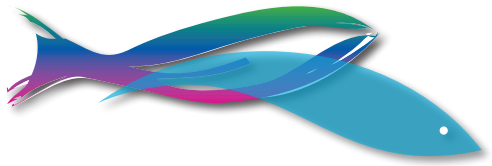
TECOmedical Group



TECO® Perch (Perciformes) Vitellogenin ELISA

For Serum, WBH & Mucus

Perch (Perciformes) Vitellogenin ELISA

Bluegill (Lepomis macrochirus)
European Perch (Perca fluviatilis)
Tilapia (Oreochromias niloticus)
Ruffe (Gymnocephalus cernua)
Goby (Neogobius sp.)
Three-spined stickleback (Gasterosteus aculeatus)

Instructions for Use
English

Catalogue No. TE1035
For Research Use Only

always your partner

Symbol Description

Kit Instructions

Lot Number

Expiry Date

Tests

Storage Temperature

Manufacturer

Intended use

TE1035

Attention

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Contents of the TECO®Perch Vitellogenin ELISA-Kit:

SYMBOL	DESCRIPTION	FORMAT
1	**96-well plate coated with pVTG Antibody** 12 break apart strips of 8 wells (12 x 8 in total), in a frame. Ready to use.	**1 plate**
S	**Standard Stock** 10 x 0.8 µg/ml	**1 x 0.2 ml**
C1	**Control C1** 10 x Concentration see Certificate of Analysis.	**1 x 0.2 ml**
C2	**Control C2** 10 x Concentration see Certificate of Analysis.	**1 x 0.2 ml**
2	**Wash Buffer 50x** Dilute 1:50 with deionized Water.	**1 x 30 ml**
3	**Dilution Buffer** Ready to use.	**1 x 55 ml**
4	**Matrix Solution** Ready to use.	**1 x 7 ml**
5	**Biotinylated Antibody (Biotin-AB)** Ready to use.	**1 x 12 ml**
6	**Streptavidin Peroxidase Conjugate (SA- HRP Conj.)** Ready to use.	**1 x 12 ml**
7	**TMB Substrate** Ready to use.	**1 x 12 ml**
8	**Stop Solution** – 1 M HCl 1 M hydrochloric acid, ready to use. 	**1 x 12 ml**
	Kit instruction	**1 x**

Storage

The kit has to be store at 2-8 °C until expiry date. Do not freeze. Store unused reagents at 2-8 °C.

Instruction for Use

The TECO®Perch (Perciformes) Vitellogenin ELISA Kit is a sensitive enzyme linked immunosorbent assay for the quantitative determination of vitellogenin (VTG) in fish serum, whole body homogenate (WBH) and mucus.

Background

In oviparous animals, vitellogenin (VTG) is an estrogen induced yolk precursor protein mainly synthesized in the liver to be deposited in the maturing oocytes, where it is split in the yolk proteins lipovitellin 1, lipovitellin 2 and phosvitin. These yolk proteins serve as nourishment storage for the developing embryos. Non-physiological induction of vitellogenin in males or in juvenile fish is thought to indicate an estrogen mediated endocrine disruption. Therefore VTG determination is one of the core endpoints in screening and testing for endocrine disrupting chemicals standardized in the OECD Guidelines for the testing of chemicals for estrogenic activity (1, 2 ,3).

Normally vitellogenin is measured in blood samples or whole body homogenate (WBH) - both sample types require invasive and destructive treatment of the fish. Blood is difficult to collect, in particular where very small fish are concerned, or in approaches where the animals must survive sampling. This is particularly important in field monitoring in order to avoid impact on the population under investigation (5).

