Single-Laboratory Validation of a homologous ELISA to quantify Vitellogenin in the fathead minnow (Pimephales promelas)

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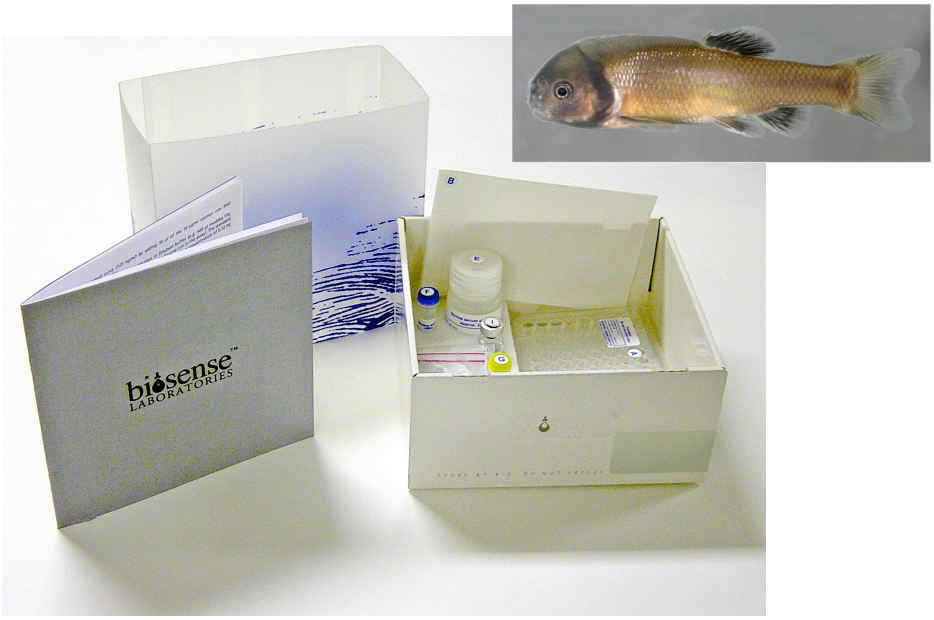

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SUMMARY

Vitellogenin (Vtg) is an established and sensitive endpoint for analysis of endocrine disruption in fish. The widespread use of Vtg in this regard has led to the need for standardised assays to quantify Vtg in fish samples. Fathead minnow (*Pimephales promelas*) is an important fish test species used in ecotoxicology laboratories across the world.

Based on monoclonal antibodies raised against fathead minnow (FHM) Vtg, we have developed a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) for FHM Vtg. A Single-Laboratory Validation was performed according to international guidelines. Study samples consisted of both plasma, whole body homogenate (WBH) and ELISA kit Dilution buffer. The results are summarised in Table 1, and show that the FHM Vtg ELISA is suitable for quantification of Vtg in both plasma and WBH samples from FHM.

Table 1: Summary of results from Single-Laboratory Validation results

Performance characteristics	Aim[1]	Value
Selectivity	Matrix blank < LOD (with the necessary dilution factor to avoid matrix effects)	No response at minimum dilution = 1:50 (plasma), 1:100 (WBH)
Calibration	Standard curve working range >10-fold, preferably 50-100 fold to be practical with the dynamic range found in Vtg levels	Standard curve working range 0.1-25 ng/ml (250-fold)
Accuracy (Recovery)	Ideally 50-200%	75-106%
Repeatability Precision RSD_r	<20%	Within-Day RSD_r: 4.5% Between-Day RSD_r: 9.9%
Limit of Detection (LOD)	<10 ng/ml	0.02 ng/ml (plasma) 0.04 ng/ml (WBH)
Limit of Quantification (LOQ)	<10 ng/ml	0.09 ng/ml (plasma) 0.11 ng/ml (WBH)
Sample LOQ (=LOQ x necessary matrix dilution)	200 – 500 ng/ml	4.68 ng/ml (plasma, 1:50) 11.35 ng/ml (WBH, 1:100)
Comparison with Biosense Carp Vtg ELISA kit		R^2>0.99
Comparison with competitor FHM Vtg ELISA kit		R^2>0.99

[1)] Goksøyr *et al* 2003

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INTRODUCTION

Vitellogenin (Vtg) is a large phospholipoglycoprotein, which functions as the egg yolk precursor in oviparous vertebrates such as fish. The Vtg protein is produced in the liver and secreted from the liver cells through the secretory pathway, enters the

blood circulation where it is transported to and taken up by growing oocytes. Endogenous oestrogen levels regulate Vtg production (Figure 1), and plasma Vtg concentrations normally indicate the maturational status of the female fish (for reviews, see Mommsen & Walsh, 1988; Arukwe & Goksøyr, 2003). More than a decade ago, several studies demonstrated that also male fish, caught in rivers and streams, had high levels of plasma Vtg (*e.g.* Purdom *et al*., 1994; Jobling *et al*., 1998), caused by chemicals present in the environment, acting like estrogens. Since then, numerous studies have shown the fish Vtg to be a highly responsive biomarker for estrogenic compounds in both *in vitro* hepatocyte cell cultures, *in vivo* aquaria studies, and field studies (for reviews, see Kime, 1995; Sumpter & Jobling, 1995; Arukwe & Goksøyr, 1998; 2003). Vtg induction in fish has now become an accepted measure of xenoestrogenic potency of chemicals, effluents, and discharges.

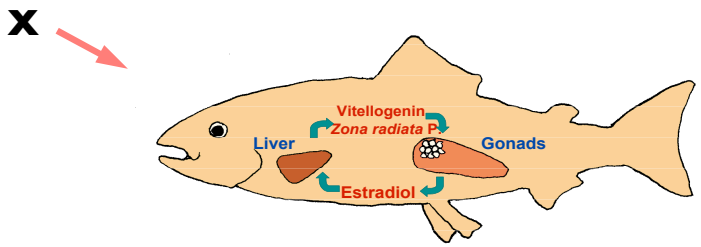

*Figure 1: In response to oestradiol or xenoestrogens (**x**), Vitellogenin (egg yolk precursor) and zona radiata proteins (egg shell precursors) are produced in the liver and transported via the blood to the gonads.*

Within international bodies such as the Organization for Economic Cooperation and Development (OECD), work is ongoing to develop screening and testing programmes for endocrine disrupting effects of new chemicals. In the focus of this development are small fish test species including the fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), and Japanese medaka (*Oryzias latipes*). These fish share several attributes that make them ideal test species for reproductive toxicity testing, including small size at maturity, relatively short generation times, asynchronous spawning, and overall ease of culture.

Against this background, there is a need for specific, sensitive, and reliable methods for measuring Vtg levels in these fish species, assays that are readily available and give reproducible results in different laboratories. The enzyme-linked immunosorbent assay (ELISA) is a sensitive laboratory technique widely used to detect and quantify antigens in a variety of biological samples. They can be quantitative (with a standard curve) or qualitative (semi-quantitative - without a standard curve). The two most widely used principles for quantitative detection of

proteins are the competitive ELISA and the sandwich ELISA techniques (Crowther, 2001).

We have previously developed quantitative sandwich Vtg ELISAs for zebrafish and Japanese medaka, as well as a homologous carp Vtg ELISA, which also works well for fathead minnow Vtg (Nilsen *et al* 2003). Here, we present Single-Laboratory (in-house) Validation data on a new, homologous quantitative sandwich ELISA for measuring Vtg in plasma and whole body homogenate samples of the fathead minnow. The validation has been carried out at Biosense, according to international guidelines for Single-Laboratory Validation (AOAC International Training Course, 2003; Thompson *et al*, 2002; Eurachem, 1998).
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THE FATHEAD MINNOW VITELLOGENIN ELISA

The fathead minnow (FHM) Vtg ELISA is based on a sandwich format, utilizing two different monoclonal mouse antibodies (Abs) raised against FHM Vtg. The antibodies were developed at the University of Florida, Gainesville, USA.

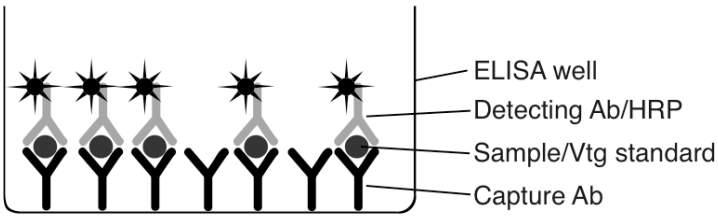

Figure 2: The principle of the fathead minnow Vtg ELISA.

