

Short communication

Development and validation of a direct homologous quantitative sandwich ELISA for fathead minnow (*Pimephales promelas*) vitellogenin

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Abstract

Vitellogenin (Vtg) is an established and sensitive endpoint for analysis of exposure to (anti-)oestrogens and their mimics in fish [Sumpter, J.P., 1995. Feminized responses in fish to environmental estrogens. *Toxicol. Lett.* 82, 737–742; Arukwe, A., Goksøyr, A., 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comp. Hepatol.* 2, 4. <http://www.comparative-hepatology.com>]. In some instances, links have been drawn between high level induction of Vtg and adverse health effects in fish [Herman, R.L., Kincaide, H.L., 1988. Pathological effects of orally administered estradiol to rainbow trout. *Aquaculture* 72, 165–172; Schwaiger, J., Spieser, O.H., Bauer, C., Ferling, H., Mallow, U., Kalbfus, W., Negele, R.D., 2000. Chronic toxicity of nonylphenol and ethinyloestriol: haematological and histopathological effects in juvenile common carp (*Cyprinus carpio*). *Aquat. Toxicol.* 51, 69–78]. The widespread use of Vtg as a biomarker has led to the development of a variety of assays to quantitatively measure Vtg concentrations in tissue samples from fish, and hence a need for a standardization of the performance criteria and validation of such assays [Goksøyr, A., Eidem, J.K., Kristiansen, S.I., Nilsen, B.M., 2003. On the need for a standardized set-up for validation studies of fish vitellogenin assays as an endpoint in endocrine disruptor testing and screening—a proposal. <http://www.biosense.com/Docs/GoksøyrEtal2003.pdf>]. One of the most popular test fish species for assessing chemical effects is the fathead minnow (*Pimephales promelas*), which is now used widely for studies into endocrine disruption [Panter, G.H., Hutchinson, T.H., Lange, R., Lye, C.M., Sumpter, J.P., Zerulla, M., Tyler, C.R., 2002. Utility of a juvenile fathead minnow screening assay for detecting (anti)estrogenic substances. *Environ. Toxicol. Chem.* 21, 319–326; Hutchinson, T.H., Yokota, H., Hagino, S., Ozato, K., 2003. Development of fish tests for endocrine disruptors. *Pure Appl. Chem.* 75, 2343–2353]. This paper describes the development and validation of a new, homologous enzyme-linked immunosorbent assay (ELISA) for quantification of Vtg in this fish species.

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Vitellogenin (Vtg) is an established and sensitive endpoint for analysis of exposure to (anti-)oestrogens and their mimics in fish (Sumpter, 1995; Arukwe and Goksøyr, 2003). In some instances, links have been drawn between high level induction of Vtg and adverse health effects in fish (Herman and Kincaide, 1988; Schwaiger et al., 2000). The widespread use of Vtg as a biomarker has led to the development of a variety of assays to quantitatively measure Vtg concentrations in tissue samples

from fish, and hence a need for a standardization of the performance criteria and validation of such assays (Goksøyr et al., 2003). One of the most popular test fish species for assessing chemical effects is the fathead minnow (*Pimephales promelas*), which is now used widely for studies into endocrine disruption (Panter et al., 2002; Hutchinson et al., 2003). This paper describes the development and validation of a new, homologous enzyme-linked immunosorbent assay (ELISA) for quantification of Vtg in this fish species.

Based on monoclonal antibodies raised against fathead minnow (FHM) Vtg, and using purified FHM Vtg for calibration, a quantitative direct sandwich ELISA was developed to measure

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Table 1
Summary of results from single-laboratory validation of the FHM Vtg ELISA kit^a

Performance characteristics	Aim ^b	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) ^c	Ideally 50–200%	Plasma: 74% (frozen once), 101% (fresh) WBH: 67% (frozen once), 102% (fresh)
Repeatability precision R.S.D. _r ^c	<20%	Within-day R.S.D. _r : 4.5% Between-day R.S.D. _r : 9.9%
Limit of detection (LOD) ^c	<10 ng/ml	<0.1 ng/ml (plasma and WBH)
Limit of quantification (LOQ) ^c	<10 ng/ml	0.1 ng/ml (plasma and WBH)
Sample LOQ = LOQ × matrix dilution ^c	200–500 ng/ml	10 ng/ml (plasma and WBH)
Comparison with Biosense Carp Vtg ELISA kit		R ² > 0.99
Comparison with Amersham FHM Vtg ELISA kit		R ² > 0.99

^a Abbreviations: FHM Vtg, Fathead minnow vitellogenin; LOD, limit of detection; WBH, whole body homogenate; R.S.D._r, relative standard deviation (under repeatability conditions); LOQ, limit of quantitation.

