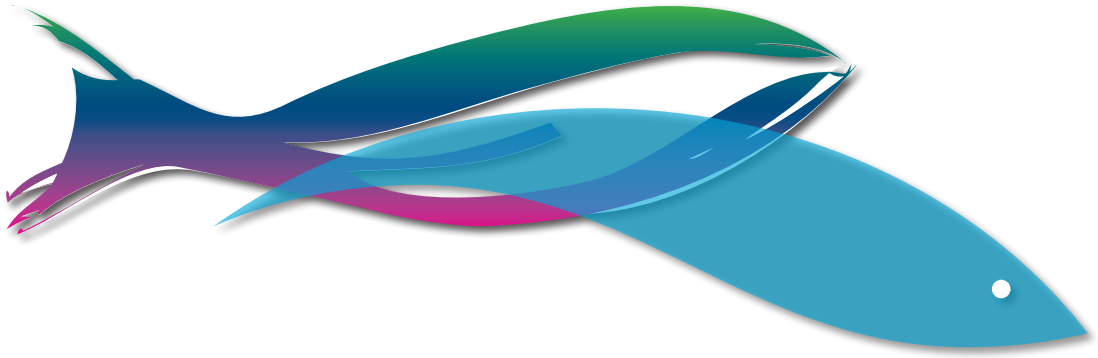
July 2015

New Generation of Vitellogenin ELISA

TECO® Cyprinid Vitellogenin ELISA
TECO® Perch (Perciformes) ELISA



Sensitive Vitellogenin Determination in Mucus, Serum and Whole Body Homogenate

- **Testing for endocrine disruptors according to OECD Guidelines**
- **Ecotox testing and monitoring**
- **Unique Mucus sample type: non-invasive, non-disruptive**
- **Two ELISA Kits for 8 Fish species**
- **For Research Use Only**

Introduction

Vitellogenin (vtg) is a precursor for the egg yolk proteins lipovitellin and phosvitin - its synthesis normally occurs only in sexually active females and therefore Vitellogenin is considered as a „female-specific protein“:

Environmental cues and endogenous factors trigger the release of the follicle-stimulating hormone by the pituitary gland, which induces the synthesis of 17ß-estradiol in the ovarian follicles. The 17ß-estradiol is then taken to the liver, where the vtg synthesis is activated. The protein is then transported back to the ovaries to mainly serve as the energy reserve of the developping embryos (6). The liver is considered to be the main production site of vtg.

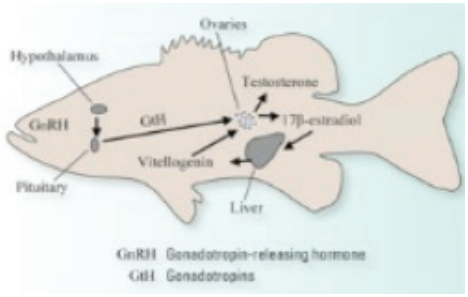


Figure 1

Endocrine system control of vtg induction in female fish

(http://mn.water.usgs.gov/projects/CED/summary.html)

The presence of vtg in blood and liver of male and immature fishes is widely used as an indicator of endocrine disruption, since the production is dependent on the presence of estrogen effective substances which should not occur in those organisms under normal circumstances. Therefore vtg is regarded as a reliable biomarker of exposure to estrogenic pollutants in both for in vitro (e.g., hepatocyte cultures) and in vivo studies.

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 vtg 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]. Even though the vtg concentration in the skin mucus is an order of magnitude lower than in blood serum or in body homogenates, the skin mucosa is very well suited as a matrix to determine exogenous vtg induction caused by environmental chemicals with affinity to estrogen receptors.

The TECO®Cyprinid and Perch (Perciformes) Vitellogenin ELISA are designed to measure vtg in serum and whole body homogenate (wbh). In combination with an unique sampling and extraction system (TECO®Mucus Collection Set; patent pending) the determination of vtg in epidermal mucosa has become a simple routine method.