The capture antibody is immobilised on ELISA microtiter plates, and binds to FHM Vtg in the standard or sample added to the ELISA well (Figure 2). After unbound components are washed away, a Detecting Ab, labelled with the enzyme horseradish peroxidase (HRP), is added. This Ab binds to a different part of the Vtg molecule, creating a sandwich of antibodies and Vtg. Addition of the HRP substrate Tetramethyl Benzidine (TMB) results in a colour reaction where the enzyme catalyses the conversion of this uncoloured substrate to a blue product. After development, the reaction is stopped by addition of a mild sulphuric acid, changing the colour from blue to yellow. The colour intensity is measured using a microplate reader with a 450 nm filter, and is proportional to the concentration of Vtg in the standard/sample.

All absorbance levels are corrected for non-specific background reading (NSB), and a calibration curve is created by plotting Vtg concentration on the x-axis and the corresponding absorbance level on the y-axis (Figure 3). A 4 parameter or a log-log curve fit can be used to describe the relationship between the concentration and

the signal. The equation for this calibration curve is then used to calculate the Vtg concentration in plasma or homogenate samples.

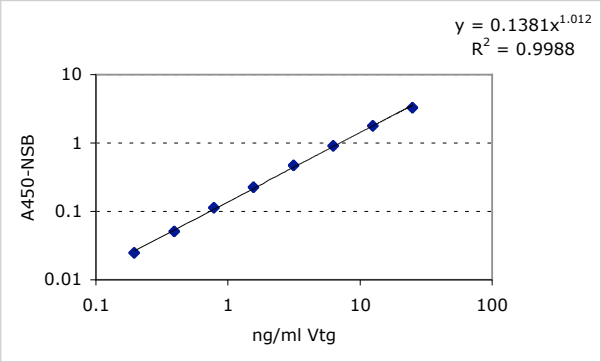

Figure 3: Fathead minnow Vtg standard curve

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BRIEF SUMMARY OF METHOD

Equipment and reagents required in addition to the FHM Vtg ELISA kit:

- 0.3M H_2SO_4 (stop solution)
- Microplate reader (wavelength 450 nm)
- Pipettes with disposable tips (5-1000 µl)
- Multi-channel pipette and reagent reservoir. Alternatively, a stepper pipette with disposable tips (100 µl) can be used.
- Test tubes (1-50 ml)
- Microplate washing device (an automatic or manual plate washer is recommended, but a squeeze bottle or a multichannel/stepper pipette can also be used)
- Vortexer
- Crushed ice

Summary of the ELISA method:

1. Thaw samples on ice.
2. Prepare dilutions of standard and samples on ice.
3. To the pre-coated plates, add 100 µl Dilution buffer to the NSB wells.
   Add 100 µl of diluted standards and samples to the remaining wells.
   Incubate at room temperature for 1.5 hour.
4. Wash the plates three times with 300 µl Washing buffer per well.
   Add 100 µl of diluted Detecting antibody to all wells.
   Incubate at room temperature for 0.5 hour.
5. Wash the plates five times with 300 µl Washing buffer per well.
   Add 100 µl Substrate solution to all wells.
   Incubate in the dark at room temperature for 20 minutes.
7. Add 100 µl of 0.3M H_2SO_4 to all wells to stop the reaction.

8. Read the absorbance at 450 nm.
9. Calculate the results.

Total sample capacity: 12 samples per plate (three dilutions of each sample, analysed in duplicates)
Total assay time: 2 hours, 20 minutes.
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PURPOSE OF THE VALIDATION

In the process of evaluating Vtg as an endpoint for endocrine disruptor testing and screening, various studies have been conducted over the recent years, involving both non-commercial laboratory methods and a few commercially available ELISA kits (for zebrafish Vtg inter-comparison study see http://abstracts.co.allenpress.com/pweb/setac2003/document/?ID=30012, for an overview of validation status in the US, see http://www.epa.gov/scipoly/oscpendo/assayvalidation/status.htm). These studies have demonstrated the variance between different Vtg standards, between different assays and between different laboratories using the same assay.

Clearly, there is a need for standardised, reproducible methods/kits that have been thoroughly characterised, with validation data describing the assay's performance, thus giving the end user reasonable expectancies. The aim of the following study was to obtain Single-Laboratory Validation data, according to international guidelines (AOAC, Eurachem, IUPAC).
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AIMS OF THE VALIDATION STUDY

In a 2003 document, Biosense took the initiative to stress the need for standardised validations of Vtg standards and assays (Goksøyr *et al*, 2003). In this document, based on experience from participation in studies comparing Vtg methods, we suggested a set of performance criteria to be met by Vtg quantification methods (Table 2).
In this Single-Laboratory Validation (SLV), a set of characteristics were analysed, chosen on the basis of guidelines set up by international bodies like AOAC, Eurachem and IUPAC. It is recommended that "Single-Laboratory validation requires the laboratory to select appropriate characteristics for evaluation from the following: applicability, selectivity, calibration, accuracy, precision, range, limit of quantification, limit of detection, sensitivity and ruggedness" (Thompson *et al* 2002).

Table 2 Pre-determined performance criteria (Goksøyr et al 2003)

Performance characteristics	Aim
Selectivity	Matrix blank < LOD (with the necessary dilution factor to avoid matrix effects)
Calibration	Standard curve working range >10-fold, preferably 50-100 fold to be practical with the dynamic range found in Vtg levels
Accuracy[1)]	Ideally 50-200%
Repeatability [2)]	<20%
Reproducibility[3)]	<50%
Limit of Detection (LOD)	<10 ng/ml
Limit of Quantification (LOQ)	<10 ng/ml
Sample LOQ (=LOQ x necessary matrix dilution)	200 – 500 ng/ml

[1)] Referred to in this document as Recovery
[2)] Referred to in this document Within-Day and Between-Day Repeatability Precision RSD_r
[3)] Referred to in this document Between-Laboratory Reproducibility Precision RSD_R

This study aimed to obtain Single-Laboratory Validation data on the following parameters:

1. Applicability
2. Calibration
3. Limit of detection (LOD), Limit of Quantification (LOQ)
4. Selectivity
5. Precision (Within-Day, Between-Day)
6. Accuracy (spiking/recovery, bias)
7. Ruggedness

In addition, comparisons with existing methods were also conducted.

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DEFINITIONS

The terminology involved in validation work is often confusing, and depends largely on which set of guidelines one looks at. The following definitions have been used in this report (see http://www.aoac.org/intaffairs/analytical_terminology.htm):

Applicability (Scope): The analytes, matrices and concentrations for which a method may be used satisfactorily.

Selectivity: The ability to measure accurately the analyte (Vtg) in the presence of components that may be expected to be present in the matrix (plasma and whole body homogenate).

Calibration, is the empirical determination of the relationship between the parameter measured (*e.g.* ELISA absorbance) and the analyte (Vtg) concentration. The range of concentrations of analyte where such relationship is established is often referred to as "calibration range" or the "standard curve working range".

Accuracy is the closeness of agreement between a test result and the accepted reference value of the property being measured.

Recovery is the proportion of the amount of analyte, present in or added to, the analytical portion, which is extracted and presented for measurement

Bias is the difference between the test results and an accepted reference value

Precision is the closeness of agreement between test results obtained under stipulated conditions.

- *Repeatability Precision* (same laboratory and operator, samples, equipment, short time intervals), separated into Within-Day and Between-Day Repeatability precision, usually expressed as relative standard deviation, RSD_r.
- *Reproducibility Precision* (different laboratories, equipment and operators, same samples). Usually expressed as relative standard deviation, RSD_R.

Limit of Detection is the smallest amount or concentration that can be reliably distinguished from zero. Defined here as reagent blank + 3x standard deviation of reagent blank. Indicates that the analyte is present, but not necessarily allowing exact quantification.

Limit of Quantification A concentration above which the analytical method can operate with an acceptable precision. Defined here as reagent blank + 10x standard deviation of reagent blank.

Sample LoQ. The LoQ corrected for minimum dilution factor necessary to avoid matrix effect.

Ruggedness. The ability of the measurement process to resist changes in results when subjected to minor changes in environmental and procedural variables.
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VITELLOGENIN STANDARD

A validation of a quantitative Vtg ELISA should address not only the assay itself, but also the Vtg standard used. Here we describe the purification, quality assurance and quantification of the Vtg standard used in the FHM Vtg ELISA.

Important issues here are

- the choice of source for purification
- the purification method
- the quality assurance
- the quantification method
- stabilization procedure

Ideally, a certified reference material (CRM) should be used as a standard in quantitative assays. In the case of a Vtg ELISA, this CRM should consist of intact Vtg purified to apparent homogeneity from the test species in question, and should

be quantified according to accepted methods for protein quantification. In addition, the Vtg should be stabilized for shipping and storage.

