Version 22-04/24

User's Manual

Zebrafish Vitellogenin ELISA Kit

REF **DEIA5068**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

Zebrafish Vitellogenin ELISA Kit is a quantitative procedure for the detection of Vitellogenin in Zebrafish body homogenate samples.

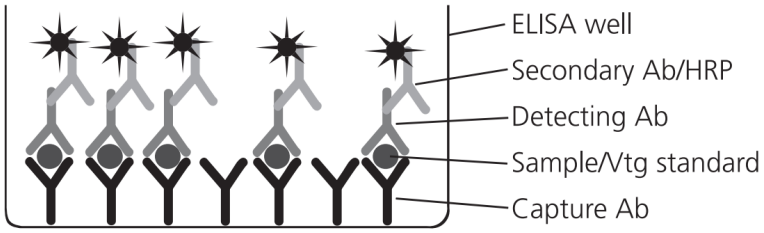
General Description

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. 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 for a standard test species used in many ecotoxicology laboratories throughout the world, the zebrafish.

Principles of Testing

This ELISA utilizes specific binding between antibodies and vitellogenin (Vtg) to quantify Vtg in samples from zebrafish. The wells of microtiter plates have been 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.



Reagents And Materials Provided

A.96 well microplates, pre-coated: 1

B.Plate sealers: 2

C.Phosphate buffered saline (PBS) tablets: 2

D.PBS/Tween tablets: 1

E.Bovine serum albumin (BSA): 2 g

F.Detecting antibody, concentrated 350x: 1 vial

G.Secondary antibody, concentrated 2000x: 1 vial, Horseradish Peroxidase (HRP) Conjugate

H.OPD-peroxidase substrate, tablet sets: 1 set

I.Zebrafish Vtg standard *: 1 vial, Purified, lyophilized Vtg from Zebrafish

* The lyophilized zebrafish Vtg standard was calibrated against purified zebrafish Vtg, quantified using amino acid analysis.

Materials Required But Not Supplied

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

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

Storage

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.

Plate Preparation

	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	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

NSB=Non-specific binding weels

S1-S11=Standards 1-11(0.12-125ng/ml Vtg)

P1-P36=Samples

Reagent Preparation

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).

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).

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.

Assay Procedure

Important notes

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.

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.

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.

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

Keep the dilutions on ice until use.

Incubation with standard and diluted samples

See suggested plate layout

3. Add 100 ul Dilution buffer to each of the two NSB wells.These wells are used to determine Non-Specific Binding (unspecific background signal).

4. Add in duplicate 100 ul of each zebrafish Vtg standard dilution.

5. Add in duplicate 100 ul of each cample dilution.

6. Seal the plates and incubate at room temperature (20-25°C) for 1 hour.

Incubation with detecting antibody:

7. Dilute the detecting antibody 1:350 by adding 31 ul to 11 ml Dilution.

8. Wash the plates three times with 200 ul Washing buffer per well.

9. Add 100 ul 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 1:2000 by adding 6 ul to 12 ml.

12. Wash the plates three times with 300 ul Washing buffer per well.

13. Add 100 ul 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 ul Washing buffer per well.

16. Add 100 ul 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 ul 2M H_2SO_4 to all wells.

19. After five minutes, read the absorbance at 492 nm with a microtiter plate reader.

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.

Calculation

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, linear or 4-parameter 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 besensitive 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.

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.

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.

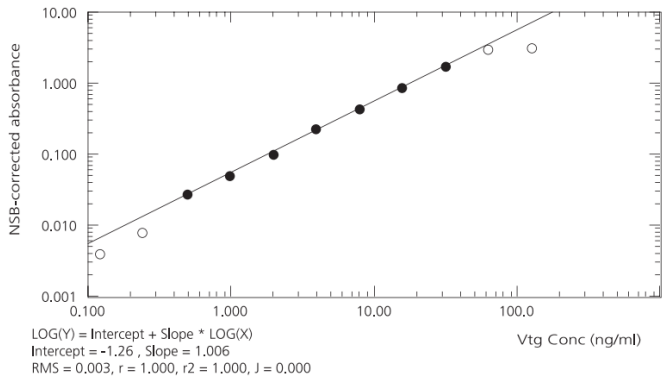
Please note: If the different dilutions yield contrasting results, care should be taken to determine which of the dilutions is the most reliable one. Samples having absorbance values close to the ends/plateaus of the standard curve should be used with care, as these parts of the standard curve are less reliable. Alternatively, the sample should be re-assayed with more dilutions.