^b Goksøyr et al. (2003).

^c Samples were diluted 1:100.

Vtg in the fathead minnow. The performance of the ELISA was validated through a single-laboratory validation (SLV), based on international guidelines for validation of analytical methods, and an inter-laboratory validation (ILV) with four participating international laboratories. The results from the two validation studies are summarized in Tables 1 and 2.

Monoclonal mouse antibodies (mAbs) specific for FHM Vtg were developed following standard methods (Denslow et al., 1999; Harlow and Lane, 1988), and among several candidates two mAbs were selected, having the necessary sensitivity and specificity to function as a pair in a sandwich ELISA without competing for binding sites.

The reference material (RM) for the calibration curve (FHM Vtg) was purified from plasma of 17β-oestradiol-treated FHM using anion exchange chromatography. The purity and identity of the RM was verified using SDS-PAGE and two-dimensional gel electrophoresis (2DE) followed by matrix assisted laser desorption ionization mass spectrophotometry (MALDI-MS) (Aebersold and Goodlett, 2001). The protein component of the FHM Vtg was quantified by amino acid analysis and the RM was stabilized for subsequent use by lyophilization.

Using these assay components, a quantitative ELISA for FHM Vtg was developed based on a direct sandwich format (Fig. 1a). Briefly, one mAb was immobilized in microplate wells,

functioning as a capture Ab to bind Vtg in standard and sample dilutions. The second mAb was labelled with the enzyme horseradish peroxidase (HRP), and functioned as a detecting Ab by binding to a different part of the Vtg molecule. Tetramethyl benzidine (TMB), a non-toxic substrate for HRP, was used for the chromogenic reaction, giving a colour intensity corresponding to the concentration of Vtg. Fig. 1b shows a representative FHM Vtg standard curve, with a linear range of 0.1–25 ng/ml, plotted using a log–log curve fit.

In the process of evaluating Vtg as an endpoint for testing and screening of chemicals for endocrine activity, various studies have been conducted to compare the available Vtg assays in a variety of fish species, including the zebrafish, medaka and fathead minnow (Hutchinson et al., 2006; Porcher, 2003; Battelle, 2003a,b). These studies demonstrated that the absolute amount of Vtg measured in the study samples varied with differing degrees, and this was dependent on the method used, and on the quality of the standard used. Furthermore, the amount of Vtg quantified often varied between laboratories using the same method and standard. In 2003, a white paper was put forward, stressing the need for standardized Vtg assays and associated standards (Goksøyr et al., 2003). This need was reinforced by the variation in performance of the Vtg assays between laboratories in the inter-laboratory studies mentioned above. The new

Table 2
Summary of results from inter-laboratory validation of the FHM Vtg ELISA kit^a

Performance characteristics	Aim ^b	Value
Between-day repeatability precision, R.S.D. _r		16.4%
Between-lab reproducibility precision, R.S.D. _R	<50%	18.6%
Accuracy (recovery) ^c	50–200%	Plasma: 77% (frozen once) WBH: 53% (frozen once)

^a Abbreviations: FHM Vtg, Fathead minnow vitellogenin; R.S.D._r, relative standard deviation (under repeatability conditions); R.S.D._R, relative standard deviation (under reproducibility conditions); WBH, whole body homogenate.

^b Goksøyr et al. (2003).

^c Samples diluted 1:100.

