



TECO[®] Ultra Sensitive Cyprinid Vitellogenin ELISA

For Cell Culture and Mucus

Ultra Sensitive Cyprinid Vitellogenin ELISA

Carp (*Caprinus carpio*)
Goldfish (*Carassius auratus*)
Zebrafish (*Danio rerio*)
Fathead Minnow (*Pimephales promelas*)
Roach (*Rutilus rutilus*)

Instructions for Use
English

Catalogue No. TE1046
For Research Use Only

Symbol Description



Kit Instructions



Lot Number



Expiry Date



96
Tests



Storage Temperature



Manufacturer



Intended use



TE1046



Attention


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Contents of the Ultra Sensitive Cyprinid Vitellogenin ELISA-Kit:

| SYMBOL | DESCRIPTION | FORMAT |
|---|--|------------------|
| 1 | 96-well plate coated with cypVTG Antibody 12 break apart strips of 8 wells (12 x 8 in total), in a frame. Ready to use. | 1 plate |
| S | Standard Stock lyophilized 35 ng | 2 x |
| C1 | Control C1 lyophilized Concentration see Certificate of Analysis. | 2 x |
| C2 | Control C2 lyophilized Concentration see Certificate of Analysis. | 2 x |
| 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 stored at 2-8 °C until expiry date. Do not freeze. Store unused reagents at 2-8 °C.

Instruction for Use

The Ultra Sensitive Cyprinid Vitellogenin ELISA Kit is a very sensitive enzyme linked immunosorbent assay for the quantitative determination of vitellogenin (VTG) in fish mucus of juvenile or male cyprinids and in cell culture. Other matrices have to be tested individually.

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 (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 chronic 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., Schaaf 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® Ultra Sensitive Cyprinid Vitellogenin EIA Kit is a 96 well immuno-capture ELISA product. Cell culture and mucus 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. The standard range of the TECO® Ultra Sensitive Cyprinid Vitellogenin EIA Kit is between 0.025 and 2.0 ng/ml.

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.

Note: As the TECO® Ultra Sensitive Cyprinid Vitellogenin ELISA kit is very sensitive, please keep the benchtop very clean and avoid contamination with stock solutions or high concentrated samples.

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 – 35 ng**

2 vials of lyophilized standard containing stabilized cyprinid vitellogenin.
Store at 2-8 °C until expiration date.
- C Cyprinid Vitellogenin Controls 1 and 2**

2 vials each of Control 1 and Control 2 containing Cyprinid 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 distilled 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 H₂O₂ 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. Add 500 µl **Dilution Buffer 3** to the vial to obtain Standard stock S. Let it stand for 15-30 minutes and then vortex. The Standard stock contains 70 ng/ml of stabilized cyprinid vitellogenin.

Preparation of the standard curve with **Dilution Buffer 3**:

| Sample | Conc. ng/ml | Dilution Buffer µl | Standard solution |
|--------------------|-------------|--------------------|--------------------------|
| Standard A (Std A) | 2.000 | 971 | 28,6 µl Standard Stock S |
| Std B | 0.667 | 400 | 200 µl Std A |
| Std C | 0.222 | 400 | 200 µl Std B |
| Std D | 0.074 | 400 | 200 µl Std C |
| Std E | 0.025 | 400 | 200 µl Std D |
| Std F | 0 | 400 | - |

Preparation Kit Controls

Cyprinid Vitellogenin Controls 1 and 2 have to be prepared freshly before use. Add 500 µl **Dilution Buffer 3** to the vial to obtain Control stocks. Let it stand for 15-30 minutes and then vortex.)

Both Cyprinid Controls have to be diluted 1:17,5 before the assay: Add 29 µl Control stock to 471 µl **Dilution Buffer 3**.

Reconstituted and diluted standards and controls are for single use only and are stable for 4 hours at room temperature (20-28°C). Do not store reconstituted standards and controls.

Preparation and Stability of Samples

Preparation of Samples

Serum, plasma and whole body homogenate (WBH)

Due to the high sensitivity of this assay, the measurement of vitellogenin in serum, plasma or WBH, especially from female fish, is not recommended.

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 vortex. For more determinations (e.g. total protein, Cortisol etc.) the swabs should be removed from each vial and discarded before vitellogenin measurement. Before pipetting sample into wells repeat vortexing the sample. In most studies, this sample dilution should be used for sample measurements as a matter of routine.