Sample Collection

Blood

For blood sampling the anaesthetized fish are killed by cervical dislocation. The dislocating cut should cause a damage of the blood vessel located ventral of the vertebrate column. The blood entering the wound is taken up with a pipette in 5-10µL steps (depending on the size of the fish) and transferred to a centrifuge tube containing 50µL 50mM Tris/HCl (pH 7,4). The sample vial is then centrifuged with 15000g for 2 min at 4 °C. The supernatant is transferred into a fresh vial and stored below -20 °C.
TECO Vitellogenin ELISA: 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.

Homogenates

Head and tail homogenates are prepared in compliance with OECD 230. The anaesthetized fish are killed by a cervical dislocating cut. Head and tail of each individual were weighed separately in an Eppendorf vial. After the addition of ice cold 50 mM Tris/HCl (pH 7,4) in a ratio of 4µL/mg the sample is homogenized with a plastic pestle. The samples are centrifuged at 15000 g for 30 min (4°C). The supernatant is then transferred into a fresh vial and stored below -20°C.
TECO Vitellogenin ELISA: Recommended sample thawing: A simple and fast method is to place the frozen homogenate samples in normal tap cold water (15- 20°C). They should be thawed within 10 to 15 minutes.

Mucus

Use the specificly designed and validated TECO®Mucus Collection Set (TE1034) for collection and extraction of mucus samples (Patent PCT/DE2014/100161 pending).

- Prepare one collection vial per sample
- Place the fish carefully in position avoiding skin injuries.
- Rub the swab gently along the body from head to tail (see Fig.2a -c) just above the lateral line; turn the swab while taking the sample.
- Place swab into the prepared collection vial (Fig.3a) and break the shaft at the breaking point (Fig.3c).
- Close the collection vial and store sample at -20°C until analysis

Figure 2a-c

Mucus sampling procedure for the TECO® Perch (Perciformes) Vitellogenin ELISA Kit and for the TECO®Cyprinid Vitellogenin ELISA Kit.

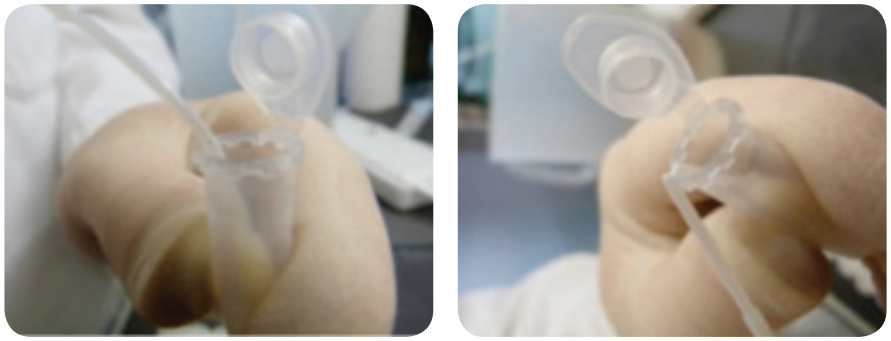


Figure 3a-b

Transfer of mucus swab into collection vial.

Mucus samples from Bluegill (Lepomis macrochirus)
After addition of Extraction Buffer, samples may be stored for at least:
- 4 days at room temperature
- 1 week at 4°C
- Avoid repeated freeze/thaw cycles.

Mucus samples from Cyprinids
After addition of Extraction Buffer, samples are stable up to 4 hours at room temperature.

Stability of mucus vitellogenin may differ significantly between species.

