
biosense®
LABORATORIES

ZEBRAFISH VITELLOGENIN ELISA KIT

PROD. NO.: V01008402

CONTENTS

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 oestrogenic effects (Arukwe & Goksøyr, 2003; Sumpter & Jobling, 1995). Measurement of Vtg has become an accepted routine screening test for oestrogenic 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 for a standard test species used in many ecotoxicology laboratories throughout the world, the zebrafish (*Danio rerio*).

FIGURE 1:
ZEBRAFISH (*DANIO RERIO*)
PHOTO: SARS CENTRE

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. The kit contains OPD (*o*-phenylenediamine) tablets. Since OPD is toxic and may be carcinogenic, contact should be avoided and gloves and suitable protective clothing should be used when handling tablets or solutions made from the tablets (see Material Safety Data Sheet [MSDS] for the OPD tablets for more details about proper handling and waste disposal). 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. Unused pre-coated microplates should be stored airtight with enclosed desiccant at 2-8°C.

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 utilises specific binding between antibodies and vitellogenin (Vtg) to quantify Vtg in samples from zebrafish (Figure 2; Nilsen *et al*, 2004). The wells of microplates have been pre-coated with a specific Capture antibody that binds to Vtg in standard and sample 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.

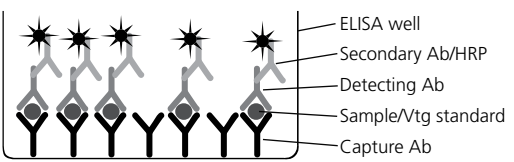

FIGURE 2: ASSAY FORMAT FOR THE SANDWICH ELISA

F. KIT CONTENTS

		1-plate kit	5-plate kit
A)	96 well microplates, pre-coated	1	5
B)	Plate sealers	2	10
C)	Phosphate buffered saline (PBS) tablets	2	10
D)	PBS/Tween tablets	1	3
E)	Bovine serum albumin (BSA)	2 g	10 g
F)	Detecting antibody, concentrated 350x	1 vial	1 vial
G)	Secondary antibody, concentrated 2000x Horseradish peroxidase (HRP) conjugate	1 vial	1 vial
H)	OPD-peroxidase substrate, tablet sets	1 set	3 sets
I)	Zebrafish Vtg standard * Purified, lyophilised Vtg from zebrafish	1 vial	2 vials

*The lyophilised zebrafish Vtg standard (Brion et al. 2002) was calibrated against purified zebrafish Vtg, quantified using 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:

- 2M H_2SO_4 (stop solution)
- Microplate reader (wavelength 492 nm)
- Pipettes with disposable tips (5-1000 µl)
- Multi-channel or stepper pipette with disposable tips (50 and 100 µl)
- Test tubes (1-50 ml)
- Microplate washing device (a manual or automatic plate washer is recommended, but a squeeze bottle or a multichannel/stepper pipette can also be used)
- Vortexer
- Crushed ice

H. IMPORTANT NOTES

1. Vtg standard.

Vtg is an *unstable* molecule, and all sample and standard dilutions should be prepared and kept on ice. Reconstituted Vtg can *not* be frozen and re-used quantitatively at a later date.

A dilution series prepared from *freshly reconstituted* Vtg standard should be run in every assay. A five-plate kit contains two vials of Vtg, sufficient to run two separate assays (for example 2+3 plates). One standard curve is sufficient for a full five-plate assay.

3. Samples.

The assay has been developed for quantification of Vtg in whole body homogenate (wbh), but may also be used with other sample types like plasma. Since compounds in the sample matrix may interfere non-specifically with the assay, usually leading to an underestimation of Vtg at low sample dilutions, the recommended minimum dilution to avoid this matrix effect is 1:500 for wbh (determined for adult zebrafish diluted 1:2 [weight:volume] during sample preparation). For other sample types and sample preparation methods, the minimum dilution factor must be determined in each case.

3. Techniques.

In order to obtain reliable results, several common sources of error should be avoided. Important factors to increase reliability are:

- Careful and precise pipetting at every step in the assay. Reverse pipetting of the Dilution buffer is recommended to increase reliability.
- Addition of sample and standard dilutions to the plate in triplicates, instead of duplicates, will increase reliability.
- Avoid shaking and excess foaming when preparing dilutions. Using a vortexer is recommended.