Due to the lack of guidelines or criteria for these issues, we will not refer to the FHM Vtg standard as CRM, but rather only as Reference Material (RM).
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SOURCE FOR RM PURIFICATION

Various sources of purified standards for Vtg assays have been used, including plasma, WBH, liver homogenate, ascites fluid, and egg yolk. For each of these sample types, the state of the Vtg will be different. For example, the liver cell will contain immature (unprocessed) Vtg that has not undergone full post-translational modifications, as well as mature Vtg ready for secretion, whereas the egg yolk will contain the lipovitellin form processed after uptake. WBH will contain a mixture of these (unless ovaries and /or liver have been removed), in addition to a high level of proteolytic activity that may act to degrade the protein during preparation.

We chose to purify Vtg from plasma obtained from estrogenized FHM, because such plasma contains intact, circulating Vtg at a high concentration (mg/ml levels). Plasma was kindly provided by Charles Tyler, University of Exeter, UK.

The matrix for RM production should be obtained from estrogenized fish exposed to 17ß-oestradiol (or another given reference oestrogen) for a given period of time. A suitable protease inhibitor (*e.g.* aprotinin or a protease inhibitor cocktail) should be added to the matrix during sampling to avoid degradation of Vtg.
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PURIFICATION OF RM

Due to the instability of Vtg, the purification procedure should be as rapid as possible while maintaining the integrity of the protein and yielding a pure product. Various methods include

- Ion exchange chromatography, with or without a following gel permeation clean-up (*e.g.* Brion *et al*., 2002).
- Selective precipitation of Vtg from plasma using $MgCl_2$. This is a rapid method that has been successfully used with some species (*e.g.* trout and carp: Norberg & Haux, 1985, and Nilsen *et al*., 2003), but appears to be less useful for other species.
- Immunoaffinity-based procedures (*e.g.* chromatography or magnetic beads). This strategy requires Vtg-specific Abs, and may give a bias in the composition of the purified product depending on the epitope specificity of the antibody used.

Different purification protocols may lead to different compositions of the Vtg holo-protein (*i.e*. different parts of the phospholipoglyco-modifications may be retained),

which also may lead to different affinities of the antibodies in an ELISA. Using either of these methods, mg quantities of purified Vtg can be obtained within a short period of time (10-30 min). To prevent degradation of the protein this process should be performed in a cold environment using cold buffers containing protease inhibitors.

We chose to use anion-exchange chromatography to purify the RM for the FHM Vtg ELISA. Using spin columns from Vivascience (Hannover, Germany), Vtg was rapidly purified in sufficient quantities from small amounts of plasma from estrogenized FHM.

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QUALITY ASSURANCE OF RM

The purity and homogeneity of the RM needs to be established using gel electrophoresis. Both SDS-PAGE and 2-dimensional electrophoresis (2-DE) were carried out to demonstrate the purity of the RM (Figure 4). In addition, Matrix assisted laser desorption ionization mass spectrophotometry (MALDI-MS) was performed to obtain more information about the proteins observed on the 2-DE gel.

Both SDS-PAGE and 2-DE shows few impurities. In SDS-PAGE, three main bands are clearly visible. All three bands are recognised by the FHM Vtg-specific mAb 1E9 in western blot.

Peptide sequencing of the spots “FHM1” and “FHM2”, obtained by MALDI-MS, were both recognised as FHM Vtg using the Mascot database (http://www.matrix-science.com). “FHM1” had hits in different parts of the protein, whereas “FHM2” only had hits in the N-terminal part, indicating that it is a breakdown product of the main 150 kDa Vtg holoprotein. “FHM3” was not recognised as Vtg, but must be seen as an unknown, low-molecular weight peptide contaminant.

All in all, the results show that the purified protein is fathead minnow Vtg, and that the preparation is satisfactory pure.

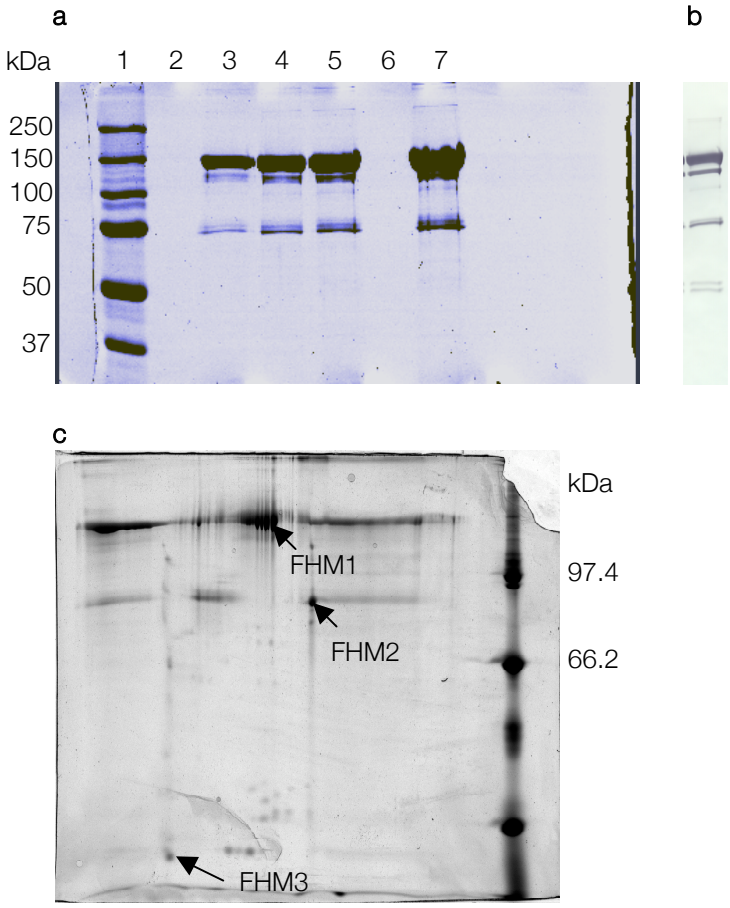

Figure 4 a: SDS-PAGE with different amounts of FHM Vtg. 7% separation gel. Lane 1: Broad range standard (Bio-Rad). Lane 2: Empty. Lane 3: 1 µg Vtg. Lane 4: 2 µg Vtg. Lane 5: 3 µg Vtg. Lane 6: Empty. Lane 7: 5 µg Vtg.
Figure 4 b: Western blot. 1 µg Vtg was applied, and mAb 1E9 was used to detect the FHM Vtg
Figure 4 c: 2-DE of FHM Vtg. 50 µg Vtg was applied. pH 3-10, 9% separation gel, low range standard (Bio-Rad). The indicated proteins (FHM 1-3) were analysed with MALDI-MS.

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QUANTIFICATION OF RM

Various methods are commonly used to quantify purified Vtg. All are dependent on a pure product.

- Staining methods such as Lowry or Bradford rely on the use of a protein standard, normally bovine serum albumin (BSA), and assume a similar staining response of the protein in question to this standard protein. In many cases, this may not be true; especially for a protein like Vtg, which contains various non-peptide groups (sugars, phosphates, lipids).

- Measurement of absorbance at 280 nm is a simple and commonly used quantification method. The method relies on the use of an extinction coefficient, which is different for each protein, due to the intramolecular environment affecting the exposure of UV absorbing aromatic amino acid residues. A theoretical extinction coefficient can be calculated from the full amino acid sequence of a protein, however posttranslational modifications will affect this value.
- A more precise method of protein quantification is quantitative amino acid analysis. This analysis can be performed by independent analytical laboratories using standard instrumentation. The amino acid composition can be compared to the theoretical composition if the sequence is known. A limitation of this method is that only the protein portion of the Vtg molecule is quantified. The lipid and phosphate parts have been reported for some species to represent 15–20% and 0.6–0.8%, respectively (*e.g.* Silversand & Haux, 1995), whereas the carbohydrate portion is not well studied.

We feel that the reliability and independence of amino acid analysis outweighs the limitations, and we chose this method for quantification of the FHM Vtg RM. Two parallel analyses were performed at the Peptide Synthesis Lab at the Biotechnology Centre of Oslo, Norway.

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STABILIZATION OF RM

Vtg is sensitive to freeze-thaw cycles (Figure 5), and needs to be stored at –80°C and shipped on dry ice. In the Biosense laboratory, we have developed a lyophilisation procedure that gives good stability to purified Vtg, with more than one year's stability of Vtg at 4°C (these stability data are "real time" data, and are so far based on other species than FHM). After lyophilisation, the RM Vtg was calibrated against non-lyophilised Vtg in the FHM ELISA.

