STUDY PLAN TO OPTIMIZE THE SLICED TESTIS STEROIDOGENESIS ASSAY

EPA Contract Number 68-W-01-023
WA 2-5, Task 15

July 25, 2002

PREPARED FOR

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Work Assignment 2-5, Task 15

Study Plan to Optimize the Sliced Testis Steroidogenesis 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. This information is summarized and presented in the following sections of this study plan narrative.

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 pre-validation 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, and 4) parenchymal viability. In addition to these most notable concerns, other factors that could potentially affect the optimal performance of this assay were identified. Thus, the present study plan and protocol describe in detail the experiments intended to provide data for setting in place the procedures and parameters that will optimize the performance of this assay.

This study plan covers the following issues:

- 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.

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 1 (Stocco, 1999).

Figure 1. 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 2 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).

Figure 2. Enzymatic Conversions of Cholesterol and Intermediate/End-Product Hormones

The second enzymatic reaction results in the conversion of pregnenolone to progesterone by the enzyme 3 -hydroxysteroid dehydrogenase/ ^{5}- ^{4} isomerase (3 -HSD). At this point, the steroidogenic pathway bifurcates into a ^{5}- hydroxysteroid pathway (starting with pregnenolone) and a ^{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.

The third enzymatic reaction involves cytochrome P450 17 -hydroxylase/C_{17-20} lyase (P450c17). For the ^{5}-hydroxysteroid pathway, P450c17 initially catalyzes the conversion of pregnenolone to 17 -hydroxypregnenolone, which is then converted to DHEA. As mentioned above, DHEA is converted to androstenedione by 3 -HSD. Likewise for the ^{4}- ketosteroids, P450c17 converts progesterone to 17 -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 as17 -hydroxysteroid dehydrogenase (17 -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 5 -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 1.

Table 1. Representative Studies Using the Sliced Testis Assay

Animal/Type of Preparation	Treatment & Stimulant	Measured Response	Reference
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 Basis for Optimization

Although the advantages and strengths, as described above, foster the decision for consideration of this assay as the screening tool for substances that alter steroidogenesis, the sliced testis assay also has some limitations, which prompted this study plan 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 impact the assay and that can be tested in order to determine their degree of impact and the setting that optimizes the analytical characteristics of the assay. Sections 2 and 3 of this study plan describe the variables that have been selected for optimization testing.

An important objective of the optimization experiments is to determine at what levels to set various factors in order to reduce to a minimum the variability of the assay. The variability of the assay can be roughly estimated using data from published research papers. To assess the extent of consistency of results across studies, comparable experimental data were extracted from various studies and compared among one another. Four studies were identified as including data

that could be compared - Laskey, et al. (1994), Fail, et al. (1994), Gray, et al. (1995), and Gurtler and Donatsch (1979). Each study utilized different test chemicals, different test chemical concentrations, and even different test chemical concentration units. Thus, only the untreated control groups were compared. Testosterone concentrations (ngT/gm Testes) were assessed at various sampling times. Cumulative standard errors of the mean were calculated under the assumption that the incremental values were independent. For each hour, where more than one study reported a cumulative mean and a cumulative standard error of the mean, a weighted one way analysis of variance test was carried out. The weights were based on the standard errors of the mean. The summary control results from the studies are presented in Table 2.

Table 2. Cumulative Mean Concentration and Standard Error of the Mean by Hour and Study

Hour	Reference[a]	n	Mean	SEM	F-value	Approx Degr Fr[b]	Approx p-value
0	F	12	130	25.0000	--	--	--
1	L	4	510	45.0000	4.14	(1,5)	0.10
	F	12	400	30.0000			
	Gr	6	216	(c)	--	--	--
2	L	4	1030	63.6396	116.23	(2, 5)	< 0.0005*
	F	12	700	75.0000			
	Gu	5	80	25.0000			
	Gr	6	417	(c)	--	--	--
3	L	4	1550	77.9423	48.91	(2, 5)	0.001
	F	12	925	80.0000			
	Gr	6	595	57.0000			
4	L	4	2100	84.6404	--	--	--
5	L	4	2620	90.8460	--	--	--

* $p < 0.005$ based on a comparison following a logarithmic transformation

a. F = Fail et al., (1994); Gr = Gray et al., (1995); Gu = Gurthler and Donatsch (1979); and L = Laskey et al., (1994).

b. The degrees of freedom associated with each study is somewhere between n-1 and 2n-1, depending on whether one or two testes per animal were used and the degree of correlation between testes from the same animal. We conservatively assumed n-1 degrees of freedom.

c. No SEM reported. Also, not used for ANOVA calculation.

The results of this comparison showed that for each hour for which several studies reported cumulative testosterone concentrations there was wide variation of mean concentrations across studies. In each case there was strong indication of significant statistical differences among studies, particularly after 2 hours and 3 hours.

For each hour where more than one study reported results, the total variance of the cumulative mean values among studies was divided into variance between studies and variance within studies. The variance within studies was estimated as the average of the squares of the within-study standard errors of the mean, based on those studies for which standard errors were reported. The variance between studies was estimated as the variance of the mean values among

studies minus the variance within studies. Table 3 displays the standard deviations among the means between studies and the standard errors of the means within studies after 1, 2, and 3 hours. The standard deviation of the means between studies is approximately 3.8 to 6.8 times the standard errors of the means within studies. This agrees with the results shown in Table 2. The variation among study means far exceeds that which is due to within-study variation.

Table 3. Standard Deviations of Between-Study and Within-Study Components of Variance by Hour

Hour	n_{mean}	n_{sem}	Std Dev of Means Between Studies	Std Err of Mean Within Studies
1	3	2	143.5	38.24
2	4	3	400.4	58.59
3	3	3	479.6	72.40

1.2.3 Prototype of the Sliced Testis Steroidogenesis Assay

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. As a starting point, so that the experimental factors and their levels that may affect the assay can be studied, the prototypical sliced testis assay is described and illustrated below.

The sliced testis assay prototype uses a 15 week old Sprague-Dawley rat, which is euthanized and its testes removed. The testes are decapsulated, weighed, and placed in cold (4°C) media. The media is medium-199 (Gibco) that has added 0.71 g sodium bicarbonate, 2.1 g HEPES, 1.0 g/L BSA, and 0.025 g/L soybean trypsin inhibitor, and is adjusted to a pH of 7.4. The time from removal to the time of slicing is held to under 1 hour. Each testis is sliced along the longitudinal axis into 4 slices. Each slice is placed in a 20 mL borosilicate scintillation vial (loosely capped) that contains 5 mL of media alone (Figure 3). The vials containing the testicular sections and media are incubated at 34°C on a shaker (low speed) in 5 percent CO_2/95 percent air. After the first period of incubation, e.g., 1 hour, the media is removed and discarded. Fresh media (5 mL) is added to the vial and an aliquot of media (0.5 mL) is collected. The sample is transferred and stored at -70°C in a siliconized plastic container. This sample is the baseline sample. Next, one half of the vials are challenged with a stimulant, e.g., hCG, and the other half are not. The final hCG concentration is 0.1 ug/mL. Additional media samples are collected from the vials at time 0 (baseline) and 1, 2, 3, and 4 hours (post challenge). These media samples are also stored frozen for later analysis. Samples are analyzed for testosterone using an RIA method.

