

**Story of the Steroidogenesis Assay**  
**in EPA's Endocrine Disruptor Screening Program**  
**by Gary Timm OSCP, OPPTS, EPA**

Background

Because it is not clear whether the *in vivo* assay that may be selected for the Tier 1 screening battery (e.g., female pubertal assay) will be adequately sensitive to detect effects on the steroid system, the *in vitro* steroidogenesis assay was devised to identify chemicals that have the ability to interfere with the synthesis of steroid hormones. In mammals, testosterone plays an essential role in sexual differentiation at a very specific time period during embryonic development. While testosterone also plays a role in females, a female *in vivo* assay would not be sufficiently sensitive to detect the effects of a steroid agonist or antagonist at the low levels that may affect sexual differentiation of the male.

Protocol Development

In developing an appropriate steroidogenesis screening assay, several different protocols were closely examined and discussed in a Detailed Review Paper (DRP). In compiling the DRP, entire protocols were dismissed for various reasons. EPA examined *ex vivo* methods (a combination of whole animal and organ methods), as well as tissue-based methods, including the testicular sliced/minced testes and minced ovary (i.e., granulosa) methods. *Ex vivo* methods were dismissed as not suitable for a screening program. It was determined that the minced ovary method was not ideal to detect chemicals that may disrupt the steroid system, as measuring steroid disruption is complicated by the large natural variation in steroid hormones due to the estrous cycle. Historically, the sliced testes method has been most successful in detecting steroid effects, and it was also judged to be the protocol most likely to be validated in a relatively short period of time.

In addition to whole animal and tissue-based methods, the DRP also examined cell line methods, which would eliminate the use of animals for this assay. Cell-based assays also offer the advantage over tissue-based methods of demonstrating enzyme induction effects in addition to competitive inhibition. While a myriad of methods were discussed and dismissed, three candidate cell line protocols were seriously considered using the following cell lines: the mouse Leydig cell tumor line (MA-10), the rat Leydig cell tumor line (R2C), and a human adrenocortical carcinoma cell line. The mouse and rat Leydig tumor cell lines lacked a critical enzyme: 17 ketosteroid reductase (17KSR), so they can only identify substances that interfere with the first half of the steroid synthesis pathway. The human adrenocortical carcinoma cell line (H295R) offers advantages over the Leydig tumor cell lines because it possesses all of the enzymes in the steroid synthesis pathway and can, therefore, identify substances that modulate the entire steroidogenesis pathway from cholesterol recruitment by STAR protein to conversion of androgens to estrogens .

Optimization of Sliced Testes Assay

Several parameters were examined for optimization of the sliced testes method. Laboratories assessed factors such as the size of the tissue slice, incubation temperature, vessel type and size, shaker speed, media volume, and the concentration of Human Chorionic Gonadotropin (HCG), which is used to stimulate hormone production in the tissue. Sampling time factors and composition of the gas in the incubation chamber were also optimized for the sliced testes protocol.

Besides the studies to optimize the parameters listed above, three other small special studies were conducted to further optimize the sliced testes protocol and to provide definitive answers to questions that arose from attempts to interpret data in the original optimization studies. First, there was a need to analyze the combination of media volume and vessel size (these had been examined as separate parameters in the first study). Second, collection and analysis of four separate samples proved to be time consuming and costly. Thus, a small experiment was carried out to determine if a single measurement could be taken at the end of the four-hour period. Because it was found that fresh media replacement was necessary, a compromise ultimately resulted: collect samples hourly but pool and analyze them at the end of the four-hour exposure period. Third, the question of the degree to which the testes fragments contribute to the variability of the assay surfaced as a key element in further reducing the variability of the assay. Therefore, an experiment was designed to examine the variability of testosterone production in fragments taken from different testes and different locations within a testes. The same experiment was used to optimize the dose level of the positive control, aminoglutethimide.

#### Additional Sliced Testes Prevalidation Studies

After optimization of the sliced testes protocol, a study was conducted to test the protocol with other test chemicals suggested in the DRP-- 2,4-dinitrophenol, flutamide, ketoconazole, vinclozolin, atrazine, dimethoate, finasteride, spironolactone, verapamil, and prochloraz-- in order to determine their effect on inhibiting the synthesis of testosterone in the sliced testes system. The results of this study will be discussed at the April 2005 Committee meeting.

The Endocrine Disruptor Methods Validation Subcommittee (EDMVS) was concerned that cytotoxicity may confound the results of the assay. In response, EPA requested Battelle to investigate several cytotoxicants including 2,4-dinitrophenol, dipropylene glycol, sodium azide and ethane dimethane sulfonate for use as possible cytotoxicant positive controls. The cytotoxicity studies using an LDH detection system showed few of the expected signs of cytotoxicity within the four-hour exposure period. Therefore, special studies further examining cytotoxicity were undertaken at EPA's laboratory in Research Triangle Park to determine what had gone wrong; for example, the LDH detection protocol may not have been sensitive enough, or the cytotoxicants may not have been acting within the four-hour time frame. The results of these studies and their implication will be discussed at the April 2005 Committee meeting.

The transferability of the protocol was also tested in other laboratories. Three laboratories participated in this study. They ran the basic assay in order to determine if they could 1) induce

testosterone production with hCG to demonstrate that they could perform the basic techniques required in the assay; 2) run the assay with the positive control, aminoglutethimide; and 3) run several the cytotoxicity positive control assay to detect potential cytotoxic chemicals. The inter- and intra-laboratory special studies were carried out several times. The fairly high variability seen in some of these studies and the multichemical study is a concern and will be a topic of discussion at the April meeting.

At the April meeting, EPA will ask the EDMVAC its recommendation regarding the next steps for this assay. Should we proceed with validation or are additional studies necessary before validation can begin? If so, what does the Committee recommend?

### H295R Assay

EPA initiated prevalidation studies for the H295R protocol in March 2004. These studies will be completed in April, 2005. EPA discussed the H295R assay at the November 2004, meeting of The Organization of Economic Cooperation and Development (OECD) non-animal test method Validation Management Group. The H295R cell line protocol has greater international acceptance than the sliced testes protocol because it does not require the use of animals. EPA invited Member Countries to join the US in validating this assay. Danish and Japanese laboratories volunteered to join the U.S. in validating the H295R assay and are beginning a demonstration of the transferability of the protocol in April 2005. Optimization should be complete in early 2005, and an interlaboratory protocol transferability study should take place in Spring 2005. Due to international involvement in the development of this protocol, the U.S. will lose some control over the schedule for the validation of this assay and the need for coordination will also lead to some delays.

### The Bottom Line

Since there is uncertainty in the H295R schedule, EPA currently plans to proceed with the validation of both assay systems. However, assuming the validation efforts on the H295R are successful, the H295R assay would replace the sliced testes assay in the U.S. and would become the basis for the OECD test guideline for steroidogenesis.

### Additional Information

For the detailed reports on the previously conducted studies mentioned in this document, please see the *Assay table* on the EDSP website at <http://www.epa.gov/scipoly/oscpendo>.