DRAFT STUDY PLAN

ON

COMPARATIVE EVALUATION OF VITELLOGENIN METHODS

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 2-19, TASK 2

FEBRUARY 26, 2002

Prepared for

LES TOUART
WORK ASSIGNMENT MANAGER
U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

Prepared by

BATTELLE
505 King Avenue
Columbus, Ohio 43201

TABLE OF CONTENTS

1.0 INTRODUCTION

The U.S. Environmental Protection Agency is implementing an Endocrine Disruptor Screening Program (EDSP) comprised of a battery of Tier 1 screening assays and Tier 2 tests. One of the Tier 1 assays under development is a short-term screening assay designed to detect substances that interact with the estrogen and androgen systems of fish. It is thought that the inclusion of the fish screening assay in Tier 1 is important because estrogenic and androgenic controls on reproduction and development in fish may differ significantly enough from that of higher vertebrates such that mammalian screening methods may not identify potential EDCs in this important class of animals. The measurement of a biochemical marker, vitellogenin (VTG) in oviparous vertebrates is generally agreed to be a good indicator for estrogenic and anti-estrogenic effects and is proposed as one of several endpoints in the fish screening assay. Vitellogenin is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually active female oviparous fishes, but can be induced to occur in males in response to estrogenic substances. Different methods are available to assess VTG production in fishes and range from enzyme-linked immunoabsorbant assay (ELISA) to mRNA detection using PCR techniques. Both plasma and whole body measurements have been proposed.

The purpose of work conducted under this assignment is to coordinate an inter-laboratory comparison of VTG analytical methods for suitability in a routine screening program. This comparison is not intended to be a validation of a given method but an evaluation across methods to ascertain the qualitative and/or quantitative comparability of the variety of methods currently available.

The specific objectives of the study are to

1) Prepare a standard evaluation series of fish plasma and tissue (e.g. whole body homogenate) to provide a range of vitellogenin and mRNA concentrations, including samples with low, intermediate, high and very high levels. This series will represent a normal male, induced male, normal female, and induced female, respectively. In addition to the standard series, a set of positive and negative control samples will be prepared. A repository of the standard evaluation series and controls will be maintained and the stability of the samples will be monitored during the study.

2) Identify laboratories to participate in the analysis of the standard evaluation series and coordinate the transfer of the samples to the participating laboratories and the collection of analytical results. The laboratories will employ the specific analytical technique routinely used to measure VTG within each laboratory. This will result in a variety of analytical methods applied to the standard series and each laboratory will provide a detailed analytical protocol and report the results of the analysis.

3) Analyze the results of the analysis and prepare a final report. The report will present and discuss the data provided by the analytical laboratories and the variability of the results.

2.0 SAMPLE PREPARATION AND HANDLING

The vitellogenin (VTG) standard evaluation series will be prepared from fathead minnow plasma and whole body homogenates. Low, intermediate, high and very high titer levels will be obtained to represent normal male, induced male, normal female, and induced female fish,

respectively. The induced male and female VTG titer will be achieved in adult male and female fathead minnows via exposure to17β-estradiol in the laboratory. Conversely an unexposed group of males and females will be used to generate normal background concentrations for male and female fish. After a 1-week exposure, when maximal VTG levels in plasma are anticipated, the fish will be sacrificed and plasma will be collected and whole body homogenate will be prepared. Several steps will be employed to aid in preserving the integrity of the samples including the use of an inhibitor (aprotinin), cold processing, and quick freezing to stabilize the VTG samples. A large number of samples will be collected individually and frozen upon collection. Following the processing of the individual fish, the group of plasma or tissue samples will be quickly thawed and combined to prepare the pooled samples. The exposed fish samples will provide the plasma and tissue samples for the male and female high VTG titer for the standard series. Samples composited from individual unexposed fish will provide the low male and female sample in the standard series. This will result in the four VTG levels of samples in the series. In addition to the samples representing four VTG levels found in plasma and whole body homogenate from normal and induced fish for the standard series, VTG will be purified from plasma from exposed male fathead minnows for controls and standards in the analytical assays.