For dose response curves or if concentrations of estrogenic induced fish are required, a further pre-dilution of mucus samples of 1:10 to 1:100 with **Dilution Buffer 3** may be necessary.

Sample Stability (Mucus samples from Cyprinids)

Mucus-containing swabs can be stored several months at <-20°C. After addition of Extraction Buffer the samples are stable up to 4 hours at room temperature (20-28°C).

Stability of sample vitellogenin may differ significantly between species and cell culture media

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 the 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 Buffers in the VTG 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 overnight 16-20 h at room temperature (20-28°C) on a shaker (500 rpm).
5. After incubation, aspirate the contents of the wells and wash 5 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 4 h ± 10 min at room temperature (20-28°C) on a shaker (500 rpm).
8. After incubation, wash the wells 5 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).

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.)

| Sample | Conc. ng/ml | OD 450 nm |
|---------------------------|--------------|--------------|
| Standard A (Std A) | 2.0 | 1.773 |
| Std B | 1.0 | 0.671 |
| Std C | 0.22 | 0.241 |
| Std D | 0.074 | 0.100 |
| Std E | 0.025 | 0.051 |
| Std F | 0 | 0.027 |

Table 1
Reader values of a typical standard curve

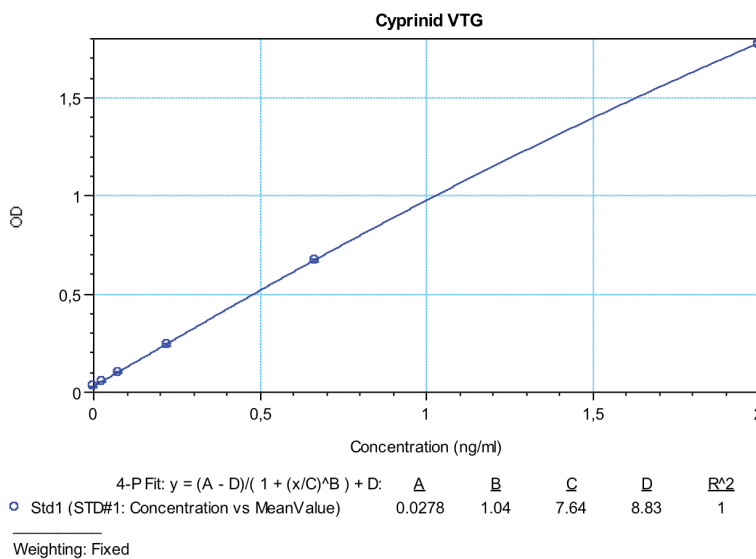


Figure 1
Standard curves by using a 4-parameter curve fit (4-PL) using measurement at 450 nm and 405 nm

Test Performance

Mucus vitellogenin concentration are expressed in ng/ml swab extract (0.5 ml buffer/swab).

Standard range: 0.025 ng/ml - 2 ng/ml (undiluted samples)

LLOQ < 0.025 ng/ml

LLD 0.002 ng/ml

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

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

| Sample | Mean | SD | CV |
|--------|-------|-------|-----|
| #1 | 0.087 | 0.004 | 5.1 |
| #2 | 1.024 | 0.034 | 3.3 |

Table 2

Intra-assay coefficient of variation (CV).

| Sample | Mean | SD | CV |
|-----------|-------|-------|-----|
| Control 1 | 0.087 | 0.005 | 5.5 |
| Control 2 | 1.024 | 0.036 | 3.5 |

Table 3

Inter-assay coefficient of variation (CV).

Table 4

Recovery of vitellogenin spiked to mucus samples of untreated zebrafish.