Figure 3. Technical Flow Illustration of the Sliced Testis Steroidogenesis Assay

2.0 PROTOCOL RESOLUTION ISSUES

2.1 INTRODUCTION

There are no issues that require resolution prior to initiation of the protocol included in this study plan. The protocol is believed to be complete and ready for implementation. However, there are some analytical assay experiments that require verification, which are included in the protocol and experimental design, and these experiments must be completed prior to initiation of the sliced testis assay optimization experiments. More specifically, the analytical assays planned for use in this study plan are the testosterone RIA assay and lactate dehydrogenase (LDH) spectrophotometric assay. Response characteristics of these two analytical assays will be verified in the media selected for use in the sliced testis assay. The following paragraphs describe the basis/method for verifying the analytical assays, as well as providing details about each of the analytical assays that are planned to be used to measure testosterone and LDH.

2.2 ANALYTICAL ASSAY VERIFICATION

A set of guidelines published in 1977 by endocrine scientists is considered the gold standard for validation of an RIA (Hafs et al. 1977). These guidelines include validation recommendations for serum and plasma, but have been modified here for use with *in vitro* culture media. The basis and methods for validation follow.

2.2.1 Analytical Assay Validation

It is good laboratory practice and science to validate an analytical assay s performance characteristics, especially when measuring the analyte in a new or different matrix. While the manufacturer may provide some information about the analytical assay, many times the supplier characterizes the analytical assay in a particular matrix, e.g., human serum or plasma, thereby necessitating further characterization by a user who uses the analytical assay in a different matrix, e.g., animal blood or culture media. Consequently, each laboratory should determine the sensitivity, reliability, repeatability, and robustness of the analytical assay for measuring an analyte in a given matrix.

Validation of an analytical assay verifies that there are not substances that interfere with the assay. For example, an RIA method that uses an antibody for testosterone but also demonstrates a high cross reactivity with androstenedione will produce erroneous results. The antibody characteristics can be a significant source of inter-laboratory variation in determining concentrations of the target hormone. There are many possible interfering substances. Examples include other tissue products, media components, the test substance, and the solvent or vehicle used to dissolve the test substance, to name just a few. Examples of interfering substances in the present study include:

- Serum proteins. These proteins are present to some extent in the testes, which are synthesized by the testis or transported to the testis by body fluids (blood, lymph). The major serum proteins are albumin, sex steroid-binding globulin, and androgen-binding protein. The type and concentration of these proteins differ among species.

Furthermore, the affinity of these proteins for a given steroid varies with sex, age, reproductive state (i.e., pregnancy, anestrous, stage of cycle, age, and presence of other steroids). The testis also produces a large number of additional proteins, which are likely to be released into the media during the incubation period. Thus, results of experiments with and without testes fragments in the media after various time periods will be evaluated for evidence of interference to the testosterone RIA and the LDH assay.

- Media components. The pH of the media, amino acid content, or ionic concentration characteristics, steroid metabolites, and/or lipid content are factors that could alter or interfere with the assay.

- Organ response. Treatment with toxic substances may alter organ responses, resulting in varying secretion patterns of proteins that otherwise would not interfere with the assay.

For these reasons, both the testosterone RIA kit and the LDH assays will be validated. The validation will initially be conducted using the prototype media, i.e., medium-199 (Gibco), 0.71 g sodium bicarbonate, 2.1 g HEPES, 1.0 g/L BSA, and 0.025 g/L soybean trypsin inhibitor, adjusted to pH 7.4. Upon completion of the sliced testis media optimization experiment, the analytical assays may need to be re-validated using the optimal media. The parameters that will be determined in the validation of the analytical assay are described below.

2.2.2 Analytical Assay Validation Parameters

Parameters that will be determined, as appropriate, for the analytical assays are listed below.

- **Accuracy**. Accuracy is a measure of the true value of the analyte and will be defined as the compared agreement between the measured and true analyte values. The criterion used for an analytical assay with acceptable accuracy is ±10 percent. The accuracy of the RIA will be estimated using indirect assessment methods - recovery and parallelism.

  Recovery. Recovery is the ability of the assay to measure spiked analyte in the matrix, i.e., media. A known amount of nonlabeled analyte (A) will be added to the media (M) and the recovery will be calculated as a concentration (C) using the following formula:

  $$(C - M)/A \times 100$$

  Parallelism. Parallelism refers to the dilutions of the samples and the accuracy is related to a comparison of the measured and true values at several volumes of fluid. When calculated quantities are converted to concentrations (ng/ml), all volumes tested should contain similar concentrations (unit per milliliter) of testosterone. Lack

of parallelism can indicate the presence of interfering substances. Lack of parallelism will be followed up using extraction or chromatography in order to isolate the testosterone from the media.

- **Sensitivity**. Sensitivity is the capacity of the assay to measure the smallest amount of analyte. The smallest quantity of analyte that can be quantified reliably will be determined and expressed as the limit of detection. Media samples containing concentrations below the level of sensitivity of the assay will be rerun using larger sample volumes, assuming that a sufficient sample volume is available.

- **Precision**. Precision (also referred to as reproducibility) is a measure of the variation in analyte measurements following repeated determination. Precision of a given analytical assay will be evaluated by determining the mean, standard deviation (sd) and coefficient of variation (CV). The precision of the assay will be determined within runs and between runs from day to day.

- **Cross Reactivity.** Cross reactivity (also referred to as specificity) occurs when the assay measures more than only the required analyte. The antibody will be characterized for binding activity with related compounds.

- **Identity of Unknowns**. Identity of an unknown will not be performed, but demonstration of its removal from the assay will be attempted. Chromatographic isolation of the analyte by HPLC (Darney et al., 1983) or other methods (differential extraction), followed by RIA or EIA, should yield values identical to concentrations found using RIA or EIA without chromatographic separation. If not, then an assessment of whether the unknown steroid is recognized by the antibody and whether it is present in similar concentrations in all samples. Otherwise, samples analysis may involve chromatographic isolation or differential extraction of the principal steroid.

- **Quality Control**. Interassay variation will be calculated from values of one or more samples included on a series of assays (standard sera). These standard or control sera will be aliquoted and frozen prior to the first assay in the series. An aliquot of these control sera will be included two or more times within each assay and on each subsequent assay. Internal quality control measures will include, where appropriate, high and low control sera and a solvent blank. From these standards, precision of the assay (inter- and intra-assay coefficients of variation) will be established.

2.3 TESTOSTERONE RIA

The end-point analyte in the sliced testis assay is testosterone and it will be measured using an RIA method. In this method, the antibody is affixed to the assay tube, and any bound antigen (testosterone), whether labeled or unlabeled, is recognized by the antibody. The labeled testosterone will be ^{125}I-testosterone and, the kit that will be used includes polypropylene tubes coated with a testosterone-specific antibody. Testosterone will be used to prepare the standard

Table 4. Characteristics of a Radioimmunoassay Validated for Determination of Testosterone in Adult Male Sprague Dawley Rat Plasma and Testicular Fluid (Contd)

curve (Sigma, St. Louis, MO; T-1500). Procedural controls (internal controls) for each assay will use media controls (two each with high or low testosterone concentrations) and reagent blanks. Separation of bound and free testosterone will be accomplished by decanting. The bound fraction (which remains in the tube) will be counted using gamma counter technology.