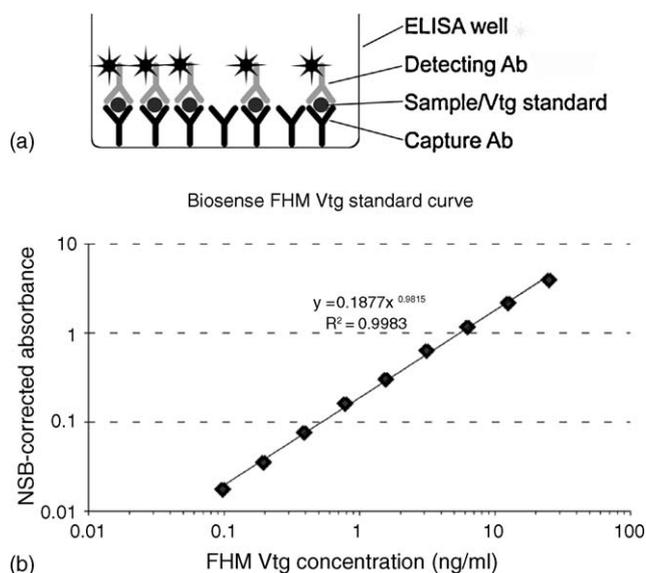


Fig. 1. (a) Schematic of the sandwich ELISA for fathead minnow vitellogenin (FHM Vtg). Vtg as standard and in samples is sandwiched between two mAbs specific for FHM Vtg. The enzyme horseradish peroxidase (HRP), conjugated to the detecting Ab, catalyses a reaction which converts an uncoloured substrate tetramethyl benzidine to a blue solution which then turns yellow when the reaction is stopped with a mild acid. The absorbance (colour intensity) is measured using a plate reader at 450 nm, and is proportional to the amount of Vtg present. (b) A representative calibration curve using purified fathead minnow Vtg. Vtg concentration (ng/ml) is plotted on the *x*-axis against absorbance values, corrected for non-specific background (NSB) absorbance, on the *y*-axis. Using a log–log curve fit (Deltasoft software), the calibration curve has a working range from 0.1 to 25 ng Vtg/ml.

FHM Vtg ELISA was therefore assessed based on performance criteria from guidelines set up by international bodies, including AOAC (2003, 2004), Eurachem (1998) and IUPAC (Thompson et al., 2002) (Tables 1 and 2).

Table 3
Summary of results assessing the robustness of the FHM Vtg ELISA kit

Condition altered	Optimal condition	Altered condition	Sample type ^a	Change in quantification (optimal – altered/optimal × 100) ^b (%)
Dilution buffer temperature	Cold	RT	Plasma WBH	–11.3 –5.7
Incubation temperature	RT ^c	30 °C	Plasma WBH	–19.0 –6.7
Incubation time Vtg	1.5 h	1 h	Plasma WBH	8.0 4.3
Incubation time detection Ab	0.5 h	1 h	Plasma WBH	–7.3 –0.3
Number of washes before development	5	3	Plasma WBH	8.7 4.3
TMB solution temperature	RT	4 °C	Plasma WBH	7.0 2.7
Development time	20 min	30 min	Plasma WBH	1.7 –3.7

^a Samples diluted 1:100.

^b Minus sign denotes that the value for the optimal condition was lower than for the altered condition.

^c Room temperature (RT) in this assay was 23 °C.

In the SLV, selectivity (matrix effect), calibration (linear range of the standard curve), accuracy (recovery), repeatability precision (inter- and intra-assay variation), limit of detection (LOD), limit of quantification (LOQ), sample LOQ and robustness were assessed for the FHM Vtg ELISA developed. The assay was further compared with two existing methods for quantifying FHM Vtg.

Components in plasma and tissue samples, not related to Vtg, can interfere with the quantification of Vtg (so-called matrix effects), and a minimum dilution factor must be determined for different sample types to avoid this problem. Selectivity (matrix effect) was measured by analyzing different dilutions of sample blanks (plasma or whole body homogenates, WBH) spiked with RM, and then determining recovery of the RM. It was found that diluting plasma by a minimum of 1:50 and WBH 1:100 avoided these matrix effects (recovery > 75% in freshly spiked samples).

Accuracy was determined by analyzing recovery of RM in plasma and WBH sample blanks, spiked at three different concentrations of FHM Vtg. Recovery was affected by both sample type, spike concentration and whether the spiked sample had been subjected to a freeze/thaw cycle (see Table 1). Spiking of samples just prior to analysis resulted in recovery close to 100% for both plasma and WBH.

