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LABORATORIES

Cod vitellogenin ELISA kit
Prod. No.: V01006401

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A. Introduction

Detection of the egg yolk precursor vitellogenin (Vtg) in blood and tissue samples of juvenile and male fish is a simple and sensitive biomarker for endocrine disrupting chemicals (EDCs) with estrogenic effects (Arukwe & Goksøyr, 2003; Sumpter & Jobling, 1995). Measurement of Vtg has become an accepted routine screening test for estrogenic and anti-androgenic effects of EDCs in fish. This Enzyme Linked Immunosorbent Assay (ELISA) can readily be combined with standard fish toxicology tests developed under the framework of governmental organizations, e.g. the OECD and the US EPA.

This ELISA is developed according to the principles outlined in ISO23893 and includes antibody raised against Cod vitellogenin as well as Cod vitellogenin standard.

Figure 1: Atlantic cod (*Gadus morhua*)

B. Safety instructions

For research use only. Not for human use or drug use. Not for clinical diagnostic use. These reagents contain sodium azide as preservative. Do not use internally or externally in humans or animals. As all chemicals should be considered potentially hazardous, it is advisable to wear suitable protective clothing during handling of this kit. Avoid contact with skin and eyes.

C. Storage and stability

Store the kit at 2-8°C upon arrival. Do not freeze. See expiry date on the kit box for stability of the kit.

D. Warranty and limitation of remedy

Biosense Laboratories AS (hereafter: Biosense) makes no warranty of any kind, expressed or implied, including, but not limited to, the warranties of fitness for a particular purpose and merchantability, which extends beyond the description of the chemicals on the face hereof, except that the material will meet our specifications at the time of delivery.

Buyer’s exclusive remedy and Biosense´s sole liability hereunder shall be limited to refund of the purchase price of, or at Biosense´s option, the replacement of, all material that does not meet our specifications. Biosense shall not be liable otherwise or for incidental or consequential damages, including, but not limited to, the costs of handling.

Said refund or replacement is conditioned on Buyer giving written notice to Biosense within thirty (30) days after arrival of the material at its destination, and Buyer treating the material as outlined in the product data sheet and/or kit insert after arrival. Failure of Buyer to give said notice within said thirty (30) days, or failure of Buyer in treating the material as outlined in the product data sheet and/or kit insert shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

The responsibility of all patent considerations in the use of our products rests solely with the user.

E. Assay principle

This ELISA utilizes specific binding between antibodies and vitellogenin (Vtg) to quantify Vtg in samples from cod (Figure 2). The wells of microtiter plates are pre-coated with a specific capture antibody that binds to Vtg in standard and samples added to the wells. A different Vtg-specific detecting antibody is added to create a sandwich of Vtg and antibody, which is detected with an enzyme-labelled secondary antibody. The enzyme activity is determined by adding a substrate that gives a coloured product, and the colour intensity is directly proportional to the amount of Vtg present.

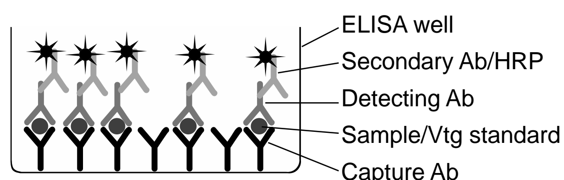

Figure 2: Assay format for the sandwich ELISA

F. Kit contents

		1-plate kit	5-plate kit
A)	96 well microtiter plates, pre-coated	1	5
B)	Plate sealers	2	10
C)	Dilution buffer (5x)	1 vial	2 vials
D)	PBS/Tween tablets	1	3
E)	Detecting antibody, concentrated 500x	1 vial	1 vial
F)	TMB substrate	1 vial	2 vials
G)	Secondary antibody, concentrated 2000x Horseradish peroxidase (HRP) conjugate	1 vial	1 vial
H)	Cod Vtg standard * Purified, lyophilized Vtg from Atlantic cod	1 vial	2 vials

*The lyophilized cod Vtg standard was calibrated against purified cod Vtg quantified by quantitative amino acid analysis.

G. Additional reagents and equipment required

In addition to the reagents supplied with the kit, the following reagents and equipment are required and/or recommended to perform the assay:

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

H. General notes

1. Read the complete procedure before starting the assay. For experienced users, a quick guide can be found on the back cover of the protocol.
2. Store kit according to instructions. Do not use kit components after the expiration date.
3. The plate sealers are used to seal the plates during incubations. Care should be taken not to contaminate the plate sealers when removing them from the plates.
4. After each wash, empty the plate completely by tapping it against a pile of paper towels until no fluid is left in the wells.
5. A dilution series of purified cod Vtg standard should be run in every assay. One standard curve is sufficient for a full five-plate assay. A five-plate kit contains two vials of Vtg, sufficient to run two separate assays (for example 2 plates + 3 plates).
6. Reconstituted Vtg should *not* be frozen and re-used.
7. NSB wells should be included on every plate. These wells are used to determine Non-Specific Binding (unspecific background signal).