Data are presented below from a study that used a specific testosterone RIA. This RIA was developed for human blood (Diagnostic Products Corporation) and was validated for use with plasma and intra-testicular (ITT) fluid testosterone analysis (Fail et al., 1992; 1995; 1996). Table 4 summarizes data for rat plasma and ITT fluid. In this study, only one assay was done so no inter-assay data were generated. In another study, estimates of variation were calculated using the internal controls from nine testosterone RIAs completed during a five-month period. Testosterone mean and SEM values were 11.37 ÿÿ0.57 ng/ml across assays for an inter-assay variation (CV) of 14.96%. The testosterone values (mean ± SEM) for the internal controls in these nine assays ranged between 9.65 ÿÿ0.13 and 14.64 ÿÿ0.62 ng/ml within assays (CV - 7.5%).

Table 4. Characteristics of a Radioimmunoassay Validated for Determination of Testosterone in Adult Male Sprague Dawley Rat Plasma and Testicular Fluid [Personal Communication, Dr. P. Fail, 2002*]

	Hormone Assay	
Parameter	Plasma Testosterone (ng/ml)	ITT Testosterone (ng/ml)
Sensitivity	3.5 pg	3.5 pg
Intra-assay Variation[a]		
blank	0/8.7%	0/7.6%
mass added	2/11.8% 8/5.2%	12.5/5.4% 25/4.4% 50/4.2%
Interassay Variation[a]	NA	NA
% Recovery of Added Mass[b]	2/92.5% 8/94.3%	12.5/60.7% 25/68.8% 50/88.5%
Index of Parallelism[c]	127.1%	112.6%
Test article cross reactivity (28 g/ml)	0%	NT

[a] Numbers are mass added/percentage variation. For interassay variation, only one run was performed. NT = not tested.

[b] Numbers are mass added/percentage recovered.

[c] Index of parallelism = concentration of low volume ÷ concentration of high volume x 100.

* The table values are most likely fluid and species specific. Thus, they should be used as representative of what may be found in characterization of other assays.

2.4 LACTATE DEHYDROGENASE (LDH) ASSAY

The enzyme, lactate dehydrogenase (LDH), is found in the cytoplasm of all cells. Cellular LDH concentrations are approximately 500 fold higher than that found in the serum. The magnitude of this gradient makes it possible to use LDH as a marker for cellular viability. Although a small amount of LDH leaks from the cells under normal conditions, high concentrations of LDH are observed when cellular damage is sufficiently detrimental that the cell ruptures and its contents are released into the extracellular environment. The normal serum LDH concentration ranges from 50 to 233 U/L (normal healthy adult human). In rat serum, historical levels are reported to range from 150 to 300 U/L (Jacobs et al., 1988; Young, 1990). In humans, elevation in serum LDH occurs following myocardial infarction, liver disease, pernicious and megaloblastic anemias, pulmonary emboli, malignancies, and muscular dystrophy, to name just a few conditions and diseases (Tietz, 1986). For these reasons, LDH was selected as a potentially useful marker in the sliced testis assay in order to provide an indication of the extent of cellular damage induced by dissection trauma, incubation conditions, and/or chemical substance testing. Repeated LDH measurements may assist in the characterization of damage over time and in determining whether cellular damage might be associated with testis fragment size, e.g., more extensive damage occurring with smaller testicular fragments. In addition, this information may be helpful in establishing the duration of the testis fragment equilibration period, i.e., the time in culture before treatment. Thus, the LDH assay is being tested as a marker to determine whether it can be used to monitor cellular/tissue viability.

The LDH assay that will be tested for its use in the sliced testis assay is based on the bioreactivity of the enzyme. The assay is commercially available as a kit and, and one such kit (LD KINETIC PROCEDURE, ThermoDMA, Arlington, Texas) is representative of the type of assay that will be used in the present study design. In the cell, LDH specifically catalyzes the oxidation of lactate to pyruvate with the simultaneous reduction of NAD to NADH. This same reaction can be used to measure the amount of LDH in a sample from a biological matrix. The rate at which NADH is formed is measured at 340 nm using a spectrophotometer, thereby providing a stoichiometric relationship that allows for the determination of LDH (Duhl and Jackson, 1978). The assay requires attention to several factors that can affect the level of variability. For example, after a 30 second incubation time, the change in absorbance per minute remains stable for 2 minutes. In addition, although the assay has been characterized at 30ÿ°C and 37ÿ°C, the assay should be consistently run at the same temperature because the LDH values change with fluctuations in temperature. Furthermore, substances and conditions in the animal have been shown to interfere with the assay. Examples include:

- Hemolysis (false elevation)
- Oxalate and ascorbic acid (decrease LDH levels)
- Drugs (increase or decrease serum LDH levels) (Young, 1990).

As a means to monitor the performance of the LDH assay, quality control procedures will be implemented in those experiments that begin to characterize LDH as a monitor for cellular viability. Normal and abnormal control sera of known LDH activities will be analyzed on a routine basis. Quality control samples are commercially available (DMA s Data-Trol N and Data-Trol A; Cat. No. 1902-605 and 1901-605). In addition to the quality control procedures, the

experimental design as described in the protocol includes evaluating the assay in the media selected. Furthermore, the laboratory will establish its own range of expected values, since differences exist among instruments, laboratories, and local populations. Finally, the accuracy, precision, and variability of the assay will be determined.

3.0 STUDY DESIGN (OPTIMIZATION OF EXPERIMENTAL FACTORS)

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 present optimization experiments that are designed to optimize the environmental, chemical, and biological components of the *in vitro* sliced testis steroidogenesis assay. The study plan includes experiments using single factor and factorial design approaches so as to identify factors that affect assay performance, as well as to test for interactions of the factors, which may be integral in uncovering those conditions essential in minimizing assay variability. This section describes the factors to be tested and the basis for the levels selected for each factor.

In order to try to make an *in vitro* assay simulate the *in vivo* environment, the culture conditions must mimic the cellular environment as closely as possible. The assay factors believed most important in accomplishing this simulation were selected for optimization in the present study plan. The designers of these experiments kept in mind that in order to make the assay usable for many different laboratories, under varied environments, and sometimes difficult situations, the study plan would need to test factors at levels that may or may not be best for the assay but are easily achieved at most laboratories, e.g., room temperature, air for the atmosphere, etc. In that way the robustness of the assay can be determined. In addition, the factors and levels selected for testing will provide information that will evaluate how to perform the assay as simply, economically, rapidly, and efficiently as possible without compromising the performance of the assay. In a related vein, some of the factors selected and the levels to be tested were based on their use by previous researchers. By including factors and levels used previously with those that have been used primarily by researcher consensus, this study plan provides the opportunity for the assay to be optimized without the confounding results that come from multiple researchers and laboratories. Thus, multiple factors and various levels have been selected for testing so as to identify optimal assay settings and conditions, as well as to define the boundaries and limitations of the assay.

Biological and chemical parameters of the assay were given consideration in identification and selection of factors for testing. The factors were categorized as those that affect incubation, the testicular parenchyma, parenchymal viability, and sample analysis. Each of the factors in these categories and the basis for the levels selected for testing are described in the following subsections.