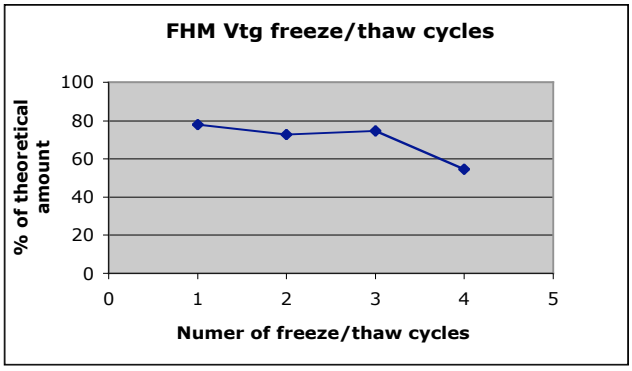

Figure 5: Stability of lyophilised FHM Vtg during repeated freeze-thaw cycles. One vial of reconstituted FHM Vtg was exposed to repeated freezing and thawing. The amount of Vtg in the vial was analysed in the FHM Vtg ELISA.

The FHM Vtg RM was compared to FHM Vtg supplied as a standard for the US EPA method study (http://www.epa.gov/scipoly/oscpendo/assayvalidation/status.htm). This Vtg had been purified using anion-exchange chromatography and had also been quantified using amino acid analyses, but the Vtg solution had been thawed once before this comparison was made. Figure 6 shows that there are between 1.2 and 1.6-fold difference between the absorbance values obtained with the two standards. This small difference is most likely due to the state of degradation and purity of the Vtg.

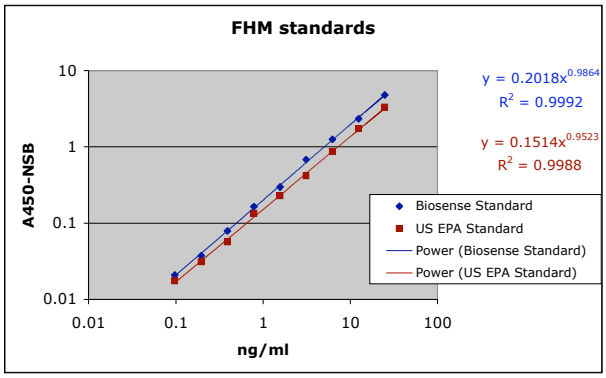

Figure 6: Comparison of the FHM Vtg RM with FHM Vtg supplied by the US EPA for Vtg assay comparison study. The standards were analysed in the FHM Vtg ELISA.

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SAMPLE PREPARATION AND SPIKING

Sample blanks:

Plasma from male fathead minnows was purchased from Fish Soup, Newberry, Florida, USA. Plasma from 34 individual males was screened, and samples having non-detectable levels of Vtg at a 1:100 dilution were pooled.

WBH from male fathead minnows was a gift from Robert Bringolf, Iowa State University, USA. The WBH showed no detectable levels of Vtg at a 1:100 dilution.

Spiked samples:

For spiking/recovery studies, Sample blanks (plasma, WBH and kit Dilution buffer) were diluted 10-fold with kit Dilution Buffer containing different concentrations of purified, non-lyophilised Vtg. Samples were mixed thoroughly, aliquoted into suitable volumes and frozen at –80°C. Concentrations in spiked samples were 5, 25 and 125 ng/ml.

Naturally incurred samples:

Plasma with low levels of Vtg was purchased from Fish Soup, Newberry, Florida, USA. WBH with low levels of Vtg was a gift from Robert Bringolf, Iowa State University, USA.

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VALIDATION RESULTS

APPLICABILITY

Applicability (Scope): The analytes, matrices and concentrations for which a method may be used satisfactorily.

This validation was performed in order to determine the characteristics of the FHM Vtg ELISA, developed for analyses of Vtg in the fathead minnow (*Pimephales promelas*), as an endpoint in endocrine disrupting chemicals (EDC) screening tests. The ability of the ELISA to accurately analyse Vtg levels in spiked (fortified) plasma, whole body homogenate (WBH) and the ELISA kit Dilution buffer was analysed. Four levels of Vtg were used in this validation (0, 0.5, 2.5 and 12.5 ng/ml Vtg), corresponding to different levels within the standard curve working range (0.1-25 ng/ml).
***

CALIBRATION

Calibration, is the empirical determination of the relationship between the parameter measured (e.g. ELISA absorbance) and the analyte (Vtg) concentration. The range of concentrations of analyte where such relationship is established is often referred to as "calibration range" or the "standard curve working range".

Calibration of the assay was performed with purified, lyophilised FHM Vtg RM. A standard serial dilution containing 11 concentration points was used, and a log-log curve fit (using Microsoft Excel) was used to define the relationship between concentration and response (absorbance).

Standard curves from 15 assays were compiled (Figure 6, Table 3 a-b). Three standard curves were run each day for five days. The standard serial dilutions were prepared fresh each day, and the same solution was used on three different ELISA plates. The Within-Day and Between-Day Precision was calculated (Figure 6).

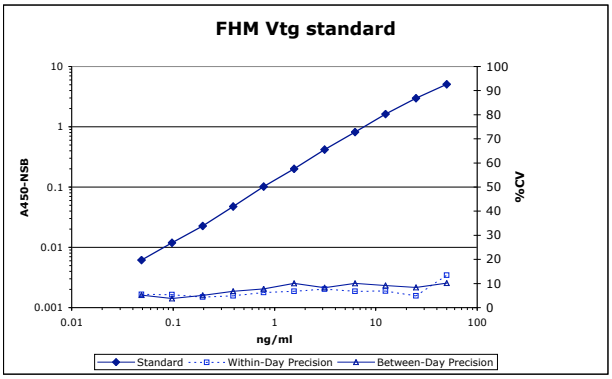

Figure 6: Combined FHM Vtg standard curve, average from 15 assays (using Microsoft Excel). The secondary y-axis shows the Within-Day and Between-Day Repeatability Precisions (RSD_r) for the standard curves.

Table 3a: Within-Day Repeatability Precision for FHM Vtg standard curves

Within Day Precision

Concentration (ng/ml)		NSB	0.05	0.10	0.20	0.39	0.78	1.56	3.13	6.25	12.50	25.00	50.00
Day 1	Average absorbance (450 nm), 3 standard curves	0.061	0.069	0.072	0.084	0.108	0.167	0.276	0.507	0.918	1.822	3.338	5.307
	%CV	8.2	11.1	8.7	5.3	4.2	4.4	4.0	4.5	4.4	2.2	2.8	16.1
Day 2	Average absorbance (450 nm), 3 standard curves	0.060	0.065	0.072	0.083	0.110	0.166	0.278	0.506	0.940	1.811	3.194	5.793
	%CV	9.0	5.9	5.3	6.2	8.7	10.2	10.1	11.5	11.0	10.0	8.7	7.3
Day 3	Average absorbance (450 nm), 3 standard curves	0.057	0.062	0.069	0.079	0.102	0.153	0.255	0.460	0.849	1.657	3.027	5.263
	%CV	5.3	3.3	5.7	3.6	3.2	4.6	6.5	6.6	5.7	5.8	5.2	13.9
Day 4	Average absorbance (450 nm), 3 standard curves	0.055	0.060	0.066	0.074	0.094	0.140	0.215	0.416	0.735	1.444	2.657	4.358
	%CV	5.6	5.0	5.9	2.3	3.7	5.8	5.1	6.3	5.2	5.1	4.2	12.4
Day 5	Average absorbance (450 nm), 3 standard curves	0.056	0.063	0.069	0.082	0.112	0.170	0.271	0.498	0.945	1.661	3.009	5.017
	%CV	4.1	2.1	1.6	4.8	4.4	6.4	8.4	9.4	7.6	11.5	3.8	17.4
Overall Within-Day Precision		6.4	5.5	5.4	4.4	4.8	6.3	6.8	7.7	6.8	6.9	4.9	13.4
Overall Within-Day Precision (working range, 0.1-25 ng/ml)		6.0											

Table 3b: Between-Day Repeatability Precision for FHM Vtg standard curves

Between-day Precision

Concentration (ng/ml)	NSB	0.05	0.10	0.20	0.39	0.78	1.56	3.13	6.25	12.50	25.00	50.00
Average absorbance (450 nm), 5 days	0.058	0.064	0.070	0.080	0.105	0.159	0.259	0.477	0.877	1.679	3.045	5.148
%CV	4.2	5.1	3.8	5.1	6.8	7.8	10.0	8.3	10.1	9.1	8.4	10.2
Overall Between-Day Precision (working range, 0.1-25 ng/ml)	7.7											

The results show that standard curves obtained on both the same day and on different days show little variability, with an average Within-Day Precision of 6.0 % and a Between-Day Precision of 7.7 %.

The standard curve working range was between 0.1 and 25 ng/ml using a log-log transformation of the data.

***

LOD AND LOQ (SENSITIVITY)

Limit of Detection is the smallest amount or concentration that can be reliably distinguished from zero. Defined here as reagent blank + 3x standard deviation of reagent blank. Indicates that the analyte is present, but not necessarily allowing exact quantification.