Recently, several cell types have been shown to produce VTG after estrogen stimulation, including those of the epidermal mucosa (4). Further studies showed that both VTG and estrogen receptor genes are expressed in epidermal cells. Immunoaffinity and mass fingerprint analysis showed induction of identical VTG peptides in liver and epidermis (6). VTG contents in the serum demonstrated a similar dose-response pattern in the epidermis and the blood using the TECO®Cyprinid Vitellogenin ELISA and TECO® Perch (Perciformes) Vitellogenin ELISA. (6). Even though the VTG concentration in the skin mucus is an order of magnitude lower than in blood serum or in body homogenates (containing liver tissue), the skin mucosa is very well suited as a matrix to determine exogenous VTG induction caused by environmental chemicals with affinity to estrogen receptors. By using a highly sensitive ELISA in combination with an unique sampling and extraction system the determination of mucosa born VTG determination has the following advantages:

- Simple and highly standardized sampling technique and sample preparation.
- Strictly defined matrix without protease contamination caused by non-target tissues or lymphatic fluid.
- Non-destructive and thereby allowing several subsequent samplings in order to record a kinetic of VTG induction with a maximum known to appear within 1-2 weeks after exposure. Therefore mucosa tests are compatible with acute as well as chronical OECD test methods.
- Epithelial organized epidermis is directly exposed to exogenous estrogens and thereby allowing a direct comparison with in vitro test using estrogen sensitive vitellogenin producing fish cell lines.
- Lower degree of interference with endogenous VTG production (in females) and bio concentration or enterohepatic circulation of the effective estrogen (xenoestrogen) and thereby showing a clear dose response relationship.
- Stability of standards and samples if prescribed storage conditions are observed.

References

[1] OECD (2009),Test No. 229
Fish Short Term Reproduction Assay. OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing.

[2] OECD (2009),Test No. 230
21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition. OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing.

[3] OECD (2011),Test No. 234
Fish Sexual Development Test. OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing.

[4] Moncaut, N., Lo Nostro, F., Maggese M. C. (2003)
Vitellogenin detection in surface mucus of the South American cichlid fish Cichlasoma dimerus (Heckel, 1840) induced by estradiol-17b. Effects on liver and gonads. Aquatic Toxicology 63, 127-137.

[5] Allner B., Gönna von der S., Griebeler E.M., Nikutowski N., Schaat A., Stahlschmidt-Allner P.(2010)
Reproductive functions of wild fish as bioindicators of reproductive toxicants in the aquatic environment. ESPR Environ. Sci. Pollut. Res., 17, 505-518.

[6] Allner B., Hennies M., Lerche C.F., Schmidt T., Schneider K, Willner M, Stahlschmidt-Allner P. (2016)
Kinetic determination of vitellogenin induction in the epidermis of cyprinid and perciform fishes: Evaluation of sensitive Enzyme-Linked Immunosorbent Assays (ELISAs). Environ Toxicol Chem. 2016 May 6. DOI: 10.1002/etc.3475. [Epub ahead of print]

Assay Principle

The TECO®Perch (Perciformes) Vitellogenin ELISA kit is a 96 well homologue immuno-capture ELISA product using homologue antibodies and homologue VTG standard material. Mucus or serum samples are incubated with the vitellogenin specific antibody coated microtiter plate. After unbound material is washed out, a polyclonal biotinylated antibody binds to the vitellogenin. In the following incubation step, a streptavidin-peroxidase conjugate binds to the biotinylated antibody. In the final substrate reaction, the color development is directly proportional to the amount of vitellogenin in the sample.

Materials required and not supplied

- Pipettes 10 µl – 1000 µl
- Multichannel pipettes for 50 µl – 100 µl
- Graduated cylinders for reconstituting or diluting reagents
- Manual Aspiration System or automatic washer for ELISA plates
- Aqua dest
- Vortex mixer
- ELISA plate reader suitable for 96 well formats and capable of measuring at 450 nm (Reference: 590-650 nm).
- ELISA plate shaker (500 rpm) (orbital shaker)
- Software package for data generation and analysis

For mucus samples:
Extraction Buffer and validated Sampling Swabs are not part of this kit. Please order "TECO® Mucus Collection Set, TE1034" separately

Warnings and Precautions

This kit is for in vitro use by professional persons only.
Follow the instructions carefully.

Observe expiration dates stated on the labels and the specified stability for reconstituted reagents. Refer to "Materials Safety Data Sheet" for more detailed safety information.

Material of animal origin used in the preparation of this kit has been obtained from animals certified as healthy but these materials should be handled as potentially infectious.

TECOmedical AG is not liable for loss or harm caused by non-observance of the Kit instructions.