3.1 INCUBATION FACTORS

The sliced testis assay involves incubation of the testicular parenchyma in media. The incubation factors selected for testing include the media type, gaseous atmosphere type, temperature, vessel type, shaker speed, media volume, and hCG stimulant concentration. Each

of these factors and their levels selected for testing are described in further detail below.

3.1.1 Incubation Media Type

A review of the literature indicates that several different types of media with supplemental components have been used in the conduct of the sliced testis assay. These media and components include:

" medium-199 (Gibco) with 0.71 g Na bicarbonate, 2.1 g HEPES, 1.0 g/L BSA, and 0.025 g/L soybean trypsin inhibitor. Adjusted to pH 7.4.

  Refs. Laskey et al., 1994; Klinefelter et al., 1994; Fail et al., 1994; 1995; Gray et al, 1995

" Eagles MEM

  Ref. Wilker et al., 1995

" RPMI-1640 medium (without phenol red) with 10% FCS and 50 ug/mL soybean trypsin inhibitor.

  Ref. Powlin et al, 1998

As is apparent above, the majority of the researchers have used medium-199 that is supplemented with buffer and reagents that reduce cellular instability and degradation. Of these researchers, none have investigated the possible effect of media type on assay performance. However, Powlin et al. (1998) did examine the effect of conducting the sliced testis assay with and without fetal calf serum (FCS), bovine serum albumin (BSA), and soybean trypsin inhibitor. Although their results indicated that the presence or absence of each of these supplemental components had no effect on testosterone production, the incubation media that they used for follow-on experiments included all three components at the concentrations listed above. Since these components were not considered critical to the assay performance, the present study plan does not include testing all combinations of media and supplemental components. The ready availability of the supplemental components and ease of combining them with the media, together with the number of experiments needed to test all the combinations and the results reported by Powlin et al (1998), precluded inclusion of such experiments in the present study plan. Thus, a decision was made in the design of the present study plan to compare the effect of the *in toto* media used by the different investigators, rather than effects of individual reagents, on the performance of the assay.

3.1.2 Incubation Gaseous Atmosphere

The types of gaseous atmosphere that have been and are used in this assay are limited. The most commonly used atmosphere is 5% CO_2/95% air. However, a commonly used atmosphere for cell culture systems is 5% CO_2/95% O_2. In addition, it is possible that the performance of the assay is not affected by the atmosphere as long as it is similar to the ambient

air, which, if correct, would simplify the requirements of performing the assay. For these reasons, the following three atmospheres were selected for testing - air (three gases); 5% CO_2/95% air; and 5% CO_2/95% O_2.

3.1.3 Incubation Temperature

The sliced testis assay is most commonly conducted using an incubation temperature of 34°C. This temperature is used because it is believed to be the temperature of the testis in the scrotum. Testicular temperature is lower than body temperature to allow spermatogenesis to occur. No report was found that determined by experimentation the temperature of the rat testes by insertion of thermal probes. It is assumed that most investigators use 34°C based on bull and human studies. In addition to testing the assay s performance at 34°C, a temperature of 25°C was selected to test whether room temperature had an effect. If not, then the assay could be performed more efficiently. A third temperature, 37°C, was chosen to determine whether whole body temperature had an effect on assay performance. Finally, 40°C was chosen as an extreme level because temperatures up to 42 °C have been used to simulate cryptorchidism in rats. Thus, the incubation temperatures selected for testing are 25, 34, 37, and 40°C.

3.1.4 Incubation Vessel

The incubation vessel used during the incubation period will be tested for its effect on assay performance. This experimental factor is included in the study plan because of the work reported by Powlin et al (1998). The sliced testis assay was conducted using a 20 mL scintillation vial or a 16 x 100 mm test tube; both were made of borosilicate glass. Their results showed a 4- and 9-fold increase in testosterone production when the scintillation vial was used relative to the test tube. They attributed this finding to greater physical dispersion of the parenchymal fragment in the scintillation vial. Based on these results and explanation for the effect, incubation vessels with varying sizes of bottom surface area were selected for testing. The levels of this factor, in increasing order of bottom surface area, that will be tested are:

- 16 x 100 mm test tube
- 20 mL scintillation vial
- 20 mL Erlenmeyer flask

3.1.5 Incubation Shaker Speed

The sliced testis assay includes shaking of the parenchyma in the media during the incubation period. The speed of the shaker is not an experimental factor that has been uniformly decided upon by researchers, nor has it been tested for its affect on the assay. Most articles refer to using a shaker during incubation but the speed of the shaker is rarely reported, if known. Powlin et al. (1998) used a shaker speed of 175 rpm, which is considered a high (or vigorous) speed. Since shaking allows the parenchyma to have greater exposure to the culture components such as media and gases, it is possible that shaking speeds may provide varying degrees of exposure between the media components and gaseous atmospheres. For these reasons, the shaking speed was selected as a factor for testing and the levels to be tested are: no shaking, and low and high speeds, where the low speed will be less than 60 rpm and the high speed

approximately 175 rpm.

3.1.6 Incubation Media Volume

The sliced testis assay includes bathing the parenchyma in media during the incubation period. The volume of media used by most researchers is generally 5 mL. This volume was arrived at by consensus rather than experimentation. The volume of media used must be balanced between two off-setting considerations. First, sufficient media must be used to keep the parenchyma in an aqueous environment, as well as to provide a means to deliver substances for toxicity testing. Varying the incubation media volume will result in different degrees of exposure to the parenchyma, as well as to the gaseous atmosphere. Second, the media must be kept to a minimum in order to preclude diluting testosterone concentrations below the level of detection of the analytical method. Thus, the media volume is a factor that is included in the study design and the levels to be tested are 2.5, 5, and 10 mL.

3.1.7 hCG Concentrations

Human chorionic gonadotropin (hCG) is used in the sliced testis assay to stimulate steroidogenesis. Concentrations used in previous studies include100 mIU/mL (Laskey et al., 1994) and 1 IU/mL (Fail et al., 1994; Powlin et al., 1998). A dose-response experiment is necessary to determine the concentrations that stimulate maximal testosterone production. From such an experiment, the optimal concentration can be selected based on the other conditions selected to conduct the assay. The optimal concentration is not necessarily the concentration that produces a maximal response. The goal of the study plan is to test several concentrations of hCG in order to identify a concentration that does not overstimulate to exhaustion the parenchymal fragment but that does provide a steady linear production of testosterone over the maximal possible incubation period. The levels selected for testing range from a concentration that is expected to have no effect to one that would likely cause exhaustion of the cells from overstimulation. The levels selected for testing are 0.001, 0.01, 0.1, 1, and 10 IU/mL.

3.2 TESTICULAR PARENCHYMA FACTORS

The sliced testis assay involves incubation of testicular parenchyma. The experimental factors selected for testing include parenchymal fragment size, time to prepare whole testis to fragment, media/temperature used during collection of whole testes, and effect of sample aliquot volume on media:tissue ratio. Each of these factors and their levels selected for testing are described in further detail below.