Limit of Quantification A concentration above which the analytical method can operate with an acceptable precision. Defined here as reagent blank + 10x standard deviation of reagent blank.

Sample LoQ. The LoQ corrected for minimum dilution factor necessary to avoid matrix effect.

Limit of Detection (LoD) and Limit of Quantification (LoQ) were determined from matrix blanks analysed during Precision studies. Since the samples analysed at a 1:100 dilution did not have detectable levels of Vtg, LoD and LoQ were determined from NSB-corrected absorbance levels and "translated" into concentration using the relevant standard curve equation (Table 4a-c).

Table 4a) LoD and LoQ for Plasma

	Replicate 1 A450nm	Replicate 2 A450nm	Replicate 3 A450nm	Average A450nm	Stdev A450nm	3x Stdev A450nm	LoD ng/ml	10x Stdev A450nm	LoQ ng/ml
Run1	0.000	-0.003	-0.002	-0.001	0.002	0.003	0.02	0.014	0.11
Run2	0.001	-0.002	-0.003	-0.001	0.002	0.005	0.03	0.019	0.12
Run3	-0.001	-0.001	-0.001	-0.001	0.000	0.000	0.00	0.003	0.02
Run4	0.001	0.002	0.000	0.001	0.001	0.005	0.04	0.013	0.12
Run5	0.000	0.000	-0.003	-0.001	0.002	0.004	0.02	0.017	0.10
Average						0.003	0.02	0.013	0.09

Table 4b) LoD and LoQ for WBH

	Replicate 1 A450nm	Replicate 2 A450nm	Replicate 3 A450nm	Average A450nm	Stdev A450nm	3x Stdev A450nm	LoD ng/ml	10x Stdev A450nm	LoQ ng/ml
Run1	-0.004	0.000	-0.001	-0.002	0.002	0.005	0.04	0.020	0.15
Run2	-0.005	-0.006	-0.007	-0.006	0.001	-0.004	ND	0.002	0.01
Run3	-0.002	-0.004	-0.003	-0.003	0.001	-0.001	ND	0.005	0.04
Run4	0.002	-0.003	-0.005	-0.002	0.004	0.009	0.08	0.034	0.32
Run5	-0.002	-0.004	-0.003	-0.003	0.001	0.000	0.00	0.008	0.05
Average						0.002	0.04	0.014	0.11

Table 4c) LoD and LoQ for Dilution buffer

	Replicate 1 A450nm	Replicate 2 A450nm	Replicate 3 A450nm	Average A450nm	Stdev A450nm	3x Stdev A450nm	LoD ng/ml	10x Stdev A450nm	LoQ ng/ml
Run1	-0.002	0.001	-0.006	-0.002	0.004	0.009	0.06	0.033	0.24
Run2	-0.009	-0.008	-0.010	-0.009	0.001	-0.005	ND	0.003	0.02
Run3	0.000	0.001	-0.001	0.000	0.001	0.003	0.02	0.009	0.07
Run4	-0.001	-0.001	-0.001	-0.001	0.000	0.000	0.00	0.003	0.03
Run5	-0.002	-0.003	0.001	-0.001	0.002	0.005	0.03	0.019	0.11
Average						0.002	0.03	0.013	0.09

The LoD varied between 0.02 and 0.04 ng/ml for different sample types, and the LoQ varied between 0.09 and 0.11 ng/ml. Thus, the LoQ is in good agreement with the lower limit of the standard curve working range (0.10 ng/ml).

***

SELECTIVITY (MATRIX EFFECT)

In order to determine the degree of interference from sample matrices on quantification of Vtg, plasma and WBH were investigated for adverse effects on the signal response, *i.e.* plasma or matrix effect. Different dilutions of matrix blanks were spiked with a range of Vtg concentrations, and recovery was measured and compared.

Figure 8 and 9 show that there is an inhibition of the signal at low dilutions of both plasma and WBH, resulting in an underestimation of Vtg at these dilutions. This effect varies somewhat between different samples, and with the spike concentration. Note that some haemolysis had happened during preparation of the plasma, which may have an effect on matrix effect.

Based on these results showed in Figure 8 and 9, we recommend 1:50 for plasma and 1:100 for WBH as the minimum dilution factors. Lower dilution factors might be used, but this should be tested in individual laboratories, and may depend on the method of sample preparartion.

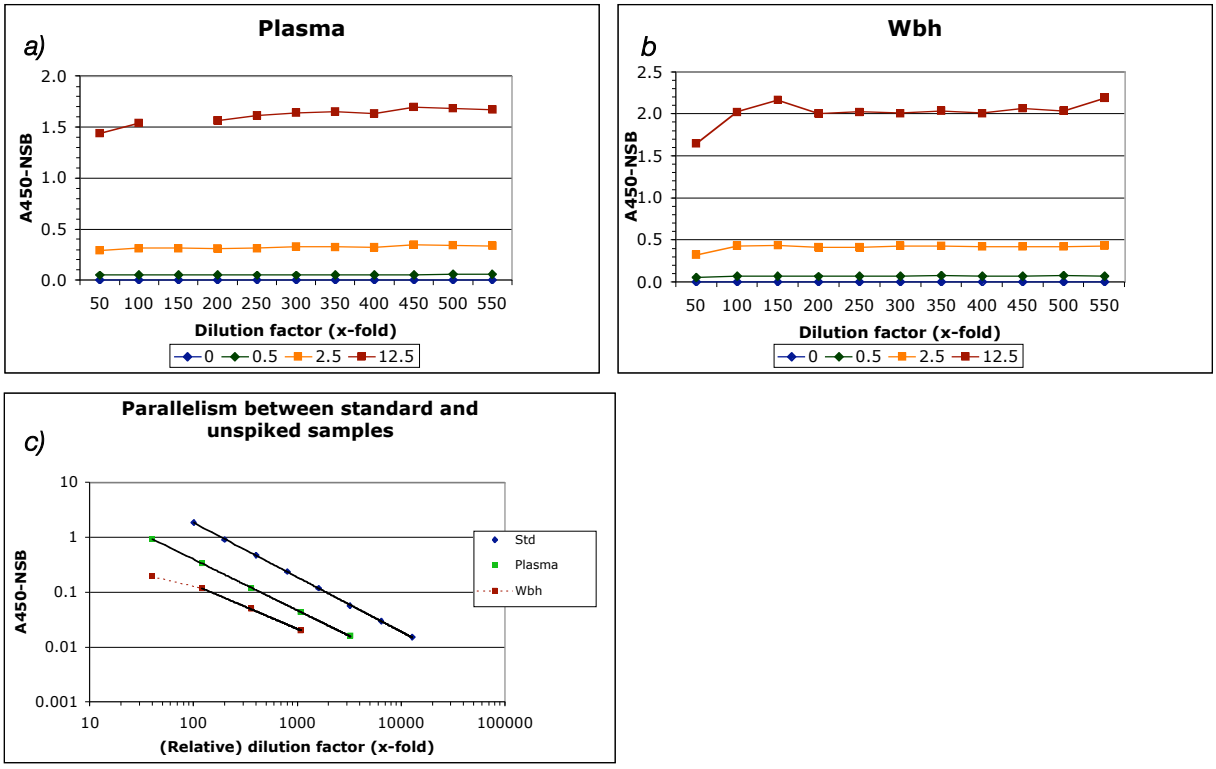

Figure 8: Effect of plasma and WBH sample dilution on detection of Vtg. Experiments were performed with spiked samples (Figure 8a-b) or with naturally incurred samples (Figure 8c), containing Vtg.