| Gender | Fish ID | Measured ng/ml | Added ng/ml | Expected ng/ml | Recovery % | Mean (%) | SD (%) |
|--------|---------|----------------|-------------|----------------|------------|----------|--------|
| Male | #1 | 0.004 | - | - | - | 102.7 | 3.8 |
| | | 0.107 | 0.102 | 0.106 | 101 | | |
| | | 1.122 | 1.057 | 1.061 | 106 | | |
| | #2 | 0.006 | - | - | - | | |
| | | 0.119 | 0.102 | 0.108 | 110 | | |
| | | 1.122 | 1.057 | 1.063 | 106 | | |
| | #3 | 0.006 | - | - | - | | |
| | | 0.113 | 0.102 | 0.108 | 105 | | |
| | | 1.088 | 1.057 | 1.063 | 102 | | |
| | #4 | 0.004 | - | - | - | | |
| | | 0.107 | 0.102 | 0.106 | 101 | | |
| | | 1.076 | 1.057 | 1.061 | 101 | | |
| | #5 | 0.002 | - | - | - | | |
| | | 0.101 | 0.102 | 0.104 | 97 | | |
| | | 1.044 | 1.057 | 1.059 | 99 | | |
| Female | #6 | 1.066 | - | - | - | | |
| | | 1.204 | 0.102 | 1.168 | 103 | | |
| | | 2.155 | 1.057 | 2.123 | 102 | | |
| | #7 | 0.022 | - | - | - | | |
| | | 0.126 | 0.102 | 0.124 | 102 | | |
| | | 1.097 | 1.057 | 1.079 | 102 | | |
| | #8 | 0.863 | - | - | - | | |
| | | 0.974 | 0.102 | 0.965 | 101 | | |
| | | 1.950 | 1.057 | 1.920 | 102 | | |

Table 5

Recovery of vitellogenin spiked to mucus samples of untreated goldfish.

| Fish ID | Added ng/ml | Expected ng/ml | Recovery % | Mean (%) | SD (%) |
|---------|-------------|----------------|------------|----------|--------|
| #1 | - | 0.324 | 104 | 106.7 | 7.2 |
| | 0.106 | 1.374 | 117 | | |
| | 1.156 | - | - | | |
| #2 | - | 1.400 | 117 | | |
| | 0.106 | 2.450 | 110 | | |
| | 1.156 | - | - | | |
| #3 | - | 0.661 | 97 | | |
| | 0.106 | 1.711 | 103 | | |
| | 1.156 | - | - | | |
| #4 | - | 0.376 | 102 | | |
| | 0.106 | 1.426 | 104 | | |
| | 1.156 | - | - | | |

Table 6

Dilution linearity in mucus samples of untreated zebrafish

| Fish ID | Measured ng/ml | Dilution | Conc. ng/ml | Dilution recovery (%) |
|---------|----------------|----------|-------------|-----------------------|
| #1 | 0.089 | neat | 0.089 | - |
| | 0.044 | 1:2 | 0.088 | 99 |
| | 0.023 | 1:4 | 0.092 | 103 |
| #2 | >> Std A | neat | - | - |
| | 2.346 | 1:2 | 4.692 | - |
| | 1.273 | 1:4 | 5.092 | 109 |
| #3 | 0.255 | neat | 0.255 | - |
| | 0.130 | 1:2 | 0.260 | 102 |
| | 0.055 | 1:4 | 0.220 | 86 |

Table 7

Dilution linearity in mucus samples of untreated goldfish.

| Fish ID | Measured ng/ml | Dilution | Conc. ng/ml | Dilution recovery (%) |
|---------|----------------|----------|-------------|-----------------------|
| #1 | 1.733 | 1:3 | 5.199 | - |
| | 0.569 | 1:9 | 5.121 | 98 |
| | 0.196 | 1:27 | 5.292 | 102 |
| | 0.065 | 1:81 | 5.265 | 101 |
| #2 | 1.015 | 1:3 | 3.045 | - |
| | 0.336 | 1:9 | 3.024 | 99 |
| | 0.116 | 1:27 | 3.132 | 103 |
| | 0.043 | 1:81 | 3.483 | 114 |
| #3 | 3.070 | 1:3 | 9.210 | - |
| | 1.037 | 1:9 | 9.333 | 101 |
| | 0.362 | 1:27 | 9.774 | 106 |
| | 0.118 | 1:81 | 9.558 | 104 |
| #4 | 0.390 | 1:3 | 1.170 | - |
| | 0.130 | 1:9 | 1.170 | 100 |
| | 0.046 | 1:27 | 1.242 | 106 |
| | 0.016 | 1:81 | 1.296 | 111 |

TECO® Ultra Sensitive Cyprinid 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® Ultra Sensitive Cyprinid Vitellogenin

Quick Guide

Prepare Standards, Controls and Samples

- Dilute Wash Buffer concentrate 1:50 with distilled 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 into wells

Incubate plate for overnight (16 - 20 h) on a shaker (500rpm) at RT (20-28°C)

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

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

Incubate plate for 4 h ± 10 minutes on a shaker (500rpm) at RT (20-28°C)

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

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

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

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

Add **100 µL** TMB Substrate 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**