1. For research use only.
2. Treat all specimen samples as potentially biohazardous material. Follow General Precautions when handling contents of this kit.
3. Disposal of containers and unused contents should be done in accordance with federal and local regulatory requirements.
4. Use the supplied reagents as an integral unit prior to the expiration date indicated on the package label.
5. Store assay reagents as indicated.
6. Do not use coated strips if pouch is punctured.
7. Test each sample in duplicate.
8. Use of multichannel pipettes or repeat pipettors is recommended to ensure the timely delivery of liquids.
9. a. 1 M hydrochloric acid is caustic and can be harmful for skin, eyes and mucosae.
   b. Handle TMB with care. Do not ingest. Avoid contact with skin, eyes, or clothing. Should there be any contact, wash with water. If ingested, call a physician.
10. A mercury-free preservative is used. Incidental contact with or ingestion of buffer solutions may cause irritation of skin, eyes or mouth. Should there be any contact, wash with water. If ingested, call a physician.

Preparation of Reagents

1 **Microtiter plate**
12 break apart strips of 8 wells (96 in total) in a frame and sealed in a foil bag. Fit strip wells firmly into the frame. After opening, return any unused wells to the original foil package and seal.
Store at 2-8 °C until expiration date.

S **Standard Stock - 10x**
1 vial of 0.2 ml standard containing stabilized perch vitellogenin (800 ng/ml).
Store at 2-8 °C until expiration date.

C **Perch Vitellogenin Controls 1 and 2 - 10x**
1 vial of Control 1 and Control 2 containing Perch vitellogenin (Concentration see Certificate of Analysis).
Store at 2-8 °C until expiration date.

2 **Wash Buffer (50x) concentrated**
1 vial of 30 ml Wash Buffer concentrate. Dilute the 50 times concentrate with deionized water up to 1500 ml. The diluted washing solution is stable for 4 weeks at 2-8 °C.
Store undiluted at 2-8 °C until expiration date.

3 **Dilution Buffer**
1 vial of 55 ml, ready for use. Store at 2-8 °C until expiration date.

4 **Matrix Solution**
1 vial of 7 ml, ready for use. Store at 2-8 °C until expiration date.

5 **Biotinylated Antibody (Biotin-AB)**
1 vial of 12 ml, ready for use. Store at 2–8 °C until expiration date.

6 **Streptavidin Peroxidase Conjugate (SA-HRP Conj.)**
1 vial of 12 ml, ready for use. Store at 2-8 °C until expiration date.

7 **TMB Substrate**
1 vial of 12 ml of H_2O_2 stabilized Tetramethylbenzidine.
Ready for use. Store at 2-8 °C until expiration date.

8 **Stop Solution – 1 M HCl**
1 vial of 12 ml of 1 M hydrochloric acid. Ready for use.
Store at 2-8°C until expiration date.

Preparation Standard curve

Standards have to be prepared freshly before use.

The Standard stock vial contains 0.8 µg/ml (800 ng/ml) of stabilized perch vitellogenin.
Preparation of the standard curve **Dilution Buffer** 3 :

ID	Concentration ng/ml	Dilution Buffer µl	Standard solution
Standard A (Std A)	80 ng/ml	450	50 µl Standard Stock S
Std B	27 ng/ml	400	200 µl Std A
Std C	9 ng/ml	400	200 µl Std B
Std D	3 ng/ml	400	200 µl Std C
Std E	1 ng/ml	400	200 µl Std D
Std F	0 ng/ml	400	---

Preparation Kit Controls

Perch Vitellogenin Controls 1 and 2 has to be diluted 1:10 before assay:
add 50 µl to 450 µl **Dilution Buffer** 3 .

Store unused Standard stock and undiluted Controls at 2-8°C. (Concentration see Certificate of Analysis)

Preparation and Stability of Samples

Preparation of Samples

Serum
Store fresh serum samples immediately after collection at -20°C or lower until assayed. Recommended sample thawing: A simple and fast method is to place the frozen serum samples in normal tap cold water (15- 20°C). They should be thawed within 10 to 15 minutes. For assay Bluegill (Lepomis macrochirus) or European perch (Perca fluviatilis) samples should be diluted 1:1000 with **Dilution Buffer** 3 , e.g. 1000 µl Dilution Buffer plus 1 µl sample. Optimal sample dilution for other species may differ. Mature female fish may have elevated vitellogenin.