3.2.1 Parenchymal Fragment Size

The sliced testis assay uses a fragment of the whole testis. Obviously, the size of the testis slice used is directly proportional with the number of Leydig cells placed in culture. However, the size of the parenchymal fragment used for testing has not been uniformly accepted. Most researchers have used quartered testis (Laskey et al., 1994; Gray et al., 1995; Fail et al., 1994, 1995) but 50 mg fragments have also been used (Powlin et al., 1998). Personal communications with researchers in this area have indicated that as the fragment size is

decreased the variability of the assay increases. However, documentation of the relationship between fragment size and assay variability is not available. Another issue that affects the fragment size is the composition of the testis. Consistent fragment sizes are difficult to obtain due to the gelatin-like consistency of the parenchyma. Once the testis is sliced in half, quartered, etc., small adjustments to the fragment size to be tested are not feasible. In addition, the parenchymal composition is such that slicing the testis to smaller and smaller sizes is no longer possible beyond 1/8 slices (~125 mg). Use of fragments less than approximately 125 mg is possible but these fragments are obtained by "scooping" the desired amount of parenchyma rather than transferring a slice of the testis to the incubation vessel. Regardless of the advantages associated with using larger fragments, it is important to determine the smallest possible amount of parenchyma that can be used without compromising assay performance so that the maximum number of assays can be conducted using a minimal number of animals, which will also reduce biological variability. Therefore, based on this information, the fragment sizes selected for testing are:

- ½ testis slice (~ 500 mg)
- ¼ testis slice (~250 mg)
- ⅛ testis slice (~ 125 mg)
- 50 mg parenchyma
- 25 mg parenchyma

3.2.2 Preparation Time

The sliced testis assay involves isolation and removal of the testis prior to processing it into fragments for testing and incubation. The duration of time that it takes to remove the testis and slice it into fragments may affect the viability of the parenchyma, thereby compromising steroidogenesis and assay performance. This experimental factor is exacerbated when several testes are used for multiple fragment preparation and testing. Since the size of the present study plan will require many days involving multiple fragment preparations, the delay time in processing the testis was considered to be an important experimental design factor for testing. Experimental data that define the limits of the testis processing procedure will allow laboratories to maximize their efforts while at the same time ensure that the organs are viable for testing. Therefore, the amounts of time that can elapse before the testis must be processed without having an effect on the assay's performance are 0.5, 1, and 2 hours.

3.2.3 Preparation Techniques of Whole Testis

Following removal of the testis and prior to processing into fragments, the testis is bathed in media. However, a variety of preparation techniques have been used to keep the testis fresh. The different procedures used by researchers primarily differ with respect to the bathing media and temperature. Few articles include a description of this procedure but, for those that do, the testis are bathed in cold phosphate-buffered saline (Gray et al., 1995) or cold saline (Powlin et al., 1998). Some papers imply that media at room or incubation temperature was used (Klinefelter et al., 1994). Thus, the following levels were selected for testing based upon the general or specific information provided by in the literature:

" Cold (4°C) phosphate buffered saline (pH 7.4)

" Warm (25°C) phosphate buffered saline (pH 7.4)

" Cold (4°C) media

" Place encapsulated testis in labeled cassette, moisten with cold physiological saline, place on ice, and cover with saline-moistened paper towels (Powlin et al., 1998).

3.2.4 Aliquot Volume

Aliquots of the media are collected during the incubation period for analyte analysis. The aliquot sample size will affect the volume of the culture system since the media is not replaced after the baseline sample is collected and the incubation period for testing begins. As the aliquots are removed during the incubation period, different tissue:media ratios exist. In addition, the size of the aliquot may have a large bearing on the tissue:media ratio, e.g., smaller aliquots taken over time allow the tissue:media ratio to remain more constant than removal of larger aliquots. For this reason, the study plan includes testing of different aliquot volumes. The selections included aliquot volumes that are typically used (0.25 and 0.50 mL) and a minimum volume (0.1 mL), which may be insufficient to conduct the RIA based on the testosterone concentration in the media.

3.3 SAMPLE STABILITY FACTORS

The sliced testis assay involves collection of samples from the media for analysis of the endpoint. For this assay, the endpoint will be testosterone. Several experimental factors could affect the measurement of testosterone; not because of the assay conditions but as a result of the storage conditions. These experiments will not require conducting the sliced testis assay to evaluate the effect of the factors on the assay. Rather, these factors will be experimentally determined by adding a known amount of the analyte, testosterone, to the media and comparing the actual concentrations to the target concentration. The concentrations to be tested will be selected from the results of the RIA verification experiments. A concentration near the level of detection or the low end range of the expected assay results will be used in order to test the limitations of the analytical method. The experimental factors selected for testing sample stability include storage container type, storage time, and storage temperature. Each of these factors and their levels selected for testing are described in further detail below.

3.3.1 Sample Storage Container

The storage container is a factor that can alter the analyte measurement. The composition of the container may react with the analyte and, if adsorption or leaching of a reactive component from the container occurs, then the analyte concentration may be compromised. For this reason, the study plan includes examining sample storage containers that are plastic with a siliconized or non-siliconized surface to determine whether testosterone concentrations are disparate.

3.3.2 Sample Storage Time

The length of sample storage will be tested to determine if there is deterioration of the testosterone over time. Spiked samples will be stored and removed for analysis in order to compare the concentrations over time. The samples will be stored in different containers so that no samples will go through freeze/thaw cycles. The storage sample times to be tested are the next day, 1 week and 1 and 3 months. The 3 month time period was selected because this was believed to be the longest time period needed before analysis could be completed for all samples from a given set of experiments.

3.3.3 Sample Storage Temperature

Some substances are known to deteriorate at different storage temperatures. For this reason, the effect of sample storage temperature on stability of the samples will be tested. Not all laboratories have access to -70°C storage facilities. Therefore, the temperatures selected for testing are -20 and -70°C. The effect of temperature on sample stability will only be determined on the samples stored for 1 and 3 months.

3.4 SAMPLE COLLECTION INTERVALS

Sample collection interval refers to the spacing of time between removing aliquots of media for testosterone analysis. In general, most researchers have taken hourly samples for 4 to 5 hours after the completion of the initial equilibration period (Laskey et al., 1994; Gray et al., 1995; Fail et al., 1995; 1995; Powlin et al., 1998). Previous investigators have shown the sliced testis assay, when stimulated with hCG, will produce testosterone at a rate of approximately 200 ng/g testis/hour for up to 3 hours (Gray et al., 1995). However, there has been no systematic investigation conducted to determine whether hourly collection intervals are the optimal time points for measuring the production of testosterone, nor if the number of time points collected are actually necessary. Perhaps fewer samples at specific time points will give the same information with increased efficiency.

In addition to experimentally determining the optimal sample collection intervals, the duration of the sliced testis assay may be able to be taken beyond 4 to 6 hours. Previous studies have shown that substances can have a delayed effect on alteration of steroidogenesis. Thoreux-Manlay and co-workers (1995) showed that the effect of lead is delayed and may not become apparent for a few hours after adding it to the incubation media. For this reason, determination of the latest period of time that the sliced testis assay produces testosterone at the established linear rate will be useful for testing those substances with possible delayed effects.

The study plan includes experiments that will determine the optimal sample collection intervals and the duration of time that the assay can be used. The time points to be tested (after equilibration) are 0.5, 1, 2, 3, 4, 8, 12, and 24 hours.