Multiple aliquots of each sample will be prepared and stored until required for analysis or for stability checks. A repository of the VTG samples will be maintained and the stability of the samples will be assessed initially and with the coordinated shipment to the participating laboratories. A sub-set of participating laboratories will check the stability of the sample when shipped. The laboratories will be advised to store the samples under conditions similar to those found in the repository and to avoid freeze-thaw cycles of the samples. A similar approach will be applied for an mRNA standard evaluation series. Adult male and female fathead minnows will be exposed to 17β-estradiol and liver samples will be collected and pooled from fish after a 2-day static renewal exposure (Korte et al., 2000). This will provide a samples with a low, intermediate, high and very high titer levels will be obtained to represent normal male, induced male, normal female, and induced female, respectively.

3.0 SAMPLE METHODS

One hundred adult male fathead minnows and 200 adult females will be exposed to 300 ng/L of 17β-estradiol in a 7-day static renewal exposure, while an additional 300 (100 male and 200 female) hundred fish will remain unexposed. On day 2, fifty exposed and unexposed fish (25 male and 25 female) will be sacrificed and the liver tissue will be harvested for the mRNA standard series. The liver tissue will be processed as rapidly as possible and flash frozen with liquid nitrogen prior to storage at -80C. After collection, this tissue will be quickly thawed and combined through homogenization to provide a uniform composite sample. This composite sample will serve as a source of tissue with very high mRNA levels for the standard series. Similarly, unexposed liver samples will be combined for the low level sample and for proportional combination with the very high sample to generate the intermediate and high level sample.

On day 7 of the exposure, the remaining exposed fish will be anesthetized with MS222 and blood will be collected from the caudal vessels into ice-chilled heparinized hematocrit tubes. The blood will be transferred to centrifuge tubes containing heparin and aprotinin and following mixing, the tubes will be centrifuged. The plasma will be harvested and frozen at -80C. Plasma will be collected from the unexposed fish in a similar manner. A subset of fish from the exposed and unexposed groups will be used to prepare whole body homogenates for analysis. For a

subset of the samples, whole body tissue homogenate will be prepared by placing the fish into ice-cold ELISA assay buffer in a 1:1 ratio. The samples will be homogenized on ice and following homogenization, the samples will be centrifuged and the supernatant will be harvested and frozen at -80C. Care will be taken in each of the steps to collect and process the samples in a timely manner under cold conditions followed by a quick freeze, to limit the time from collection to storage. Individual plasma and tissue homogenate samples will be thawed and pooled on ice to create the composite samples for the series.

Multiple aliquots of each sample will be created, coded and entered into the repository management system. The number of samples will vary depending upon the amount of material obtained from the fish for each type of sample, however, the number of samples will ensure a supply of samples for analysis by the total number of laboratories in the study, including backup samples for contingencies, and for stability checks. The samples will be assigned unique numbers and will be provided to the participating laboratories with the low, intermediate, high and very high description. As concentrations of VTG in fish plasma and homogenate samples can span eight orders of magnitude, this general designation will allow the laboratories to anticipate the degree to which the sample must be diluted to run in their assay. Based on the type of assay and volume needs that a testing laboratory has, the appropriate aliquot will be packaged and shipped to the test facility. The stability of the samples will be monitored by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and Western blotting. The samples will be reduced with β-mercaptoethanol, separated on SDS gels and the separated proteins will be transferred to a PVDF membrane for Western blotting.

For purification of VTG, two hundred adult male fish will be exposed to 300 ng/L of 17β-estradiol in a 7-day static renewal exposure. On day 7, plasma will be collected as described for the standard series and pooled for purification by anion exchange chromatography on DEAE agarose. The purified VTG will be used for standards in the analytical assays and to create a positive control sample within the range of the standard series.