Whole Body Homogenate (WBH)
Store fresh WBH samples immediately after preparation below -20°C until assayed. For assay, WBH samples should be pre-diluted dependent on fish species with Dilution Buffer 3, e.g. 1:1,000.

Mucus
Collect mucus as described in the TECO® Mucus Collection Set TE1034. For assay, add 500 µl Extraction Buffer (**TECO® Mucus Collection Set, TE1034)** to the swab 15-30 min before pipetting and vortex. In most studies, this sample dilution should be used for sample measurements as a matter of routine. A further dilution for the assay is **not** recommended.

Sample Stability (Mucus samples from Bluegill (Lepomis macrochirus))
Mucus-containing swabs can be stored several months at <-20°C. After addition of Extraction Buffer, samples may be stored for at least:

- 4 days at room temperature (20-28°C)
- 1 week at 4°C

Stability of mucus vitellogenin from other species may differ significantly
Avoid repeated freeze/thaw cycles.

Correction of vitellogenin results by the protein concentration
Independently from the assay procedure, various factors may influence the final amount of biological samples added into the Vitellogenin ELISA (e.g. total amount of blood collected into prefilled sample tubes; effectiveness of homogenization; amount of mucus on the swab etc.). In order to obtain the correct analytical result, all samples may be corrected by the protein concentration by using in parallel a colorimetric protein determination. The Dilution Buffer and the Extraction Buffer in the vitellogenin kits are protein free and may be used as standard buffers and for sample dilution in the protein assays. This sample dilution may differ from the optimal sample dilution in the vitellogenin assay.

Assay Procedure

All determinations (Standards, controls and samples) should be assayed in duplicate. When performing the assay, the standards, controls and samples should be pipetted as fast as possible (<15 minutes).

To avoid distortions due to differences in incubation times, HRP Conjugate, Substrate Solution and Stop Solution should be added to the plate in the same order and with the same time interval as the samples. A multichannel pipette is essential.

Allow all reagents to stand at room temperature (20-28°C) for at least 30 minutes. During all incubation steps, plates should be sealed with the adhesive foil or a plastic cover. For light protection, incubate in a dark chamber or cover plate with aluminum foil.

1. Allocate the wells of the Microtiter plate **1** for standards, controls and samples.
2. Pipette 50 µl Matrix solution **4** (multichannel pipette) into all wells.
3. Add 50 µl of each prepared standard (**A** - **F**), prepared controls (**C1** and **C2**) and (pre-diluted) samples into the corresponding wells.
4. Cover the wells and incubate the plate for 120 ± 5 min at room temperature (20-28°C) on a shaker (500 rpm).
5. After incubation, aspirate the contents of the wells and wash 3 times with 350 µl diluted Wash Buffer **2** . The use of an automatic plate washer is recommended.
6. Following the last washing step, pipette 100 µl of the Biotinylated AB **5** in each well (multichannel pipette).
7. Cover the wells and incubate the plate for 60±5 min at room temperature (20-28°C) on a shaker (500 rpm).
8. After incubation, wash the wells 3 times with Wash Buffer as described in step 5.
9. Following the last washing step, pipette 100 µl of the SA-HRP Conjugate **6** in each well (multichannel pipette).
10. Cover the wells and incubate the plate for 30±5 min at room temperature (20-28°C) on a shaker (500 rpm).
11. After incubation, wash the wells 5 times with Wash Buffer as described in step 5.
12. Pipette 100 µl of the TMB Substrate Solution **7** in each well (multichannel pipette).
13. Incubate the plate for 15-30 min, in the dark, at room temperature (20-28°C) on a shaker (500 rpm).
14. Stop the reaction by adding 100 µl of Stop Solution **8** (multichannel pipette).
15. Measure the color reaction within 10 minutes at 450 nm (reference filter between 590–650 nm). If the extinction of the Standard A (80 ng/ml) exceeds 3.0, the measurement may be repeated at 405 nm.