   The assay has been developed for quantification of Vtg in plasma samples. For

samples prepared otherwise (*e.g.* homogenates of juvenile fish or samples diluted differently during sample preparation), the optimal minimum dilution factor should be determined in each case.

8. Unused pre-coated microtiter plates should be stored airtight with enclosed desiccant at 2-8°C.
9. In order to obtain reliable results, several common sources of error should be avoided. Important factors to increase reliability are:
   - Careful and precise pipetting is essential at every step in the assay. Reverse pipetting of the Dilution buffer is recommended to increase precision.
   - Adding sample and standard dilutions to the plate in triplicates, instead of duplicates, will increase precision.
   - Avoid shaking and excess foaming when preparing dilutions. Using a vortexer is recommended.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NSB	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
B	-	-	-	-	-	-	-	-	-	-	-	-
C	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
D	-	-	-	-	-	-	-	-	-	-	-	-
E	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24
F	-	-	-	-	-	-	-	-	-	-	-	-
G	P25	P26	P27	P28	P29	P30	P31	P32	P33	P34	P35	P36
H	-	-	-	-	-	-	-	-	-	-	-	-

Figure 3: Suggested plate layout
NSB = Non-specific Binding wells
S1-S11 = Standards 1-11 (0.24-250 ng/ml Vtg)
P1-P36 = Samples

I. Preparation of buffers/reagents

1. Dilution buffer, 5x concentrate:

   Dilute the concentrated buffer (vial C) with distilled water, e.g. 15 ml 5x dilution buffer + 60 ml distilled water.

   Store at 2-8°C (stable for at least 7 days).
2. Washing buffer (PBS, 0.05% Tween-20):

   Dissolve one buffer tablet (bag D) in 1000 ml distilled water.

   Store at 2-8°C (stable for at least one month).
3. TMB Substrate solution (vial F) is ready to use. The solution should be brought to room temperature before the development reaction. Therefore, before starting the assay, measure out the required volume (12 ml per plate) into a new, clan vial and place in the dark at room temperature. Keep the reamaining TMB at 2-8°C.
4. 0.3 M H_2SO_4 (stop solution not included in the kit):

   1.6 ml H_2SO_4 (95-97%) in 100 ml distilled water.

   Note: Beware of the hazardous nature of sulphuric acid. Please refer to the manufacturers Material Data Sheet for appropriate handling of this reagent.

J. Assay procedure

Preparing dilutions of standard and samples:

Please note: Vtg is an *unstable* molecule, and all standard and sample dilutions should be prepared and kept on ice.

1. Dilution of the Vtg standard:

   Dissolve the content of one vial of cod Vtg standard (vial H) in 1.0 ml cold Dilution buffer.

   Please note: Release the vacuum in the vial *carefully*. Do not remove the grey rubber stopper completely, but add the buffer through the opening at the side. Recap the vial and mix carefully by tipping and vortexing. Avoid foaming. Ensure that all material in the vial is dissolved.

   Calculate the concentration of Vtg in this stock solution based on the Vtg amount specified on the vial (µg per vial). Prepare the first dilution step for the standard curve by diluting 50 µl of the stock solution in an appropriate volume of cold Dilution buffer to give a solution of 250 ng cod Vtg/ml (see example below).

   Example: A vial containing 10 µg Vtg dissolved in 1.0 ml Blocking/Dilution buffer gives a solution of 10 µg cod Vtg/ml. Prepare the first dilution step for the standard curve (250 ng/ml) by adding 50 µl of the 10 µg/ml solution into 1950 µl Dilution buffer.

   Prepare a two-fold serial dilution in Dilution buffer (*e.g.* 500 µl cod Vtg dilution + 500 µl buffer for each standard curve run in the assay). The standard series should include 11 dilution steps, ending with a concentration of 0.24 ng cod Vtg/ml.

   Keep the dilutions on ice until use.

2. Dilution of plasma samples:

   Given the wide range of Vtg levels found in experimental studies, we recommend preparing at least three different dilutions of each sample in order to hit the linear part of the standard curve. Mix the plasma samples well before preparing the dilutions.

   We recommend preparing a 1:50 dilution (add 10 µl sample to 490 µl cold Dilution buffer), a 1: 2 500 dilution (add 20 µl of the 1:50 dilution to 980 µl cold Dilution buffer) and a 1: 125 000 dilution (add 20 µl of the 1: 2 500 dilution to 990 µl cold Dilution buffer).