I. PREPARATION OF BUFFERS/REAGENTS

1. Dilution buffer: PBS (Phosphate buffered saline, pH 7.3), 1% BSA:
   Dissolve one buffer tablet (bag C) and 1.0 g BSA (vial E) per 100 ml distilled water.
   Store at 2-8°C (stable for 2-3 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. Substrate solution (*prepare just prior to use*):
   Dissolve one Urea Hydrogen Peroxide tablet in 20 ml distilled water (dissolves slowly, 10-15 minutes with gentle shaking), then add one OPD tablet and let it dissolve. The substrate solution should be used within 30 minutes.
   Warning: *OPD (o-phenylenediamine) is toxic and may be carcinogenic. Avoid contact. Use gloves and suitable protective clothing when handling tablets and substrate solution. See MSDS for proper disposal of hazardous waste.*

J. ASSAY PROCEDURE

Please note: *Read the complete procedure before starting the assay. For experienced users, a quick guide can be found on the inside back cover of the protocol.*

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. Frozen samples should be thawed on ice.*

1. Dilution of the Vtg standard:
   Dissolve the content of one vial of zebrafish Vtg standard (vial I) in 1.0 ml cold Dilution buffer.
   Please note: *Release the vacuum in the vial carefully. Add buffer 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 125 ng zebrafish Vtg/ml (see example below).

Example: *A vial containing 10 µg Vtg dissolved in 1.0 ml Dilution buffer gives a solution of 10 µg zebrafish Vtg/ml. Prepare the first dilution step for the standard curve (125 ng/ml) by adding 50 µl of the 10 µg/ml solution into 3950 µl Dilution buffer.*

Prepare a two-fold serial dilution in Dilution buffer (*e.g.* 500 µl zebrafish 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 0.12 ng zebrafish Vtg/ml.

Keep the dilutions on ice until use.

2. Dilution of whole body homogenate 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.

Please note: *Mix the samples well before preparing the dilutions and between each dilution.*

We recommend preparing a 1:500 dilution (add 5 µl sample to 2495 µl cold Dilution buffer), a 1:30 000 dilution (add 10 µl of the 1:500 dilution to 590 µl cold Dilution buffer) and a 1:1 800 000 dilution (add 10 µl of the 1:30 000 dilution to 590 µl cold Dilution buffer).

Keep the dilutions on ice until use.

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.

	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 (0.12-125 NG/ML VTG)
P1-P36 = SAMPLES

3. Add 100 µl Dilution buffer to each of the two NSB wells.
   ***Please note**: NSB wells should be included on every plate. These wells are used to determine Non-Specific Binding (unspecific background signal).*
4. Add in duplicate 100 µl of each zebrafish Vtg standard dilution.
5. Add in duplicate 100 µl of each sample dilution.
6. Seal the plates and incubate at room temperature (20-25°C) for 1 hour.

***Please note**: Ensure equal incubation time when running more than one plate. Addition of standards and samples often take several minutes. If there is a substantial time difference between plates, they can be synchronised at this point: after one hour incubation, wash the plate (step 8 below), seal it and leave at room temperature until all plates have been washed. Then proceed with addition of Detecting antibody (step 9 below).*

Incubation with Detecting antibody:

7. Dilute the Detecting antibody (vial F) 1:350 by adding 31 µl to 11 ml Dilution buffer for each plate run in the assay.
8. Wash the plates three times with 300 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 (20-25°C) for 1 hour.

Incubation with Secondary antibody:

11. Dilute the Secondary antibody (vial G) 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 300 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 (20-25°C) for 1 hour.

Development

***Please note:** The Substrate solution should be prepared just before proceeding to the next step.*

15. Wash the plates *five* times with 300 µl Washing buffer per well.
16. Add 100 µl Substrate solution to all wells.
17. Incubate in the dark (cover the plates with *e.g.* aluminium foil) at room temperature (20-25°C) for 30 minutes.
18. Stop the reaction by adding 50 µl 2M H_2SO_4 to all wells.
19. After five minutes, read the absorbance at 492 nm with a microplate 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 defined 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.

Calculation of Vtg concentration in the 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 2-3).
6. Multiply the Vtg concentration in the diluted sample with the dilution factor to get the Vtg concentration in the original sample.
   Use the following guidelines when determining the Vtg concentration in the samples:

- 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.
- If more than one dilution of a sample fall within the standard curve working range, the mean Vtg concentration should be calculated.