Result Analysis

Establishing the Standard Curve

A calibration curve can be established by plotting standard concentration on the x-axis (linear scale) against the absorbance of the standards on the y-axis (linear scale). The vitellogenin concentrations in mucus can then be read off the calibration curve. A 4-parameter curve fit should be used for automatic data reduction. If samples were pre-diluted, the concentration will be obtained by multiplying the value read off the calibration curve by the dilution factor. There is no dilution correction for mucus necessary, if the 0.5 ml Extraction buffer is added to the swab. Samples with higher absorbance values than standard A should be tested again pre-diluted with Dilution Buffer. This additional dilution has to be taken in account for the concentration calculation.

Typical Results

(Example only, not for use in calculation of actual results.)

ID	Concentration (ng/ml)	OD (450 nm)
Standard A (Std A)	80	3,723
Std B	27	1,935
Std C	9	0,830
Std D	3	0,399
Std E	1	0,241
Std F	0	0,160

Table 1
Reader values of a typical standard curve.

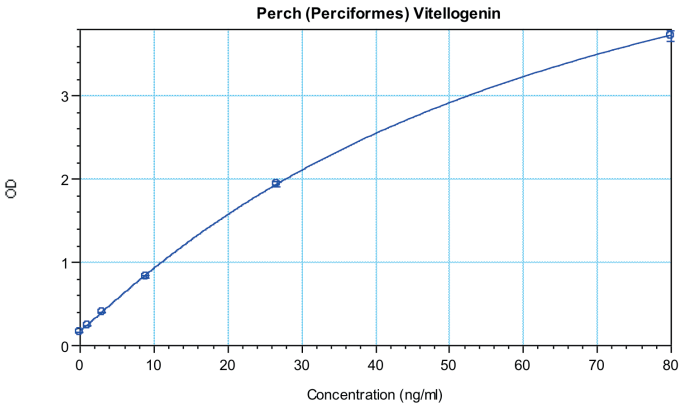

Figure 1
Standard curve by using a 4-parameter curve fit (4-PL).

Test Performance

Data obtained by using Bluegill mucus samples. Mucus vitellogenin concentration are expressed in ng/ml swab extract (0.5 ml Buffer/swab). For serum or WHB samples, concentration has been corrected by pre-dilution.

Standard range: 1 ng/ml – 80 ng/ml (undiluted samples)

LLOQ < 1 ng/ml

LLD < 0.22 ng/ml

(The LLD (lower limit of detection) is defined as the corresponding concentration of the mean OD zero standard plus 3 SD.)

The mean coefficient of determination (R2) of 10 standard curves was 1.0.

Sample	Mean	SD	CV
Pool 1	**16,2**	**0,17**	**1,1**
Pool 2	**11,9**	**0,31**	**2,6**
Pool 3	**1,3**	**0,06**	**4,6**
Pool 4	**2,9**	**0,15**	**5,2**

Table 2
Intra-assay coefficient of variation (CV) of 6 replicates.

Sample	Mean	SD	CV
Control 1	**7,1**	**0,51**	**7,3**
Control 2	**34,9**	**2,16**	**6,2**

Table 3
Intra-assay coefficient of variation (CV) in 13 assay runs.

Sample	Dilution	Measured ng/ml	Expected ng/ml	Recovery %
Pool A + 45 ng/ml	1	48		
	2	22	24	**90**
	4	10	12	**86**
Pool B + 45 ng/ml	1	48		
	2	25	24	**102**
	4	12	12	**96**

Table 4
Dilution Linearity (pooled mucus plus standard vitellogenin).

Sample	Concentration ng/ml	Added ng/ml	Measured ng/ml	Expected ng/ml	Spike Recovery %
Pool C	1,4	3,0	4,4	4,4	**100**
		11,0	12,5	12,4	**101**
		41,7	41,7	43,1	**97**
Pool D	2,8	3,0	5,7	5,8	**98**
		11,0	13,7	13,8	**99**
		41,7	53,5	44,5	**120**

Table 5
Recovery (pooled mucus plus standard vitellogenin).