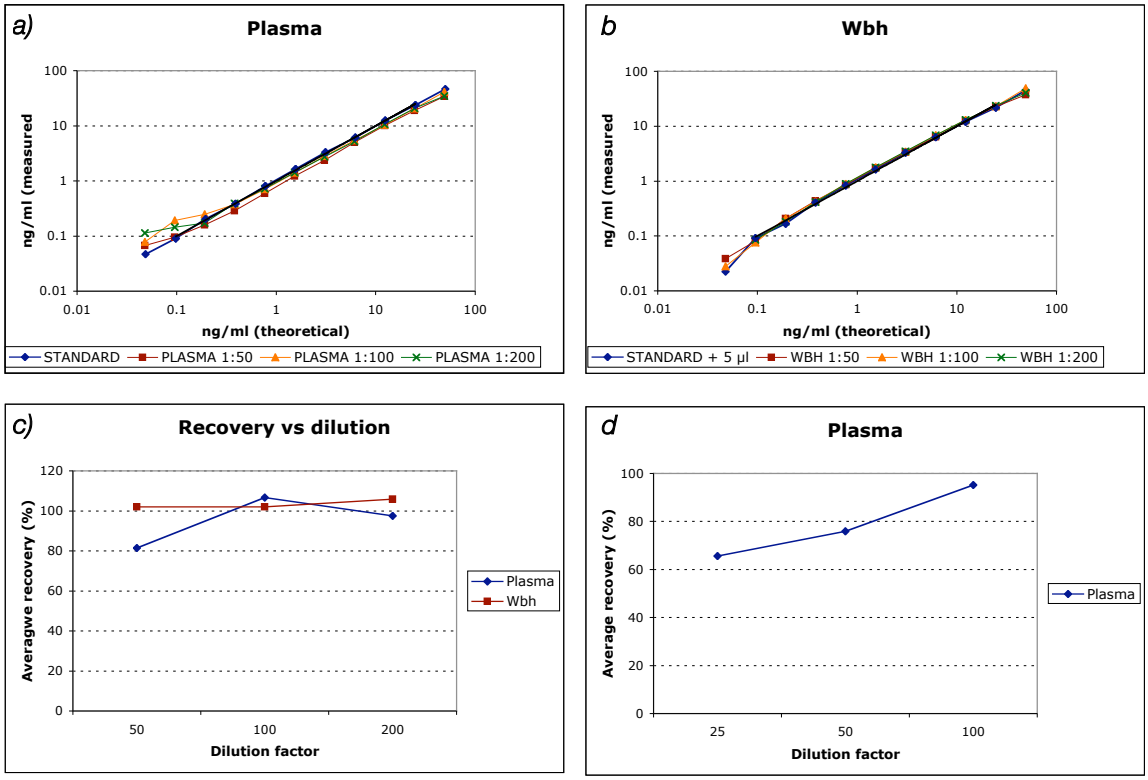

Figure 9: Effect of plasma and WBH sample dilution on quantification of Vtg. Different amounts of plasma or WBH were added to standard dilutions (Figure 9a-b). The average recovery for each sample dilution factor was cacluated (Figure 9c). An additional experiment was conducted with even lower dilution of plasma, tested with three different Vtg levels (Figure 9d).

***

REPEATABILITY PRECISION (INTRA- AND INTERASSAY VARIATION), RSD_r

Precision is the closeness of agreement between test results obtained under stipulated conditions.

- *Repeatability Precision (same laboratory and operator, samples, equipment, short time intervals), separated into Within-Day and Between-Day Repeatability precision, usually expressed as relative standard deviation, RSD_r.*

Matrix blanks from plasma and WBH, as well as ELISA kit Dilution buffer were analysed, spiked with three different concentrations of Vtg corresponding to the low, medium and high parts of the standard curve working range. In addition, unspiked material was analysed. All samples had been aliquoted and stored at –80°C until analysis. On five successive days, three aliquots were thawed and analysed in triplicates at a 1:100 final dilution.

Within-Day and Between-Day Repeatability Precision (RSD_r), often referred to as intra- and interassay variation, were calculated (Table 5a-c and Table 6a-c).

Within-Day Repeatability Precision: %CV between the three aliquots analysed in one day.
Overall Within-Day Repeatability Precision: average of the individual Precision values for five days

Table 5a) Within-Day Precision for spiked Plasma samples

Run #	Spike concentration ng/ml	Replicate 1 ng/ml	Replicate 2 ng/ml	Replicate 3 ng/ml	Average ng/ml	RSDr %
1	0.5	0.3	0.3	0.3	0.3	0.7
	2.5	1.9	1.8	1.9	1.9	3.0
	12.5	10.8	10.5	10.8	10.7	1.5
2	0.5	0.3	0.3	0.3	0.3	2.6
	2.5	1.8	1.7	1.7	1.7	3.4
	12.5	9.9	9.4	9.2	9.5	3.7
3	0.5	0.4	0.3	0.3	0.3	8.8
	2.5	2.0	1.9	1.9	1.9	3.7
	12.5	10.9	10.3	10.7	10.6	2.8
4	0.5	0.4	0.4	0.3	0.4	5.8
	2.5	2.1	1.9	1.9	2.0	4.0
	12.5	12.6	11.3	11.8	11.9	5.3
5	0.5	0.3	0.3	0.3	0.3	7.8
	2.5	1.6	1.7	1.6	1.6	3.1
	12.5	9.9	10.0	9.8	9.9	0.6
Overall Within-Day Precision (%)						3.8

Table 5b) Within-Day Precision for spiked WBH samples

Run #	Spike concentration ng/ml	Replicate 1 ng/ml	Replicate 2 ng/ml	Replicate 3 ng/ml	Average ng/ml	RSDr %
1	0.5	0.3	0.3	0.3	0.3	4.6
	2.5	1.6	1.5	1.5	1.6	4.1
	12.5	8.0	7.2	7.4	7.5	5.5
2	0.5	0.3	0.3	0.3	0.3	4.7
	2.5	1.5	1.5	1.5	1.5	3.3
	12.5	7.9	7.4	7.5	7.6	3.4
3	0.5	0.3	0.3	0.3	0.3	4.4
	2.5	1.8	1.9	1.8	1.8	2.4
	12.5	9.1	9.5	9.5	9.4	2.6
4	0.5	0.4	0.3	0.3	0.4	7.0
	2.5	2.0	1.7	1.8	1.8	7.7
	12.5	10.3	9.0	9.6	9.7	6.5
5	0.5	0.3	0.3	0.3	0.3	10.1
	2.5	1.9	1.7	1.7	1.8	7.3
	12.5	9.6	9.1	8.8	9.2	4.3
Overall Within-Day Precision (%)						4.6

Table 5c) Within-Day Precision for spiked Dilution buffer samples

Run #	Spike concentration ng/ml	Replicate 1 ng/ml	Replicate 2 ng/ml	Replicate 3 ng/ml	Average ng/ml	RSDr %
1	0.5	0.5	0.4	0.4	0.4	10.3
	2.5	2.1	2.2	2.1	2.1	2.3
	12.5	11.1	11.1	11.3	11.1	0.9
2	0.5	0.3	0.3	0.3	0.3	3.7
	2.5	1.8	1.8	1.9	1.9	2.5
	12.5	11.2	10.6	10.3	10.7	4.2
3	0.5	0.4	0.4	0.4	0.4	3.1
	2.5	2.2	2.2	2.2	2.2	1.2
	12.5	11.4	11.1	11.4	11.3	1.9
4	0.5	0.4	0.4	0.4	0.4	2.4
	2.5	2.3	2.3	2.4	2.3	2.3
	12.5	11.6	11.7	12.2	11.8	2.6
5	0.5	0.4	0.4	0.3	0.4	4.2
	2.5	2.2	2.1	1.5	2.0	20.8
	12.5	12.3	11.4	11.0	11.6	5.9
Overall Within-Day Precision (%)						**4.6**

Overall Within-Day Precision for all sample types and concentrations	**4.5**

Between-Day Repeatability Precision: % CV between the average measured concentrations from each day.
Overall Between-Day Repeatability Precision: Average Precision for all concentrations

Table 6a) Between-Day Precision for Plasma samples

Spike concentration ng/ml)	Average (ng/ml)	RSD, %
0.5	0.3	11.5
2.5	1.8	8.2
12.5	10.3	8.8
Overall Between-Day Precision		**9.5**

Table 6b) Between-Day Precision for WBH samples

Spike concentration ng/ml)	Average (ng/ml)	RSD, %
0.5	1.8	1.9
2.5	1.6	10.3
12.5	5.8	17.4
Overall Between-Day Precision		**9.9**

Table 6c) Between-Day Precision for Dilution buffer samples

Spike concentration ng/ml)	Average (ng/ml)	RSD, %
0.5	0.4	14.7
2.5	2.1	11.4
12.5	11.1	4.7
Oerall Between-Day Precision		10.3

Overall Between-Day Precision for all sample types and concentrations	9.9

The results show low variation in quantification, both within the same day (Overall Within-Day Precision = 4.5 %) and between successive days (Overall Between-Day Precision = 9.9 %).

***

ACCURACY

Accuracy is the closeness of agreement between a test result and the accepted reference value of the property being measured.

Recovery is the proportion of the amount of analyte, present in or added to, the analytical portion, which is extracted and presented for measurement

Bias is the difference between the test results and an accepted reference value

Recovery and bias

The concentrations measured in the spiked samples during Precision studies were compared to the theoretical values and Recovery and Bias were determined using the following formulas (Table 7-8):

Recovery = (C1-C2)/C3 x 100

Where C1= concentration measured in spiked sample, C2= concentration measured in unspiked sample, C3= theoretical concentration.