4.0 ANALYTICAL METHODS

A number of methods have been developed for the quantification of VTG in blood plasma, liver tissue, or whole-body homogenates. The various methods differ in sensitivity, specificity, and technical difficulty. Currently, the most popular approach to measure VTG is some form of an enzyme linked immunosorbent assay (ELISA), although radioimmunoassay (RIA) may occasionally be employed.

The principle of RIA is based on the reversible reaction between the antigen (the VTG protein of interest) and a specific antibody that has been raised against the antigen. In the assay, a limited amount of specific antibody is reacted with the corresponding steroid labeled with a radioisotope (tritium or iodine) and differing amounts of VTG in solution. Increasing the amount of unlabeled VTG competing for the antibody results in progressively less radiolabeled VTG bound to the antibody. The free VTG and that bound to the antibody can be separated, quantified with a radioactive detector, and these two fractions used to construct a standard curve against which unknown samples can be compared.

A number of VTG RIAs for different fish species have been reported from individual research laboratories (So et al., 1985; Diamond & Oris, 1995; Goolish et al., 1998; Kim & Cooper, 1999), based on the first VTG RIA developed for rainbow trout (Sumpter, 1985). A commercial fish VTG RIA has never been marketed.

In addition to the radioimmunoassay (RIA), VTG has been measured by other immunoassay techniques including enzyme linked immunosorbent assay (ELISA), immunodiffusion and immunoblotting. The ELISA employs enzyme-linked antibodies and an adsorbent surface to detect specific antigens in solution. The ELISA has been widely used to quantify VTG in teleosts due to the ease in use and unlike the RIA, ELISA does not require the use of radioactive isotopes. There are a variety of ELISA designs that typically fall into three general assay formats including competitive, sandwich and direct ELISAs. Competitive ELISAs incorporate a step in which the samples and antibody (antibody-capture) or labeled antigen (antigen-capture) are incubated together prior to adding the sample on the test plate. This non-equilibrium design is often used to enhance sensitivity and counteracts potential preferential binding (Edmunds et al., 2000). In a direct antibody-capture ELISA, the sample and standards are adsorbed directly on the surface of the microwell plate. After incubation, the wells are blocked and anti-VTG antibody is added to bind to the VTG attached to the well. As with other ELISAs, subsequent steps culminate in the development of color reflective of the amount of antigen present in the sample. Sandwich ELISAs employ two antibody preparations to detect the antigen. The antigens can recognize different epitopes on the target analyte, thereby providing a large degree of specificity and sensitivity

Mass spectrometry (MS) offers exciting future possibilities for becoming a reference method for VTG and for combining multiple protein analysis from a single tissue sample. In general, MS approaches to protein quantification attempt to measure the protein largely in its intact form or rely on digestion procedures (chemical or enzymatic) to reduce the size of the protein into smaller fragments. The MS technique allows both the direct measurement of the VTG mass and generation of peptide-fingerprinting data for further identification (Wunschel and Wahl, 2002).

An alternative to measuring the VTG protein is to quantify the messenger ribonucleic acid (mRNA) for VTG that codes for the protein. Two methods for quantifying fish VTG mRNA have emerged, the ribonuclease protection assay (RPA) and quantitative reverse transcription-polymerase chain reaction (QRT-PCR), although other methods exist (e.g., Northern blot, slot-blot), they have drawbacks relative to sample throughput or sensitivity. All methods can be used for absolute or relative quantification of mRNA.