Vitellogenin (ng/ml) in Bluegill mucus after Estradiol (E2) and ethinyl estradiol (EE2) treatment

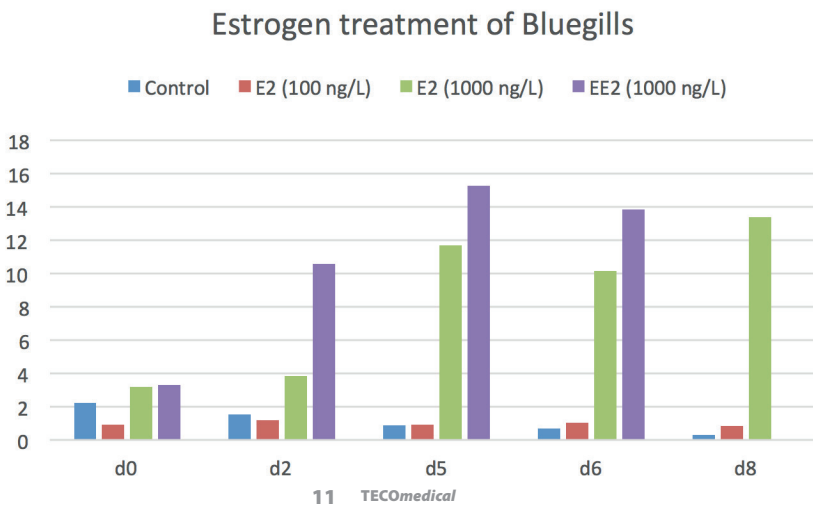

Figure 2
Three individuals per treatment group were maintained per aquarium and were exposed to estradiol (E2) or ethinyl estradiol (EE2) under semi-static conditions. Fish were selected from a cohort including dominant elder juveniles and subordinate immatures. Considering that estrone is known to be present in the rearing water, the elevated initial values of VTG may be due to these pre-exposure conditions.

Vitellogenin (ng/ml) in Bluegill mucus after Bisphenol A treatment kit

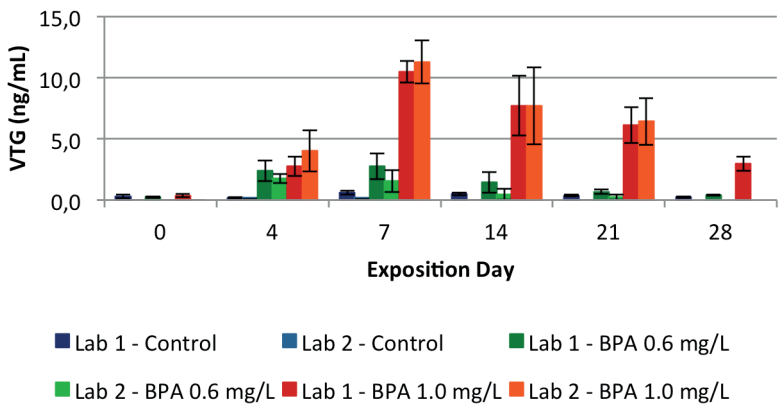

Figure 3
Three groups of five fish were exposed for 28 days under semi-static conditions to 0, 600 and 1000 ng/L bisphenol A (BPA). Mucosa sampling were performed using one swab for each body side at day 0, 4, 7, 14, 21, 28. The swaps from the left and the right body site were measured independently in two different laboratories.

In this experiment, every animal group was maintained in an individual aquarium before the start of the experiment, so that initial VTG values are considered to be unaffected by extraneous influences

	Control			Estradiol treated	
Fisch ID	**Mucus ng/ml**	**Serum µg/ml**	**Fisch ID**	**Mucus ng/ml**	**Serum µg/ml**
c1	1,2	0,0	**e1**	11,6	17,3
c2	0,6	0,0	**e2**	9,8	17,9
c3	0,7	0,0	**e3**	6,9	17,5
c4	0,6	1,1	**e4**	12,1	19,4
c5	0,5	0,0	**e5**	10,4	16,0
c6	0,9	0,0	**e6**	13,6	16,8
c7	0,2	0,0	**e7**	14,6	16,7
Mean	**0,7**	**0,2**		**11,3**	**17,4**
SD	**0,3**	**0,4**		**2,6**	**1,1**

Table 6
Comparison of vitellogenin in Bluegill mucus and serum after 7 days of semi-static exposure to estradiol (1000 ng/L).