Typical Standard Curve

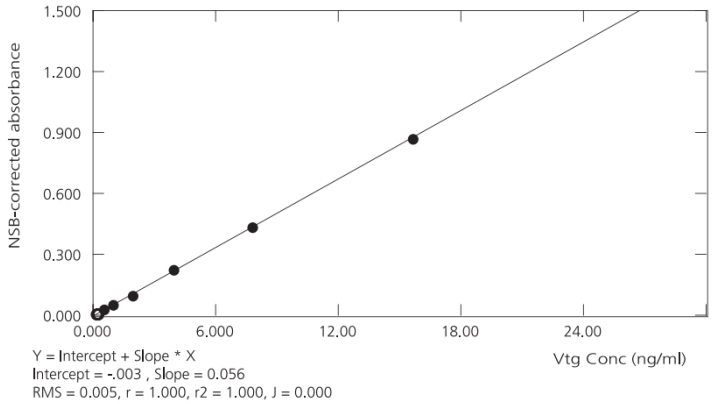
Zebrafish Vtg standard

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

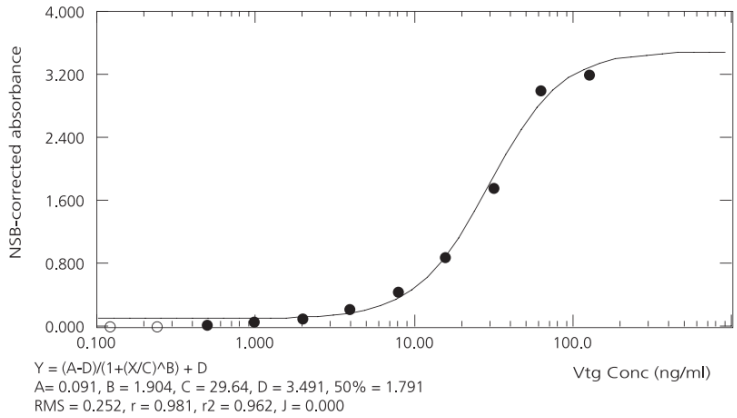
**) Mean NSB absorbance value: 0.125*



Log-Log curve fit



Linear curve fit



4-parameter curve fit

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

I.e. depending on the choice of curve fitting, the concentration of Vtg in the whole body homogenate sample is 7.56-10.3 mg/ml.

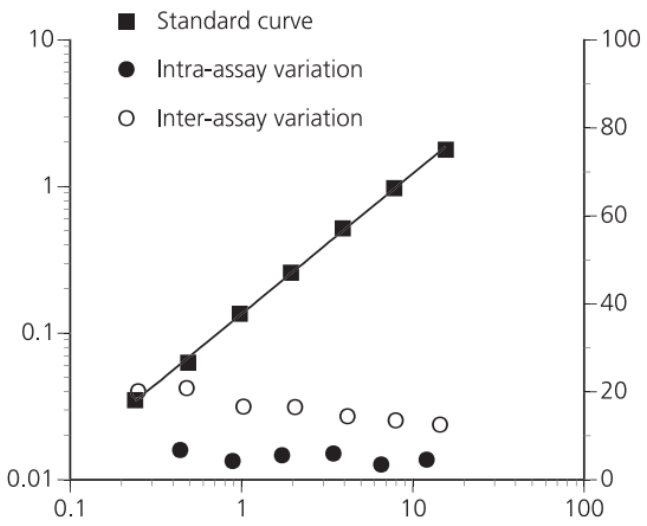
Precision

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

Specificity

Zebrafish

Reproducibility



Precautions

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.

References

1.Arukwe A. & Goksøyr A. (2003) Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation. Comp. Hepatol. 2:4. http://www.comparative- hepatology.com/content/2/1/4.

2.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.

3.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.

4.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.