Within-day and between-day repeatability precision (relative standard deviation, R.S.D._r) were both determined from repeated analysis of spiked samples (three replicate analyses in five separate assays), and these were 4.5 and 9.9%, respectively.

LOD was measured for each sample type as 3 × the standard deviation of sample blanks (three replicate analyses in five separate assays). LOQ was similarly determined as 10 × the standard deviation. LOD was below, and LOQ was equal to the lowest end of the linear range of the standard curve (0.1 ng/ml), and were thus not limiting for the working range of the assay. Correcting for sample dilution, which was 1:100 for both plasma and WBH

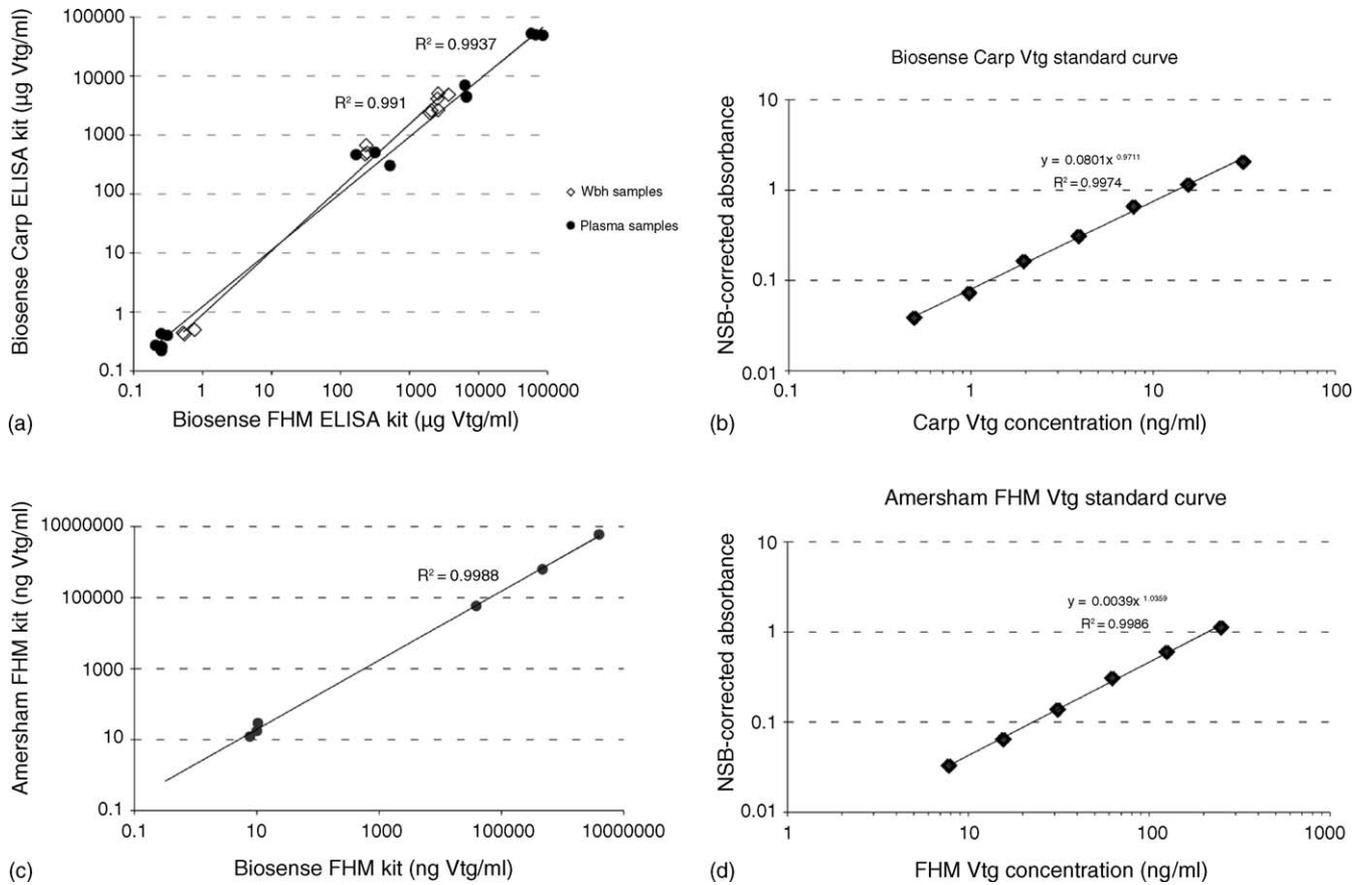


Fig. 2. (a) Correlation between Vtg concentrations ($\mu\text{g/ml}$) in a set of FHM plasma (closed circles) and WBH (open diamonds) samples, quantified using the new FHM Vtg ELISA (Biosense FHM ELISA kit) and the Biosense Carp Vtg ELISA kit. (b) A representative standard curve for the Biosense Carp Vtg ELISA kit, using purified carp Vtg as standard. (c) Correlation between Vtg concentrations (ng/ml) in a set of FHM samples, quantified using the new FHM Vtg ELISA kit (Biosense FHM kit) and the Amersham FHM Vtg ELISA kit. (d) A representative standard curve for the Amersham FHM Vtg ELISA kit.

in these experiments, the LOQ was 10 ng/ml. Theoretically, for plasma diluted 1:50 the LOQ would be 5 ng/ml.

The robustness test assesses the ability of the ELISA method to withstand alterations in seven parameters combined in eight assays (Wernimont, 1985). Table 3 shows the effect of altering these parameters on quantification of Vtg in plasma and WBH samples. The data show that the temperature for incubation and the temperature of the dilution buffer affected the performance of the assay more than the other factors tested.

The FHM Vtg ELISA was compared with two other commercially available ELISA kits used to quantify FHM Vtg, a heterologous Carp Vtg ELISA kit (Biosense Laboratories AS, prod. no. V01003401; Nilsen et al., 2004) and a homologous FHM Vtg ELISA kit (Amersham Biotech., prod. no. 25900132). WBH and plasma samples from an inter-laboratory comparison study for FHM Vtg methods (Battelle, 2003a,b), previously analyzed in the Biosense Carp Vtg ELISA, were re-analyzed in the new Biosense FHM ELISA (Fig. 2a and