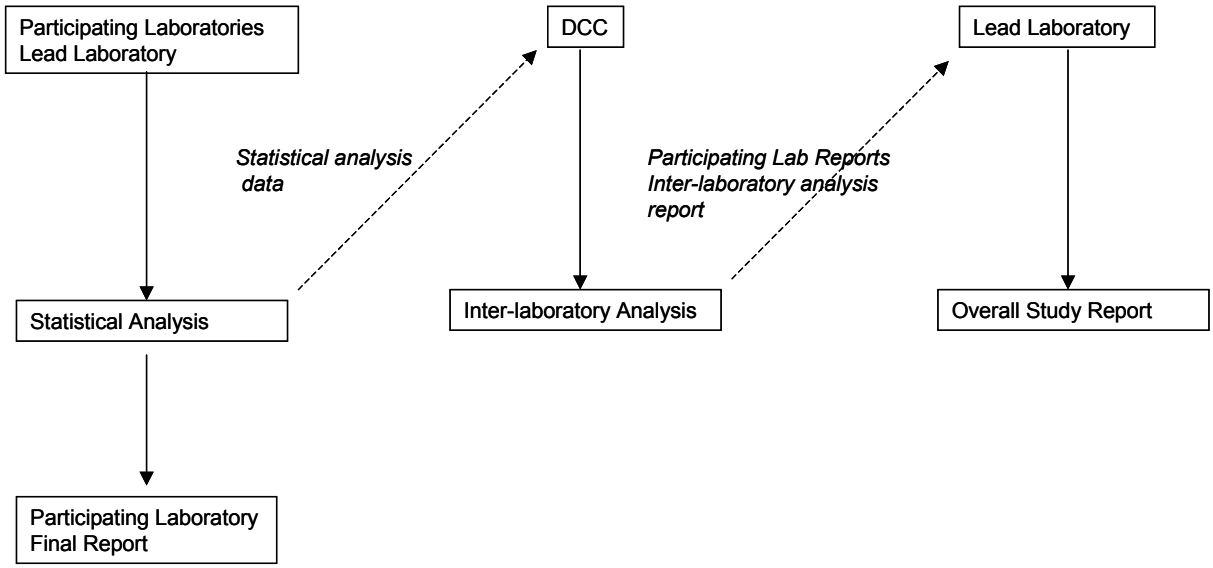


Figure B-2. Data Flow for Final Report Submission

It is anticipated that the participating laboratories and the lead laboratory need to provide data to the DCC at interim points for two reasons:

1) The results of the inter-laboratory analysis at the end of the baseline and positive control experiments may delay submission of the final report.

2) EPA desires to receive data soon after generation for purposes of reviewing the data for additional information based on their experience and the goals of the EDSP.