3.5 CHARACTERIZATION OF VIABILITY

The viability of the parenchymal fragment used in the sliced testis assay is an important consideration if it is used to evaluate steroidogenic altering effects of a substance. Viability refers to the capacity of the parenchymal fragment to simulate its *in vivo* function in an *in vitro* environment. In regard to the sliced testis assay, a distinction must be made between testosterone

released as a result of basal operation or hCG stimulation versus testosterone release due to cellular damage and death. A marker is needed that will allow the integrity of the parenchyma to be monitored. Several techniques have been used in *in vitro* assays to evaluate viability. Klinefelter et al., (1987) described how to stain for 3 -HSD activity, and many investigators now use this technique to demonstrate viability of Leydig cell cultures. Other techniques that assess cell viability include Trypan blue, which is excluded by intact cells, histological examination of the cells (Klinefelter et al., 1991), quantification of [^{35}S] methionine incorporation into proteins synthesized *de novo* (Kelce et al., 1991), and a colorometric assay using tetrazolium salt MTT, which is reduced by succinate dehydrogenase (a mitochondrial enzyme) to formazan (Thoreux-Manlay et al., 1995). Other viability markers include cytokine release and the ATP bioluminescence assay (Dr. Jerome Goldman, personal communication). Lactate dehydrogenase (LDH), a cytoplasmic enzyme, is also used as a marker for cell damage (as described in Section 2). In the present study, LDH will be evaluated as a marker of parenchymal damage in the sliced testis assay. The LDH assay was selected because it is inexpensive to perform, uses equipment commonly found in laboratories, has a fast through-put, and is well established.

Characterization of the marker assay is the objective of the experiments included in this part of the study plan. These experiments will be conducted using the optimized assay. The experiment will evaluate the viability of the parenchyma and capacity of LDH to detect damage and potential factors that may affect viability, i.e., vehicle type and concentration. In addition, the LDH marker may be useful for characterizing the actual time needed for parenchymal equilibration. Each of these experiments and the basis for their selection are described in further detail below.

3.5.1 LDH - Evaluation as a Marker

Experiments will be conducted to evaluate LDH as a marker for parenchymal fragment viability. Viability will have to be compromised in order to show that LDH is released and can be measured as a result of parenchymal fragment damage. The study plan will use trauma (blunt instrument) and/or heat (45°C for the duration of the incubation sampling period) and/or chemicals (EDS - ethane dimethanesulfonate at 500 or 3000 µM) as sources of potential cellular damage. Initially, the intent will be to inflict sufficient damage to the fragment such that a clear LDH response can be demonstrated. Later, a gradation of the damage will be examined in order to assess the extent of damage needed in order to elicit a response. The endpoints during these viability tests will include testosterone and LDH. In that way, the parenchymal responsiveness based on its viability can be compared with the change in LDH concentrations over time.

3.5.2 Equilibration Period

The sliced testis assay includes an equilibration period. This period begins when the parenchymal fragment is placed in the incubation vessel and media is added. In general, most researchers use a 1 hour equilibration period. Equilibration is needed in order for the parenchymal fragment to recover after being transferred from an *in vivo* to an *in vitro* environment. It is also during this time that any release of testosterone and other intracellular components are discharged into the media. The media used during the equilibration period is discarded at the conclusion of the equilibration period. New media is added and it is generally

agreed upon that only basal or hCG stimulated testosterone is released into the media from the Leydig cells after the equilibration period is over.

The objective of these experiments will be to characterize parenchymal viability during the equilibration period. LDH will be measured at various time points to determine whether a 1-hour equilibration period is sufficient or necessary. The information obtained from this experiment will provide a basis for enhancing assay performance, e.g., longer time period is needed, or increased efficiency, e.g., a shorter time period is adequate. The sliced testis assay will be conducted by taking samples during the equilibration period. The sampling times selected are 15, 30, and 45 minutes and 1, 1.5, and 2 hours.

3.5.3 Vehicle Type and Concentration

The sliced testis assay is being optimized for use as a screening tool of chemicals with possible steroidogenic altering effects. The vehicle represents a component that is added to the media, which has the potential to cause damage to the integrity of the parenchymal fragment. These experiments will test the effect of vehicles and concentrations of the vehicles that are commonly used to deliver to the incubation vessel the substances being tested. A wide range of concentrations of the vehicles will be evaluated because solubility of the test substance can often be a limiting factor in using *in vitro* assays. These experiments will provide the information needed about the limitations of the vehicles as it pertains to parenchymal viability. Both testosterone and LDH will be measured at specified time points after the equilibration period. The vehicles and the concentrations selected for testing are ethanol, DMSO, and Tween 20 at 0.5, 1, 5, and 10 percent (v/v).

4.0 RECOMMENDED TEST SUBSTANCES

The purpose of this study plan is to design experiments that will evaluate factors for the optimal performance of the sliced testis assay. For this reason, there will be no substance testing included in this study plan. Once the optimization experiments are completed, the data evaluated, and the assay further reviewed for its potential to meet the objectives as a screening tool, then a pre-validation study plan will be developed. In the pre-validation study plan, the optimized sliced testis assay would be evaluated for its capacity to detect an effect of a substance on steroidogenesis.

5.0 STUDY PROTOCOL

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

6.0 STATISTICAL METHODS

6.1 INTRODUCTION

The study plan for testing the factors described in the previous sections involves two phases and utilizes single factor and factorial experimental designs. A diagram of the experimental design for this study plan is illustrated in Figure 4

The study plan is divided into Phases 1 and 2.

In Phase 1, the Preliminary Experimental Phase, the analytical assays planned for use will be verified and three factors that may affect the performance of the assay will be tested. The reasoning for including these three factors in the preliminary phase was to establish early whether a given level of each factor was going to affect assay performance. Although any factor listed in the study plan could be rationalized to fit such a criteria, inclusion in the preliminary phase also required that the factor be unlikely to have an interaction, or at best a minimal interaction, with another experimental factor. Although subjective, these three factors were believed to best fit these criteria. Furthermore, it was believed essential to establish the optimal level for each of these factors before proceeding with the factorial experiments since an effect of one of these would require additional verification experiments after sensitivity analysis. Finally, by establishing the media type early on in the experiment, the analytical assay verification testing (Phase 1) and Optimization of Sample Testing (Phase II) could be initiated earlier in the study milestone schedule.

In Phase II, Primary Experimental Phase, the majority of the experiments designed to evaluate the factors that may affect assay performance will be tested. Phase II is divided into six different sections. Each section is comprised of those factors that may produce interactions. The groupings allow the interactions to be identified and estimated. It is not possible to test all factors and their levels for all possible interactions, nor is it prudent. For example, some factors will absolutely not result in an interaction, e.g., incubation vessel type and sample storage container type. Others are unlikely to result in an interaction, e.g., incubation temperature and time taken to process the testis into fragments. Others were sufficiently likely to interact that it was believed necessary to include them in two different optimization experiments, e.g., hCG concentration testing with both the incubation condition and the testis preparation factors. Thus, an attempt was made to categorize factors according to type and whether a possible interaction needed to be identified. These experiments comprise the first four sections of Phase II.