Bias = (C3-(C1-C2))/C3 x 100

Where C1= concentration measured in spiked sample, C2= concentration measured in unspiked sample, C3= theoretical concentration

Table 7: Recovery (expressed as % of theoretical concentration)

Spike concentration (ng/ml)	Plasma	WBH	Spiked buffer
0.5	64.2	63.3	75.9
2.5	72.8	68.0	83.9
12.5	84.3	69.4	90.5
Overall Recovery (%)	73.8	66.9	83.4
Overall Recovery for all sample types and concentrations (%)			74.7

Table 8: Bias (expressed as % difference from theoretical value)

Spike concentration (ng/ml)	Plasma	WBH	Spiked buffer
0.5	-35.8	-36.7	-24.1
2.5	-27.2	-32.0	-16.1
12.5	-15.7	-30.6	-9.5
Overall Bias (%)	-26.3	-33.1	-16.6
Overall Bias for all sample types and concentrations (%)			-25.3

The results show that the recovery and bias depend on both sample type and spike concentration, with an overall 25% underestimation of spike concentrations in the sample.

Using freshly spiked samples, without previous freezing/thawing of the samples, Recovery was measured during the Selectivity study (see Figure 9). Results from these experiments, using several different concentrations of Vtg (standard curve dilution series), and different dilutions of plasma and WBH, gave the Recovery results much closer to 100% (Table 9a-c).

Table 9a: Recovery in WBH and plasma: *Average Recovery for 11 different spike concentrations (expressed as % of theoretical value)*

Dilution factor	Plasma	WBH
1:50	81.4	102.0
1:100	106.7	102.0
1:200	97.6	105.9

Table 9b: Recovery in WBH and plasma: *Average Recovery for three different spike concentrations (expressed as % of theoretical value)*

Dilution factor	Plasma	WBH
1:25	65.6	nd
1:50	75.9	84.8
1:100	95.2	102.5

Table 9c: Recovery in WBH and plasma, average of two experiments *(see Table 9a and b)*

Dilution factor	Plasma	WBH
1:25	65.6	nd
1:50	78.7	93.4
1:100	101.0	102.3
1:200	97.6	105.9

***

RUGGEDNESS

Ruggedness. The ability of the measurement process to resist changes in results when subjected to minor changes in environmental and procedural variables.

In order to investigate the stability of the assay when exposed to variations in the environment and assay procedure, seven parameters were combined in eight assays to determine their effect on quantification in the FHM Vtg ELISA (according to Youden & Steiner, 1975).

The effect on quantification of the three matrix blanks, spiked with three different concentrations, was analysed. Table 10 shows the compiled results.

Table 10: Ruggedness test*. Standard conditions are shown in bold. Alterations higher than 10% are highlighted.*

Condition altered	Value of condition	Sample type	Sample concentration level			
			low	med	high	
			Difference (%) [1]			Average (%) [1]
Buffer temperature	**Cold**					
	RT	Buffer	7	6	4	6
		Plasma	-11	-10	-13	-11
		WBH	4	-9	-12	-5
Incubation temperature	**RT[2]**					
	30C	Buffer	12	11	4	9
		Plasma	-18	-18	-21	-19
		WBH	-7	-5	-8	-7
Standard/sample incubation time	**1.5 h**					
	1 h	Buffer	-2	1	7	2
		Plasma	6	7	11	8
		WBH	1	4	8	4
Detection Ab incubation time	**0.5 h**					
	1 h	Buffer	5	4	-3	2
		Plasma	-1	-10	-11	-7
		WBH	3	1	-5	0
Number of washes before development	**5**					
	3	Buffer	8	3	-6	2
		Plasma	16	11	-1	9
		WBH	11	7	-5	4
TMB solution temperature	**RT**					
	4C	Buffer	7	0	1	3
		Plasma	9	8	4	7
		WBH	4	3	1	3
Development time	**20 min**					
	30 min	Buffer	-8	-5	1	-4
		Plasma	-2	1	6	2
		WBH	-7	-4	0	-3

[1] Minus sign denotes that the value for the unaltered condition was lower than for the altered condition
[2] RT in this assay was 23°C

The results show that buffer and incubation temperature have the highest influence on Vtg quantification in the FHM Vtg ELISA. The effect of buffer temperature was studied in separate assays, and showed that room-tempered buffer had a stronger effect on the samples than on the standard, increasing absorbance and the measured Vtg concentration in the samples (data not shown). These results demonstrate the importance of keeping the Dilution buffer cold and to keep the room temperature relatively low (20-25°C). On the other hand, deviations to the incubation times have less influence on the quantification of samples.

***

COMPARISON WITH EXISTING METHODS

No established reference method for measuring FHM Vtg exists today, so the new FHM Vtg ELISA was therefore compared with two other commercially available ELISA kits, a Carp Vtg ELISA (Biosense Laboratories) and a FHM Vtg ELISA (competitor).

***

Carp Vtg ELISA (Biosense Laboratories AS)

Plasma and WBH samples from the US EPA FHM Vtg assay comparison study (2003) were tested in the FHM Vtg ELISA. These samples had previously been tested in the Biosense Carp Vtg ELISA, using both the kit (carp) Vtg standard and the FHM Vtg standard supplied with the study samples. Figure 10a and 10d show the results compared. The Carp Vtg ELISA utilises one monoclonal and one polyclonal carp Vtg-specific antibodies, as well as carp Vtg standard, and the assay is therefore heterologous for the FHM. However, the antibodies show excellent cross-reactivity with FHM Vtg (Nilsen *et al* 2004), reflected in values varying less than 2-fold between the two assays and a good correlation between Vtg levels measured in the samples ($R^2 > 0.99$).

***

Competitor FHM ELISA kit

The Biosense and a competitor FHM Vtg ELISA kit were compared using spiked and unspiked samples with different Vtg levels. Figure 10b, c and e show the two standard curves and the results from the analysed samples. Although the absolute values varies on average less than 2-fold, due to differences in standards and antibodies, the results show very good correlation ($R^2 > 0.99$). The largest difference is the sensitivity of the assays, with a 78 times more sensitive standard curve in the Biosense FHM ELISA, and a corresponding lack of detection of Vtg in samples spiked with low Vtg concentrations in the competitor ELISA kit.

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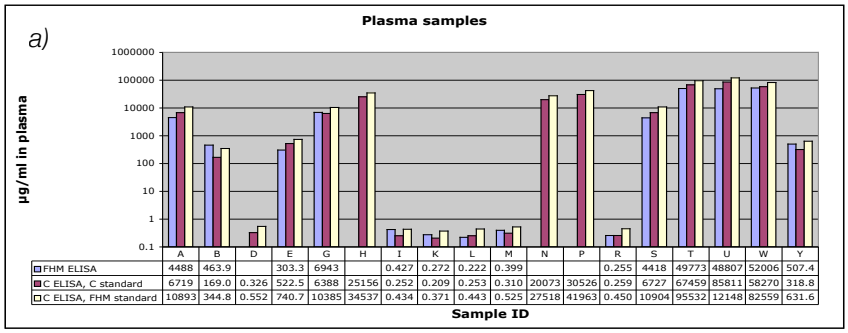

