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LABORATORIES

# ZEBRAFISH VITELLOGENIN ELISA KIT

## PROD. NO.: V01008403

**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 EnzymeLinked 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 antibodies raised against Zebrafish vitellogenin as well as Zebrafish vitellogenin standard.

**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. 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 i ncidental 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). 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, labelled with the enzyme horseradish peroxidase (HRP), is added to create a sandwich of Vtg and antibodies. 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.

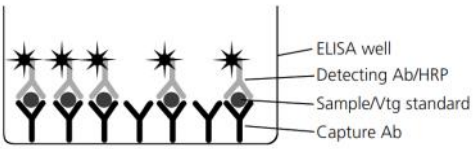


**FIGURE 2:** ASSAY FORMAT FOR THE SANDWICH ELISA

## F. KIT CONTENT

| | | 1-plate kit | 5-plate kit |
|---|---|---|---|
| A) | 96 well microplates, 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) | Zebrafish Vtg standard*<br>Purified, lyophilised Vtg from Zebrafish | 1 vial | 2 vials |

*The lyophilised Zebrafish Vtg standard was calibrated against purified Zebrafish vtg, quantified using 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:

- 0.3M $H_2SO_4$ (stop solution)
- Microplate reader (wavelength 450nm)
- Pipettes with disposable tipd (5-1000 µl)
- Multi-channel or stepper pipette with plastic tips (100 µl)
- 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

## 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.

### 2. Samples

The assay has been developed for quantification of Vtg in plasma and whole body homogenate (wbh) samples, but may also be used with other sample types like liver homogenates. 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 dilutions to avoid this matrix effect are 1:50 for both plasma and 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. Techiques

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, 5x concentrate:
   Dilute the concentrated buffer (vial C) with distilled water, e.g. *15 ml 5x dilution buffer + 60 ml destilled 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 (12ml per plate) into a new, clean vial and place in the dark at room temperature. Keep any remaining TMB at 2-8°C.

## J. ASSAY PROCEDURE

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

**Preparing dilutions of standards 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 content of one vial of zebrafish Vtg standard (vial G) 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 sthis stock 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 the solution of 500 ng Zebrafish Vtg/ml (see example next page).

***Example:*** *A vial containing 5,0 µg Vtg dissolved in 1.0 ml dilution buffer gives a solution of 5,0 µg Zebrafish Vtg/ml. Prepare the first dilution step for the standard curve (500 ng/ml) by adding 100 µl of the 5 µg/ml solution into 900 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 0,49 ng Zebrafish Vtg/ml.
Keep the dilutions on ice until use.

**2.** Dilution of plasma and whole body homogenate samples:
Given the wide range of Vtg levels found in experimental studies, we recommend preparing at least three different dilutions for 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 1:50 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 980 µl cold dilution buffer).
Keep the dilutions on ice until use
***Please note:*** *For samples with high Vtg concentrations (more than 8 mg/ml) it may be necessary to dilute the samples more than 1:250 000.*

**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 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:**
RECOMMENDED PLATE LAYOUT
NSB= NON-SPECIFIC BINDING WELLS
S1-S11 = STANDARDS 1-11 (0.05-50 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 1 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 E) 1:500 by adding 24 µl to 12 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.

**Development:**

**11.** Wash the plates *five* times with 300 µl Washing buffer per well.
**12.** Add 100 µl room tempered TMB substrate solution to all wells.
**13.** Incubate in the dark (cover the plates with *e.g.* aluminium foil) at room temperature (20-25°C) for 20 minutes.
**14.** Stop the reaction by adding 100 µl 0.3M $H_2SO_4$ to all wells.
**15.** After 5 minutes, read the absorbance at 450 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 a 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 ajusted standard curve determined above (pt 2-3).
***Please note:*** *Only sample dilutions with absorbance values that fall within the standard curve working range should be used (see example next page). 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 are less reliable.*
*Alternatively, the sample should be re-assayed with more dilutions.*

**Example:**
**Zebrafish 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*

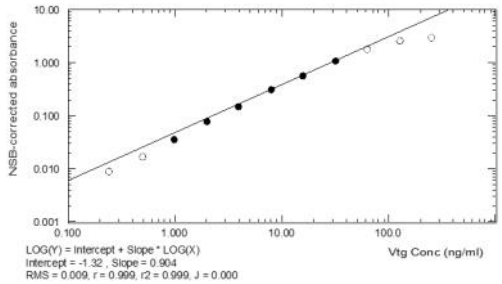


**Figure 4A):** Log-log curve fit. Omitted data points are shown as open circles.

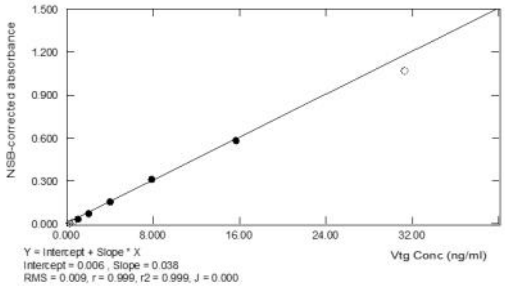


**Figure 4B):**
Linear curve fit. Omitted data points are shown as open circles.

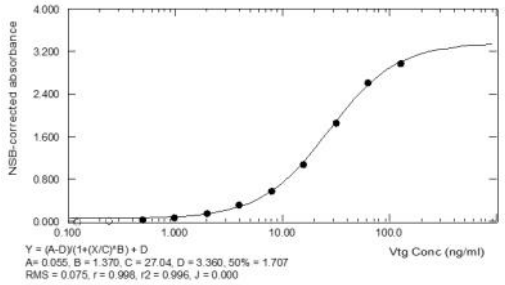


**Figure 4C):**
4-parameter curve fit (see note under pt. 2). Omitted data points are shown as open circles.

**Plasma and whole body homogenate 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 is calculated to be in the range 116-155 µg/ml.

## L. REPRODUCIBILITY OF THE ASSAY

**Table 1:** Intra– and inter-assay variation and Accuracy recovery. The Assays were performed with wbh samles from zebrafish.

| | | n |
|---|---|---|
| Intra-assay variation | 8,0 %CV | 15 |
| Inter-assay variation | 15,1 %CV | 15 |
| Accuracy recovery | 88,2 % | 15 |

## M. REFERENCES


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## N. QUICK GUIDE

1. Thaw samples on ice
2. Prepare dilutions of standards 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 5 times with 300 µl Washing buffer per well.
   Add 100 µl Substrate solution to all wells.
6. 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. After 5 minutes, read the absorbance at 450 nm.
9. Calculate the results.

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