Sensitivity analysis is the fifth section of Phase II. Briefly, this analysis involves the development of a response surface model based on the results of all the optimization experiments. The response surface model describes the variation in response level as a function of the variation in the totality of experimental factors. This analysis is described in further detail later in this section. Upon completion of this analysis, selected verification experiments may be needed to evaluate any untested interactions and verify the model predictions.

Upon completion of the sensitivity analysis, the sliced testis assay will be sufficiently characterized to describe the optimal settings of factors and levels. The assay will then be used to characterize whether LDH can be used as a marker to assess viability of the parenchymal fragment. In addition, other factors will be tested during this stage of Phase II that may affect the viability of the fragment.

The statistical methods used in the design of this study plan are described below.

6.2 ASSUMPTIONS AND RESPONSE ASSESSMENT

Assumptions used in the experimental design include:

- The prototypical design of the sliced assay is considered a good assay. Slight modifications of this design will serve as the the initial reference design and the point of comparison.
- All combinations of specified factor levels are *a priori* sensible.
- While it is believed that the optimum conditions will be in the vicinity of the prototypical conditions, it is also possible that any combination of the specified factor levels may be better. Thus, the entire response region needs to be explored.
- All factors can be varied with equal ease. Thus, the order of the tests (within experimental sections) should be completely randomized.
- Approximately 20 runs can be performed a day and approximately 60 testosterone RIA samples can be analyzed a day.

The principal response that will be measured is the rate of production of testosterone (ng T/mg testis/hr). This will be measured both in unenhanced medium (baseline) and in medium stimulated with hCG. Responses will be assessed at 1, 2, 3, and 4 hours following parenchymal equilibration. From these measurements, the objective will be to determine the combination(s) of factors that result in baseline and hCG-enhanced testosterone production response curves that are

- Individually linear
- Individually steep
- Well separated from one another.

These objectives will be evaluated by determining the slope and the curvature of the baseline trend and the hCG-enhanced trend at each set of experimental conditions. At the optimum conditions the slopes are large and different and the curvature is small.

6.3 PRELIMINARY EXPERIMENTAL PHASE (PHASE I)

The conduct of the optimization experiments in this phase will involve setting all factors to their prototypical levels (See Section 1.2.3). The three factors to be tested will be varied one at a time. It is assumed that effects of variation of these factors will not interact among one another or with the remaining experimental factors. For example, if a particular incubation atmosphere produces the best results with the remaining factors set at the prototypical conditions, then it will also produce the best results for all combinations of conditions in the region of the optimum.

The preliminary experiments include testing:

1. Media Type (3 levels)
2. Incubation atmospheres (3 levels)
3. Rat age (3 levels)

4. Testosterone RIA - verify claims of the commercial supplier
5. LDH assay - verify claims of the commercial supplier.

Each level of these preliminary experiments above, i.e. 1, 2, and 3, will be repeated three times with true replicates (with independently generated media and testis combinations). These replicate determinations will provide some initial indication of whether the differences in responses at the various factor levels represent real effects or may just be due to experimental variation.

6.4 PRIMARY EXPERIMENTAL PHASE (PHASE II)

6.4.1 Optimization of Incubation Conditions

The incubation factors experiment includes six factors, each varied across three, four, or five levels. It is assumed that these factors may interact among one another but will not interact with the factors discussed in the other parts of the design. All factors not included in this part of the design will be held constant at their prototypical levels. The incubation factors to be studied and their ranges of variation are as follows:

Table 5. Summary of Experimental Incubation Factors for Optimization

			Experimental Levels				
Factor Identification	**Units**	**Factor Designation**	**1**	**2**	**3**	**4**	**5**
Incubation Temperature	^{0}C	X_1	25	34*	37	40	--
Incubation Vessel Type	--	X_2	--	scintillation* vial	Erlenmeyer flask	test tube	--
Incubation Shaker Speed	--	X_3	--	none	low	high*	--
Incubation Media Volume	ml	X_4	--	2.5	5*	10	--
hCG Concentration	IU/ml	X_5	0.001	0.01	0.1*	1	10
Fragment Size	mg	X_6	25	50	125	250*	500

* The central levels of the factors.

The principal portion of the design will consist of two 2^{6-1} factorial parts in the "central" levels of the factors (i.e., levels 2,3,4 - except for incubation temperature, for which levels 1,2, and 4 will be run). The prototypical level of each factor, i.e., indicated with an asterisk, will be included in both parts. The other central levels will be included in just one. Thus, more information will be obtained about the prototypical levels of the factors than about perturbations about these levels. The principal portion of the design will permit estimation of linear main

effects and linear by linear two factor interactions for each of the six variables. Quadratic main effects will be estimable under the assumption that they do not interact with other factors.

In addition, a center point will be run, axial points will be run for variables 1, 5, and 6, and 9 replicate points will be spaced over the design space. All variables, other than those that are included in the primary incubation factor experiment will be set to the prototypical settings.

The experimental matrix is divided into five parts.

- Part 1. 2^{6-1} fractional factorial design. 32 runs. This includes the prototypical level of each factor and one of the central factor levels. This permits estimation of all linear main effects and linear by linear interactions among the levels of the factors included in this portion of the design.

- Part 2. 2^{6-1} fractional factorial design. 32 runs. This includes the prototypical level of each factor and the central factor level that was not included in Part 1. This permits estimation of all linear main effects and linear by linear interactions among the levels of the factors included in this portion of the design. Part 2, in conjunction with Part 1, permits estimation of quadratic main effects in each of the factors, under the assumption that the quadratic effects do not interact with the other factors.

- Part 3. Center Point. This estimates the response when all factors are set to their prototypical levels. This does not require any additional runs since Part 1, Run 19, is already at the center point.

- Part 4. Axial Points. 5 runs. These points explore additional factor levels for variables 1, 5, and 6. They provide additional estimates of the nature and extent of nonlinearity in the main effects of these variables.

- Part 5. Replicates. 9 runs. These points provide estimates of the reproducibility of the response at the center point of the design and at various extremes of the design space.

The total number of runs is 78, which will be performed for both the non-stimulated and the hCG stimulated assays, which is a grand total of 156 runs. The order of the runs will be randomized.

6.4.2 Optimization of Testis Preparation Factors

The testis preparation experiment includes four factors, each varied across three or four levels. Interactions of these factors among one another will be studied; however, interactions of these factors included in other portions of the design will not. All factors not included in this part of the design will be held constant at their prototypical values. The testis preparation factors to be studied and their ranges of variation are as follows:

Table 6. Summary of Experimental Testis Preparation Factors for Optimization

Factor Identification	Units	Factor Designation	Experimental Levels			
			1	2	3	4
hCG Concentration	IU/ml	X_1	0.01	0.1*	1	--
Time Delay	hr	X_2	0.5	1*	2	--
Organ Preparation Techniques	--	X_3	Cold buffered saline	Warm buffered saline	Cold media*	Cold physiological saline
Sample Aliquot Volume	ml	X_4	0.1	0.25	0.5*	--

* The central levels of the factors.

The design will consist of four parts, with a total of 46 runs. Both non-stimulated and hCG stimulated will be performed for a grand total of 92 runs. The order of the 92 runs will be randomized.