b). For the Amersham Biotech FHM ELISA, WBH, plasma and spiked dilution buffer samples, prepared for the ILV, were analyzed (Fig. 2c and d). Based on the working range of the standard curve for the different assays, in this study the new Biosense FHM ELISA was 4.9 times and 78 times more sensitive than the Carp Vtg ELISA and the Amersham Biotech FHM Vtg ELISA, respec-

tively. Although the absolute values measured in the samples varied somewhat, and some of the samples were below the detection limit using the alternative assays, quantification across the different assays correlated well.

In order to determine the performance of the FHM Vtg ELISA across different laboratories an ILV study was carried out with four international laboratories, all of which had previous experience performing Vtg ELISAs. Eighteen samples were analyzed, including both plasma and WBH containing naturally induced Vtg, and plasma, WBH and Dilution buffer spiked with three different concentrations of FHM Vtg. The results are summarized in Table 2.

Between-day repeatability precision ($R.S.D._r$) was calculated from two replicate samples analyzed on different days. The $R.S.D._r$ varied from 7.7 to 37.4% for the different laboratories, with an overall $R.S.D._r$ of 16.4%. Between-laboratory reproducibility precision ($R.S.D._R$) for the four different laboratories varied between 7.4 and 37.6% for the different samples, with an overall $R.S.D._R$ of 18.6%.

Accuracy varied somewhat with sample type and spike concentration. An overall recovery of 69.4% was obtained from all sample types and spike concentrations. Given that Vtg is known to be unstable and prone to degradation (Arukwe and Goksøyr, 2003), spiking and analyses of samples for recovery assessments

should ideally be done just prior to analysis to avoid freezing and thawing of spiked samples. In order to obtain data for determination of between-day and between-laboratory precision, however, samples had to be spiked, aliquoted and frozen before analysis and this freeze/thaw cycle is likely to have affected the recovery of Vtg in spiked samples (see SLV).

In summary, a sensitive and robust homologous FHM Vtg ELISA has been developed, which can be reliably applied for the quantification of Vtg in plasma and WBH samples from the fathead minnow.

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