The quantification of VTG mRNA by RPA relies on hybridization in solution of a homologous antisense RNA probe (either radiolabeled or non-isotopically labeled) with sample RNA extracted from the liver of the test species. Probes are designed to be 200 to 400 nucleotides long. After the hybridization step, any RNA that has not hybridized to the probe (i.e., any single-stranded RNA) is degraded using ribonucleases. The remaining hybridized RNA fragments (target + probe) are electrophoresed on an acrylamide gel and the amount of radioactivity quantified. In the case of a non-isotopically labeled probe, the samples are transferred from the gel to a membrane and measured with a secondary detection method (e.g., chemoluminescence). Molecular biological kits to conduct RPA are commercially available (e.g., Ambion) and have been adapted for fish VTG mRNA measurement (Kishida & Callard, 2001) or developed in-house from published methods on the technique (Spitsbergen et al., 2000).

In QRT-PCR, all the mRNAs in a liver RNA sample from the test fish are copied into complementary DNAs (cDNAs) using oligo-dT primer and reverse transcriptase (RT). Alternatively, a species-specific VTG 3=-primer can be used in the RT reaction to yield only VTG cDNA. Independent of the RT method used, the next step uses polymerase chain reaction

(PCR) to preferentially amplify the cDNA using VTG sequence-specific primers. Subsequent detection of VTG PCR products currently follows two routes. The first involves electrophoresis of the DNA products on an agarose gel. If radiolabeled nucleotides or primers were used, the gel is dried and some means of detection (e.g., phosphoimager, X-ray film) is used to quantify the radioactivity associated with the DNA products. If not isotopically labeled, the DNA products are detected using DNA-binding dyes (e.g., ethidium bromide, SYBR green) (Van Den et al., 1987). The other detection method uses real-time PCR in which a fluorescent probe, specific to the amplified DNA sequence, is used to detect the amount of product produced (Yasuda et al., 2000). The amount of fluorescence is monitored at the end of each cycle of PCR (i.e., real-time), and the amplification curve is recorded by computer. With either detection scheme, the amount of DNA product measured is assumed to be directly proportional to the amount of starting mRNA. This measurement is compared with known amounts of VTG mRNA that have been included in the assay to determine the amount of starting mRNA target. A number of commercially available QRT-PCR kits (e.g., Life Technologies; Applied Biosystems) are currently on the market that could be adapted for species-specific VTG mRNA measurements.

Specific protocols employed by the participating laboratories will be applied to the samples in this study. The analysis will fall into the general categories of vitellogenin and mRNA as the target analyte. Within these categories, multiple methods will be applied to the sample series. The participating labs will receive multiple aliquots of the standard series as a contingency to prevent the need for sample freeze thaw cycles and to limit the number of shipments to each laboratory. Each laboratory will be asked to analyze the samples with a minimum of four replicates.

5.0 REPORTING FORMAT TO BE USED BY ALL PARTICIPANTS

The results of analysis provided by the individual laboratories will include a minimum of the following information:

Laboratory Identification
Protocol Reference ID
For each sample:
- Sample Code
- Raw data series
- n=
- Mean
- Standard Deviation
- % Coefficient of Variation

The results of the analysis will be tabulated in the following manner:

Target Analyte	Vitellogenin	mRNA
Participating Lab (A, B, C, etc)	Testing Lab	Testing Lab
Analytical method (e.g., ELISA)	Method	Method
Standard Series	Results (including Mean, Standard Deviation, and % Coefficient of Variation)	Results (including Mean, Standard Deviation, and % Coefficient of Variation)
Sample Code ("Blank")	Reported Results	Reported Results
Sample Code (Low)	Reported Results	Reported Results
Sample Code (Intermediate)	Reported Results	Reported Results
Sample Code (High)	Reported Results	Reported Results
Sample Code (Very High)	Reported Results	Reported Results

6.0 PARTICIPATING LABORATORIES

The laboratories participating in this study will be selected based upon their previous experience in the measurement of vitellogenin or mRNA. The laboratories should have well established protocols in routine use and should be willing to commit to analysis during the study period. It is anticipated that the laboratories in this "ring study" will include representatives from academia, government and private industry. The laboratories will be invited to participate irrespective of the specific VTG or mRNA method employed, however, every effort will be made to limit the redundancy of the various methods.