   Keep the dilutions on ice until use.

   Please note: For samples with very low Vtg-concentrations it is possible to dilute the plasma sample as little as 1:20 without risk of unspecific interference (plasma effect). For samples with high Vtg concentrations (more than 8 mg/ml) it may be necessary to dilute the sample more than 1:125 000.

Incubation with standard and diluted samples:

Please note: When more than one plate is run in the assay, complete the addition of both standard and sample dilutions on one plate before proceeding to the next plate.

See suggested plate layout in Figure 3.

3. Add 100 µl Dilution buffer to each of the two NSB wells.
4. Add in duplicate 100 µl of each cod Vtg standard dilution.
5. Add in duplicate 100 µl of each sample dilution.
6. Seal the plates and incubate at room temperaure (20-25°C) for 1 hour.

Incubation with detecting antibody:

7. Dilute the detecting antibody (vial E) 1:500 by adding 22 µl to 11 ml Dilution buffer for each plate run in the assay.

8. Wash the plates three times with 200 µl Washing buffer per well.
9. Add 100 µl of the diluted detecting antibody to all wells.
10. Seal the plates and incubate at room temperature for 1 hour.

Incubation with secondary antibody:

11. Dilute the secondary antibody (vial F) 1:2000 by adding 6 µl to 12 ml Dilution buffer for each plate run in the assay.
12. Wash the plates three times with 200 µl Washing buffer per well,
13. Add 100 µl of the diluted secondary antibody to all wells.
14. Seal the plates and incubate at room temperature for 30 minutes.

Development

Please note: The substrate solution should be adjusted to room temperature before use.

15. Wash the plates *five* times with 200 µl Washing buffer per well
16. Add 100 µl TMB substrate solution to all wells.
17. Incubate in the dark (cover the plates with *e.g.* aluminium foil) at room temperature for 15 minutes.
18. Stop the reaction by adding 100 µl 0.3M H_2SO_4 to all wells.
19. After 5 minutes, read the absorbance at 450 nm with a microtiter plate reader.

K. Calculation of results

Subtraction of NSB absorbance values:

On each plate, calculate the mean of the absorbance values of the two NSB wells and subtract this value from the absorbance values of all other wells on the same plate. This gives the NSB-corrected absorbance values for standard and sample dilutions.

Preparation of the standard curve:

1. Calculate the mean of the NSB-corrected absorbance values for each set of standard duplicates.
2. Plot absorbance values against the Vtg concentration. Perform a regression analysis, using for example log-log (Figure 4a), linear (Figure 4b) or 4-parameter (Figure 4c) transformation of the data.

   Please note: A 4-parameter transformation will often give a wide working range, but is best suited for standard curves with clear plateaus (as in competitive assays). Care should be taken when employing such a model in this assay. The model will be sensitive to the exclusion of data points, and the upper and lower ends of the curve should be used with care.
3. To determine the working range of the standard curve, omit data points using the following guidelines (see also example below):
   - The correlation coefficient (R^2) should be higher than 0.990 (a perfect regression has an R^2 value of 1.0). If the R^2 value is lower than 0.990, exclude points that deviate from the line (usually at the ends) until it is above 0.990.
   - Data points that *clearly* deviate from the regression line should not be included, even if the R^2 value is above 0.990.
   - Data points with NSB-corrected absorbance values lower than 0.020 should *not* be included in the working range.

Calculating the concentration of Vtg in unknown samples:

4. Calculate the mean of the NSB-corrected absorbance values for each set of sample

duplicates.

5. Calculate the Vtg-concentration in the diluted sample using the equation for the adjusted standard curve determined above (pt 2). Use the following guidelines when determining the Vtg concentration in the samples:

   Please note: Only sample dilutions with absorbance values that fall within the standard curve working range should be used (see example below). If all dilutions of a sample give absorbance values outside the working range, the sample should be re-assayed at different dilutions.

6. Multiply the Vtg-concentration in the diluted sample with the dilution factor to get the Vtg concentration in the original sample.

   Please note: If more than one dilution of a sample fall within the standard curve working range, the mean Vtg-concentration should be calculated. If the different dilutions yield contrasting results, care should be taken to determine which of the two dilutions is the most reliable one. Samples having absorbance values close to the ends/plateaus of the standard curve should be used with care, as these parts of the standard curve is less reliable.

   Alternatively, the sample should be re-assayed with more dilutions.