- Part 1. 3^{4-1} fractional factorial design. 27 runs. Three of the four levels of Organ Preparation Techniques - 1, 2, 3 - will be included. This portion of the design permits estimation of linear and quadratic main effects of factors X_1, X_2, X_4 independent of 2 factor interactions; contrasts among levels 1, 2, 3 of factor X_3 independent of 2 factor interactions; and all pairwise interactions among factors X_1, X_2, X_3. Interactions with Sample Aliquot Volume cannot be estimated.

- Part 2. 3^{3-1} fractional factorial design. 9 runs. Part 2 will be run at level 4 of Organ Preparation Techniques, which was omitted from Part 1. This portion of the design permits estimation of the linear and quadratic main effects of factors X_1, X_2, X_4 at level 4 of Organ Preparation Techniques, independent of 2 factor interactions. This, in conjunction with Part 1 permits estimation of pairwise interactions among factors X_1, X_2, and all levels of X_3.

- Part 3. Center Point. 1 run. This estimates the response when all factors are set to their "conventional" levels.

- Part 4. Replicates. 9 runs. These points provide estimates of the reproducibility of the response at the center point of the design and at various extremes of the design space.

6.4.3 Optimization of Sample Factors

The sample factors experiment is a stability test of the constituency of the medium or testosterone in the medium. Stability is assessed as a function of sample handling factors. The incubation medium used will be that which was found to be best in the preliminary experiments

(Phase I). It will be spiked with testosterone to known concentration(s), to be specified. The testosterone concentration at the end of the test run will be compared to the known spike level and the response will be the percentage recovery.

The sample factors experiment includes four factors, each varied over multiple levels. The factors to be studied and their ranges of values are as follows:

Table 7. Summary of the Experimental Sample Stability Factors for Optimization

			Experimental Levels			
Factor Identification	**Units**	**Factor Designation**	**1**	**2**	**3**	**4**
Sample Storage Container	--	X_1	Siliconized plastic*	Non-siliconized plastic	--	--
Sample Storage Time		X_2	1 day	1 week	1 month*	3 months
Sample Storage Temperature (1 and/or 3 months only)	^{0}C	X_3	--	--	-20^{0}C	-70^{0}C*

* The central levels of the factors.

Since sample storage time and sample storage temperature are not fully crossed, they are expressed as a single variable with six levels, namely:

		Experimental Levels					
Factor Identification	**Factor Designation**	**1**	**2**	**3**	**4**	**5**	**6**
Sample Storage Time/Temp	$X_{2/3}$	1 day	1 week	1 month/ -20^{0}C	1 month/ -70^{0}C*	3 months/ -20^{0}C	3 months/ -70^{0}C

The design will consist of a full 2 X 6 factorial layout in 12 runs and 9 replicate points, for a total of 21 runs. Both the non-stimulated and hCG stimulated assays will be run for a grand total of 42 runs. The order of the runs will be randomized.

6.4.4 Optimization of Sampling Time

In the tests discussed above (Sections 6.4.1 to 6.4.3) testosterone concentrations will be determined for the baseline (Time 0) and at 1, 2, 3, and 4 hours after equilibration. Tests will be carried out at the optimum conditions to determine whether the sampling times can be shortened or lengthened. Shortening the sampling times will improve assay efficiency. Lengthening the sampling times will permit application of the assay to test substances that require a relatively lengthy time period to display their effects on testosterone production.

Tests will be carried out at the previously determined optimum conditions. The medium will be sampled for testosterone concentration at a succession of sampling times, from short to long, e.g. 0.5, 1, 2, 3, 4, 8, 12, and 24 hours. Five independent replicate determinations will be made at each time point. The baseline concentration and the hCG-induced concentration will be determined at each time point. Average concentration and coefficient of variation will be determined as a function of sampling time. Of interest is how long the trend in testosterone concentration remains linear and how soon the coefficient of variation settles down to an approximately constant value.

6.4.5 Sensitivity Analysis

The experimental runs carried out in Sections 6.4.1 through 6.4.4 will be used to develop a response surface model that describes the variation in response level as a function of the variation in the totality of experimental factors. Sensitivity analyses will initially be carried out based on predictions from the response surface model. Changes in response from the predicted optimum will be estimated as a function of changes in experimental variables from their optimum values. The linear combination of experimental factors corresponding to the eigenvector associated with the minimum, i.e., the most negative, eigenvalue of the matrix of second partial derivatives at the maximum will be studied to assess the effect on reaction sensitivity of perturbations in experimental conditions in that direction.

Categorical variables will be varied across their possible levels. Quantitative variables will be varied by, e.g., ± 5%, ± 10%, ± 20%, and ± 30% from their optimum values. Those individual variables or combinations of variables that have the greatest influence on response will be determined. The predicted changes in sensitivity due to changes in these variables will be verified experimentally. The variables that are verified to in fact to be the most influential on assay efficacy will need to be controlled the most tightly.

Those individual variables or combinations of variables that have the least influence on response are also of interest. These variables can be changed relatively large amounts without substantially affecting assay efficacy. Model predictions of the least sensitive individual variables or combinations of variables will be verified experimentally. The variables that are verified in fact to be the least influential on assay efficacy may suggest ways to increase assay efficiency. For example, if sample storage at -20^{0}C results in nearly as good stability as sample storage at -70^{0}C, then there is a way to simplify sample storage without much impact on results.

6.4.6 Characterization of Viability

The experiments described below will be conducted after the preceding sections are completed, evaluated, and an optimized assay is identified. These experiments will be single factor experiments. Each level for a given factor will be run in triplicate.

1. Effect of potential solvents that will be used as carriers or vehicles for test substances. The solvents to be tested include ethanol, DMSO, and tween 20. In addition, each of the solvents will be tested at concentrations of 0.5, 1, 5, and 10 percent.

2. Time needed to achieve parenchymal equilibration (the minimum amount of time needed to allow the parenchyma to stop releasing testosterone from damaged cells). The times tested will include 15, 30, and 60 minutes.

3. Use of LDH to characterize cellular damage. The assay will be conducted using both testosterone and LDH as endpoints. It will include sampling before and after damage to parenchyma. Damage will be induced using trauma and/or heat.

4. Variability assessment. Evaluate optimized assay for day-to-day variability (the same technician conducts the assay on different days).

6.5 STATISTICAL ANALYSIS

Statistical analysis will be based on multiple regression analysis. Preliminary graphical displays will be used to identify the nature of the trends, the nature of the response variability, and the need for transformations of the response or of the primary experimental variables. Full quadratic response surface models will be fitted to the data. Residuals from the model will be examined graphically and numerically to identify outlying observations, heterogeneity of variability, and trend departures from model assumptions.

If several individual factors or combinations of factors exhibit particularly strong influence on the response outcome, consideration will be given to augmenting the design with supplemental runs to study the trends or interactions in these directions in greater detail.

The final response surface model will be optimized to determine the experimental conditions associated with the optimum response. A confidence region will be constructed around the optimum by considering the set of experimental factors whose associated responses do not differ significantly from that at the maximum. The optimum condition may occur at the interior of the design space or at a boundary. In the former case the optimum and an associated confidence region will be reported. In the latter case it may be possible to further improve the efficiency of the reaction by extrapolating outside the design space. Consideration will be given to extending the experimental region in the direction of the (increasing) response gradient to determine the extent of possible improvement in efficiency.

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