7.0 REFERENCES

Diamond, S. A. & Oris, J. 1995, "An Inexpensive Fathead Minnow Egg Incubation and Toxicant Exposure System," *Environmental Toxicology and Chemistry*, Vol. 14, No. 8, pp. 1387-1388.

Edmunds, J. Stewart, McCarthy, Robert A., Ramsdell, John S., 2000, "Permanent and functional male-to-female sex reversal in d-rR strain medaka (*Oryzias latipes*) following egg microinjection of o,p'-DDT," *Environmental Health Perspectives*, Vol. 108, Issue 3, pp 219-224.

Goolish, E. M., Evans, R., Okutake, K., & Max, R. 1998, "Chamber Volume Requirements for Reproduction of the Zenbrafish Danio Rerio," *Progressive Fish-culturist*, Vol. 60, No. 2, pp. 127-132.

Kim, Y. & Cooper, K. R. 1999, "Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and Polychlorinated Biphenyls (Pcbs) in the Embryos and Newly Hatched Larvae of the Japanese Medaka (*Oryzias latipes*)," *Chemosphere*, Vol. 39, No. 3, pp. 527-538.

Kishida, M. & Callard, G., 2001, "Distinct Cytochrome P450 Aromatase Isoforms in Zebrafish (Danio Rerio) Brain and Ovary Are Differentially Programmed and Estrogen Regulated During

Early Development," *Endocrinology*, Vol. 142, No. 2, pp. 740-750.

Korte, J.J., Kalh, M., Jensen, K., Pasha, M., Parks. L., LeBlance, G., and Anlkey, G., 2000, "Fathead Minnow Vitellogenin: Compllementary DNA Sequence and Messenger RNA and Protein Expression after 17β-Estradiol Treatment," *Environmental Toxicology and Chemistry*, Vol. 19, No. 4, pp. 972-981.

So, Y. P., Idler, D. R., Truscott, B., & Walsh, J. M. 1985, "Progestogens, Androgens and Their Glucuronides in the Terminal Stages of Oocyte Maturation in Landlocked Atlantic Salmon," *Journal of Steroid Biochemistry and Molecular Biology*, Vol. 23, No. 5a, pp. 583-591.

Spitsbergen, J. M., Tsai, H., Reddy, A., Miller, T., Arbogast Dan, Hendricks, J. D., & Bailey, G. S. 2000, "Neoplasia in Zebrafish (Danio Rerio) Treated with N-methyl-n'-nitro-n-nitrosoguanidine by Three Exposure Routes at Different Developmental Stages," *Toxicologic Pathology*, Vol. 28, No. 5, pp. 716-725.

Sumpter, J. P. 1985, "The Purification and Radioimmunoassay of Vitellogenin from the Rainbow Trout.," in *Current Trends in Comparative Endocrinology*, B. Lofts & W. M. Holmes, Eds., Hong Kong University Press, Hong Kong, pp. 355-357.

Van Den, H. U. R. K., Schoonen, W. G. E. J., Van Zoelen, G. A., & Lambert, J. G. D. 1987, "The Biosynthesis of Steroid Glucuronides in the Testis of the Zebrafish Brachydanio-rerio and Their Pheromonal Function as Ovulation Inducers," *Gen Comp Endocrinol*, Vol. 68 (2). 1987. 179-188..

Wunschel, D.S., and Wahl, K.L. 2002, "Deglycosylation and Analysis of Vitellogenin and Ovalbumin by Mass Spectrometry." Unpublished studies. Advanced Analytical and Organic Methods Group, Pacific Northwest National Laboratory, Richland, WA.

Yasuda, T., Aoki, K., Matsumoto, A., & Ishikawa, Y. 2000, "Heritable Malformations Induced by X-irradiation in the Medaka," *Journal of Radiation Research*, Vol. 41, No. 4, P. 484.