BPA treatment of European perch (Perca fluviatilis)

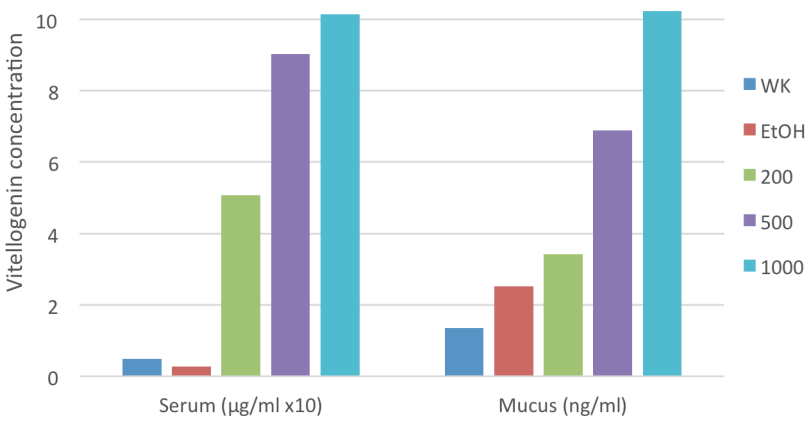

Figure 4
Comparison of vitellogenin in European perch mucus and serum after 6 days of semi-static exposure to estradiol (500 ng/L). Following mucus collection at day 6, blood were taken from the dislocating cut after cervical dislocation and diluted 1:1000 before assay.

TECO®Perch (Perciformes) Vitellogenin

Mucus sample preparation: Quick Guide

Mucus samples have to be collected using the validated TECO Mucus Collection Set TE1034. This collection set also contains the required Extraction Buffer for sample extraction before assay run.

Procedure

Take frozen sample swab tips (vials) selected for testing in the Vitellogenin ELISA out of the freezer.

Open all vials.

Add **500 µl** Extraction Buffer* (TECO Mucus Collection Set, TE1034) into each vial and wait **15-30 min.**

Vortex the closed vials extensively.
For more determinations (e.g. total protein, Cortisol etc.) remove the swabs before vitellogenin assay and discard.

Before pipetting repeat vortexing the sample.

Please read Kit instruction before using the Quick Guide

*If necessary, the sensitivity of the vitellogenin determination may be increased by using 250 µl instead of 500 µl Extraction Buffer (TECO Mucus Collection Set, TE1034) into each vial and wait for 15-30 min. In order to correct the dilution factor, divide the final result obtained from the standard curve by factor 2.

TECO®Perch (Perciformes) Vitellogenin

Quick Guide

Prepare Standards, Controls and Samples

- Dilute Wash Buffer concentrate 1:50 with destilled water
- Allow all reagents to stand at room temperature (20-28°C) for at least 30 minutes

Assay Procedure

Add **50 µL** Matrix solution **4** into each well (multichannel pipette)

Add **50 µL** of each standard **A** - **F**, prepared controls (**C1**, **C2**) and (pre-diluted) samples in wells

Incubate plate for 120±10 minutes on a shaker (500rpm) at RT (20-28°C)

*Wash plate **3 times** using Wash Buffer* **2**

Add **100 µL** Biotinylated AB **5** in each well (multichannel pipette)

Incubate plate for 60±5 minutes on a shaker (500rpm) at RT (20-28°C)

*Wash plate **3 times** using Wash Buffer* **2**

Add **100 µL** SA-HRP-Conjugate **6** in each well (multichannel pipette)

Incubate plate for 30±5 minuten on a shaker (500rpm) at RT (20-28°C)

*Wash plate **5 times** using Wash Buffer* **2**

Add **100 µL** TMB-Substrat Solution **7** in each well (multichannel pipette)

Incubate the plate for 15-30 minutes, in the dark, on a shaker (500rpm) at RT (20-28°C)

Add **100 µL** Stop-Solution **8** in each well (multichannel pipette)

Measure the color reaction within 10 minutes at 450nm
(reference filter between 560 - 650 nm)

A 4-parameter curve fit should be used for automatic data reduction

Please read Kit instruction before using the Quick Guide