  Please note: *If the different dilutions yield contrasting results, care should be taken to determine which of the 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 are less reliable. Alternatively, the sample should be re-assayed with more dilutions.*

Example:

Zebrafish Vtg standard

Vtg concentration (ng/ml)	Absorbance at 492 nm	NSB-corrected absorbance *)
125	3.321	3.196
62.5	3.127	3.002
31.3	1.880	1.755
15.6	0.998	0.873
7.81	0.559	0.434
3.91	0.349	0.224
1.95	0.225	0.100
0.98	0.176	0.051
0.49	0.152	0.027
0.24	0.133	0.008
0.12	0.129	0.004

**) Mean NSB absorbance value: 0.125*

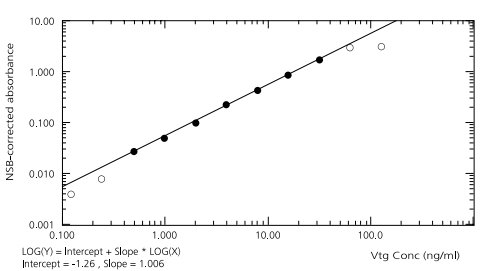

FIGURE 4A:
LOG-LOG CURVE FIT.
OMITTED DATA POINTS ARE SHOWN AS OPEN CIRCLES.

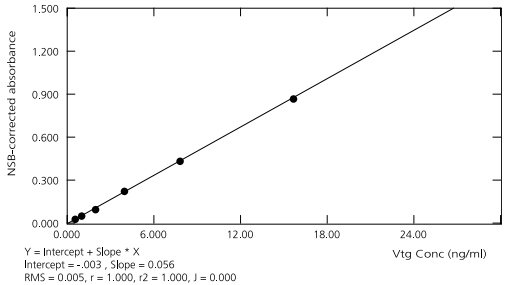

FIGURE 4B:
LINEAR CURVE FIT.

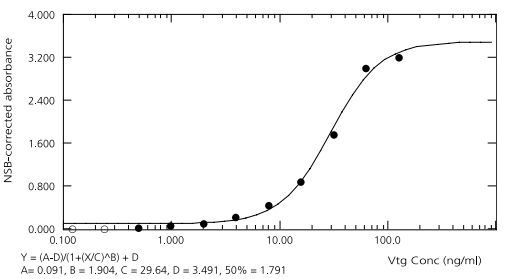

FIGURE 4C:
4-PARAMETER CURVE FIT (SEE NOTE UNDER PT. 2).

OMITTED DATA POINTS ARE SHOWN AS OPEN CIRCLES.

Whole body homogenate sample

Dilution factor	Absorbance at 492 nm	NSB-corrected absorbance	Concentration in original sample (ng/ml)		
			Linear	Log-log	4-parameter
500	3.412	3.287	over	over	over
30 000	3.372	3.247	over	over	over
1 800 000	0.358	0.233	7 566 000	7 564 000	10 280 000

Calculations were made using Deltasoft software. Depending on the choice of curve fitting, the concentration of Vtg in the whole body homogenate sample is 7.56-10.3 mg/ml.

L. REPRODUCIBILITY OF THE ASSAY

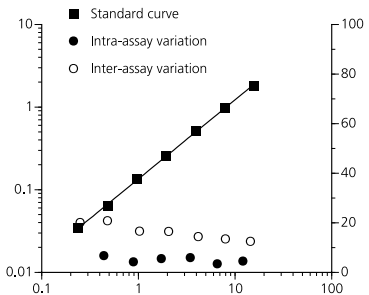

FIGURE 5:
INTRA- AND INTER-ASSAY VARIATION.

THE ASSAYS WERE PERFORMED WITH A WHOLE BODY HOMOGENATE SAMPLE FROM AN OESTRADIOL-INDUCED ZEBRAFISH.

	%CV	n
Intra-assay variation	3.6-6.8	12
Inter-assay variation	13-21	10

M. REFERENCES

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- Brion F., Nilsen B.M., Eidem J.K., Goksøyr A. & Porcher J.M. (2002). Development and validation of an enzyme-linked immunosorbent assay to measure vitellogenin in the zebrafish (*Danio rerio*). *Environ. Toxicol. Chem.* 28, 1699-1708.
- 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 estrogenic contamination of 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 300 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 300 µl Washing buffer per well.
   Add 100 µl of diluted Secondary antibody to all wells.
   Incubate at room temperature for 1 hour.
6. Wash the plates 5 times with 300 µl Washing buffer per well.
   Add 100 µl Substrate solution to all wells.
   Incubate in the dark at room temperature for 30 min.
7. Add 50 µl of 2M H_2SO_4 to all wells to stop the reaction.
8. After 5 minutes, read the absorbance at 492 nm.
9. Calculate the results.

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