Example:

Cod Vtg standard

Vtg concentration (ng/ml)	Absorbance at 450 nm	NSB-corrected absorbance *)
500	3.166	2.972
250	2.808	2.614
125	2.052	1.858
62.5	1.268	1.074
31.3	0.781	0.587
15.6	0.507	0.313
7.81	0.348	0.154
3.91	0.272	0.078
1.95	0.231	0.037
0.98	0.211	0.017
0.49	0.203	0.009

**) Mean NSB absorbance value: 0.194*

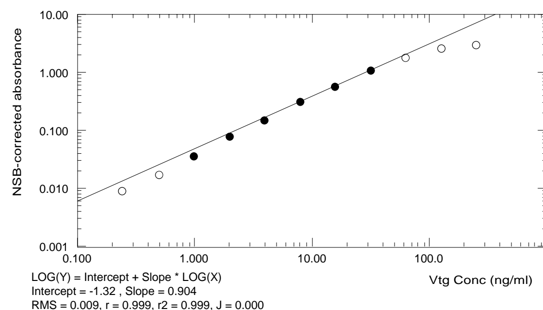

Figure 4a): Log-log curve fit. Omitted data points are shown as open circles.

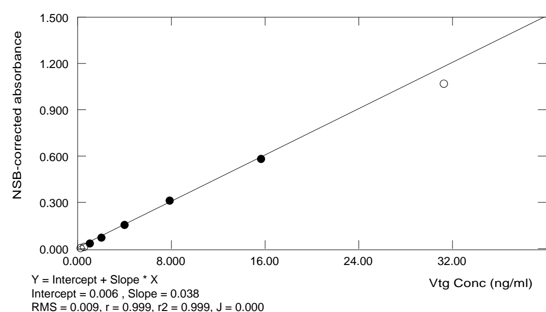

Figure 4b): Linear curve fit. Omitted data points are shown as open circles.

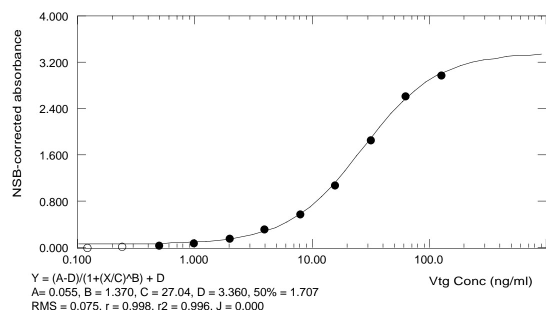

Figure 4c): 4-parameter curve fit (see note under pt. 2). Omitted data points are shown as open circles.

Plasma sample

Dilution factor	Absorbance at 450 nm	NSB-corrected absorbance	Concentration in original sample (ng/ml)		
			Linear	Log-log	4-parameter
50	3.170	2.976	*over*	*over*	*over*
2 500	2.058	1.864	123 686	143 056	155 310
125 000	0.218	0.024	117 056	116 090	*under*

I.e. depending on the choice of curve fitting, the concentration of Vtg in the plasma sample is calculated to be in the range 116-155 µg/ml.

L. Reproducibility of the assay

Table1: Intra- and inter-assay variation

The assays were performed with a plasma sample from an estradiol-induced cod.

	%CV	n
Intra-assay variation	4.3-16.1	12
Inter-assay variation	13.2-23.0	10

M. References

Arukwe A. & Goksøyr A. (2003) Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation. *Comparative Hepatology* 2:4. http://www.comparative-hepatology.com

Nilsen B.M., Berg K., Eidem J.K., Kristiansen S.I., Brion F., Porcher J.M. & Goksøyr A. (2004). Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening. *Anal. & Bioanal. Chem*. 378, 621-633.

Sumpter J.P. & Jobling S. (1995). Vitellogenesis as a biomarker for oestrogenic contaminants in the aquatic environment. *Environ. Health. Persp*. 103 (suppl. 7), 173-178.

N. Quick guide

1. Thaw samples on ice.
2. Prepare dilutions of standard and samples.
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 hour.
4. Wash the plates 3 times with 200 µl washing buffer per well.
   Add 100 µl of diluted detecting antibody to all wells.
   Incubate at room temperature for 1 hour.
5. Wash the plates 3 times with 200 µl washing buffer per well.
   Add 100 µl of diluted secondary antibody to all wells.
   Incubate at room temperatur for 30 minutes.
6. Wash the plates 5 times with 200 µl washing buffer per well.
   Add 100 µl Substrate solution to all wells.
   Incubate in the dark at room temperature for 15 min.
7. Add 100 µl of 0.3M H_2SO_4 to all wells to stop the reaction.
8. After 5 minutes, read the absorbance at 450 nm.
9. Calculate the results.

V – 06.04.22

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