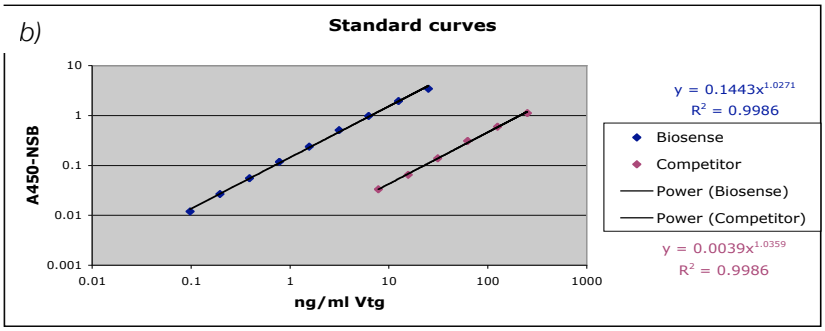

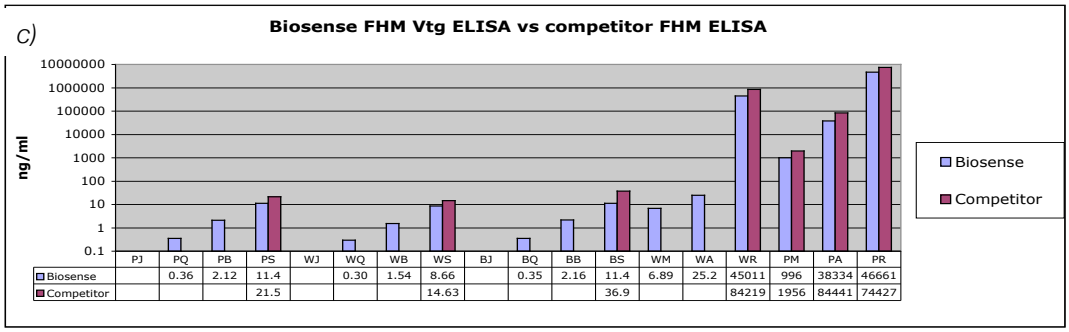

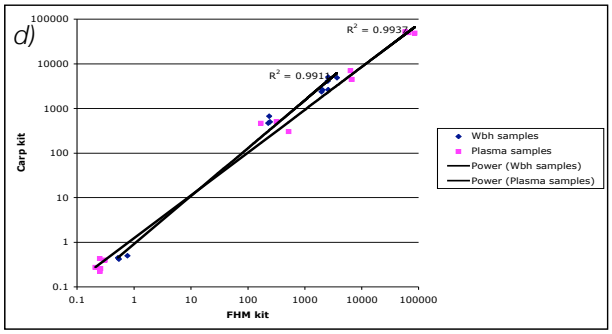

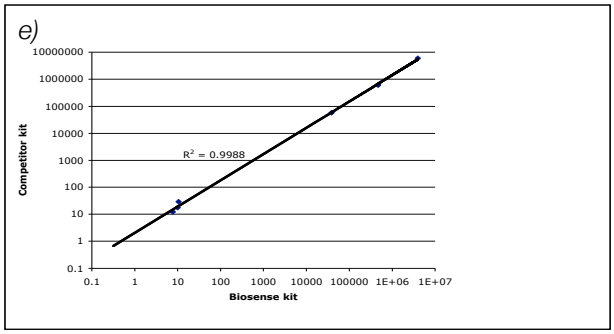

Figure 9: Comparison of the Biosense FHM Vtg kit with two methods. Figure 9a: Samples from a US EPA method comparison study were compared in the Biosense Carp (C) and FHM Vtg ELISA kits, using both C and FHM Vtg standard in the C ELISA. Figure 9b: FHM Vtg kit standard curves in the Biosense and competitor FHM Vtg ELISA kit. Figure 9c: Identical samples analysed in the Biosense and competitor FHM Vtg ELISA kit. 9d: Correlation between results obtained with Biosense Carp and FHM Vtg ELISA kits. 9e: Correlation between Biosense and competitor FHM Vtg ELISA kits.

***

DISCUSSION

A new sandwich ELISA, based on monoclonal antibodies raised against FHM Vtg, has been developed to quantify Vtg in samples from FHM. Vtg was purified from FHM plasma to be used as standard in the ELISA,. The Vtg was further characterised and quantified, and was found to be of sufficient purity to be used reliably as Reference Material (RM) in the FHM ELISA.

A Single-Laboratory Validation was performed to validate the performance of the new Biosense FHM Vtg ELISA. A set of plasma, WBH and ELISA kit Dilution buffer samples, both spiked, unspiked and naturally incurred (containing Vtg), were analysed on different days. Variation within days and between days was determined, as well as the ability of the assay to measure correct values in spiked samples.

A calibration curve was run, prepared from a serial dilution of the FHM Vtg RM, in each assay. Using log-log transformation of the standard curves, the working range was from 0.1-25 ng/ml (250-fold). For 15 standard curves (three separate standard curves per day for five days), the Within-Run and Between-Run Repeatability Precision (RSD_r) within the working range was 6.0 and 7.7%, respectively. This shows that the variability between different vials of Vtg RM is low, as is the variability between standard curves prepared on different days. Also, the 250-fold working range is well within our aim (100-fold, Goksøyr *et al*, 2003) Vtg concentrations in samples can vary over several orders of magnitude, and a broad working range is important because fewer dilutions from each sample of unknown Vtg concentration are necessary in order to “hit” the standard curve working range.

Important parameteres for a quantitative assay is the Limit of Detection (LoD) and Limit of Quantification (LoQ). The FHM assay is highly sensitive, with an average LoD of 0.03 ng/ml and an LoQ equal to the lower limit of the standard curve working range, 0.10 ng/ml. With minimum recommended sample dilutions of 1:50 for plasma and 1:100 for WBH, the sample LOQs were 4.7 and 11.4 ng/ml, respectively. Both plasma and WBH from unexposed male fish (from the US EPA Study) were easily quantified by the FHM ELISA.

In order to determine the Within-Day and Between-Day Repeatability Precision (RSD_r) of the ELISA, three replicates of each sample were analysed on five different days. Within-Day RSD_r values were between 3.8 and 4.6 % for the different sample types, with an overall RSD_r of 4.5%. The Between-Day RSD_r was between 9.5 and 10.3%, with an overall RSD_r of 9.9%. These values show that the assay shows little variability between runs, and are well within the aims defined by Goksøyr *et al* (2003).

The assay’s ability to accurately quantify Vtg in spiked samples was analysed by comparing concentrations measured in the ELISA with the theoretical concentrations. Samples with three different concentrations of Vtg were analysed,

and the Recovery varied between 63 and 91%, depending on concentration and sample type, with an overall Recovery of 75% (Bias = -25%).

Vtg is an unstable molecule, prone to degradation (Arukwe and Goksøyr 2003). For this reason, spiking of samples should ideally be done on the day of analysis to avoid freeze-thaw cycles and degradation. However, in order to obtain data for determination of Between-Day Repeatability Precision, samples had to be diluted, aliquoted and frozen. This is likely to have affected the Recovery of Vtg in spiked samples. Spiking/recovery experiments performed without freezing and thawing of the spiked samples, gave Recovery in both plasma and WBH between 79-106%, supporting this theory.

The Ruggedness test revealed that small changes to factors such as incubation time and temperatures affected the FHM ELISA's ability to quantify Vtg to different degrees. Seven factors were modified and tested together in different combinations according to Youden & Steiner (1975), and the factors having highest impact on Vtg quantification were elevated buffer and room temperatures.

Since no official Reference Method for FHM Vtg quantification is established, the new Biosense FHM ELISA was compared to two exisiting Vtg ELISA kits. The Biosense Carp Vtg ELISA, although heterologous to the FHM, has successfully been applied on samples from FHM (Nilsen *et al*, 2004). A set of FHM samples from the US EPA method comparison study (2003) was re-analysed in the FHM ELISA. The absolute values obtained with the two kits differed less than 2-fold, and the correlation (R^2) between the results were >0.99, showing that the two kits give comparable results. An alternative FHM ELISA kit was also tested, using a set of spiked and unspiked samples. The Biosense FHM kit standard curve was 78-fold more sensitive than the competitor ELISA Vtg standard, but the absolute values varied less than 2-fold and the correlation between the results from the two kits was equally good, with R^2>0.99.

The results from these two comparisons show that although absolute values may differ, due to factors such as differences in antibodies, Vtg standard quantification, purity, the assays still yield similar correlating results and suit their purpose of differentiating between control and exposed groups of fish in studies of endocrine disruption.

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CONCLUSION

A successful Single-Laboratory Validation was performed on the Biosense FHM Vtg ELISA. The results, summarised in Table 11, show that our pre-defined aims (Goksøyr *et al* 2003) were met with good margin. The kit is sensitive and reliable, and is therefore a good tool which fits its purpose for quantitative analysis of Vtg in the fathead minnow.

Table 11: Summary of Single-Laboratory Validation results for the Biosense FHM Vtg ELISA kit

Performance characteristics	Aim[1]	Value
Selectivity	Matrix blank < LOD (with the necessary dilution factor to avoid matrix effects)	No response at minimum dilution Minimum dilution = 1:50 (plasma), 1:100 (WBH)
Calibration	Standard curve working range >10-fold, preferably 50-100 fold to be practical with the dynamic range found in Vtg levels	Standard curve working range 0.1-25 ng/ml (250-fold)
Accuracy (Recovery)	Ideally 50-200%	75%[2] 79-106%[3]
Repeatability[4]	<20%	Within-Day RSD_r: 4.5% Between-Day RSD_r: 9.9%
Limit of Detection (LOD)	<10 ng/ml	0.02 ng/ml (plasma) 0.04 ng/ml (WBH) 0.03 ng/ml (buffer)
Limit of Quantification (LOQ)	<10 ng/ml	0.09 ng/ml (plasma) 0.11 ng/ml (WBH) 0.09 ng/ml (buffer)
Sample LOQ (=LOQ x necessary matrix dilution)	200 – 500 ng/ml	4.68 ng/ml (plasma, 1:50) 11.35 ng/ml (WBH, 1:100)

[1)] Goksøyr *et al* 2003
[2)] From Precision studies, samples frozen and thawed once, dilution 1:100
[3)] From Selectivity studies, samples freshly spiked and not frozen, dilutions 1:50-1:100
[4)] Referred to in this document Within-Day and Between-Day Repeatability Precision, RSD_r

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