Benefits of Vitellogenin determination in epidermal mucosa

The determination of vtg in epidermal mucosa has important advantages:

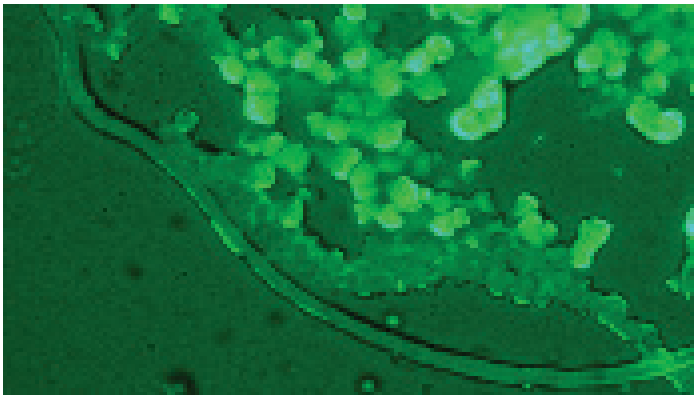
- Simple and highly standardized sampling technique and sample preparation.
- Strictly defined matrix without protease contamination caused by non-target tissues or lymphatic fluid.
- Non-invasive and non-destructive sampling method - easy to perform also in non- anaesthetized fish under field conditions.
- Non-destructive and thereby allowing several subsequent samplings in order to record a kinetic of vtg induction with a maximum known to appear after 7 days of exposure. Therefore Mucosa test are fully 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 vtg producing fish cell lines.
- Repeated sampling possible without affecting population in e.g. ecotoxicological studies.
- Determination of sex and status of sexual maturation.
- New tool to establish vtg profiles also in individual fish.
- A minimum of 2 samples per fish (from each side) can be taken.
- Additional sample type to serum and wbh.
- Mucus-containing swabs can be stored several months at -20°C.

Due to the non-invasive and non-destructive character of sampling, the vtg determination in mucus has clear advantages in terms of animal welfare and also the potential to reduce the numbers of fish used in OECD and experimental testing.

TECO®Cyprinid Vitellogenin ELISA

The TECO® Cyprinid Vitellogenin ELISA is a sensitive enzyme immune-sorbent assay for the quantitative determination of vtg in Carp (Carinus caprio), Goldfish (Carassius auratus), Zebrafish (Danio rerio), Medaka, Japanese rice fish (Oryzias latipes), Fathead Minnow (Pimephales promelas) and Roach (Rutilus rutilus).
Validated sample types are serum, whole body homogenate (wbh) and epidermal mucus.

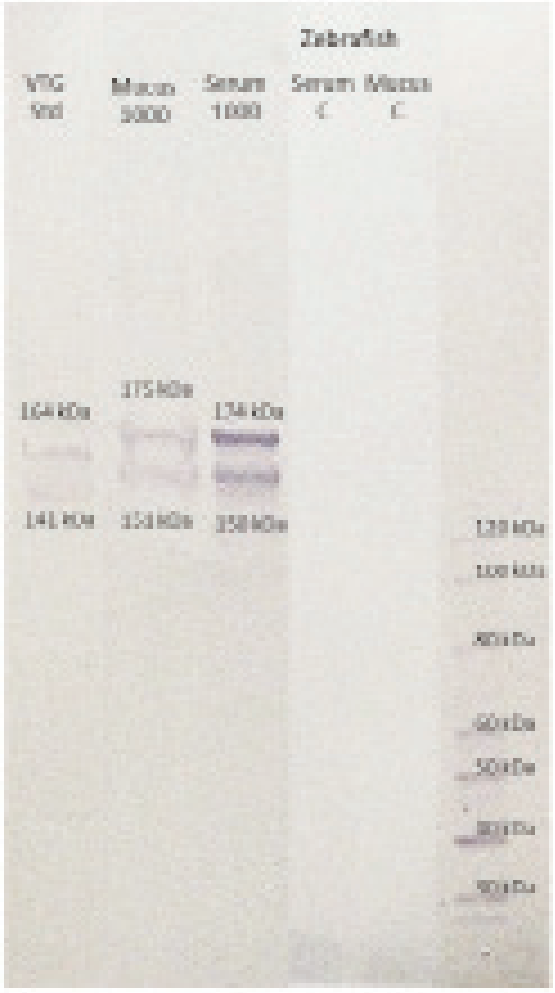
The antibodies used in this ELISA were also used in immune histological staining and in a western blot (see Picture 1 and 2).



Picture 1

Ovulated carp egg: Immune histological staining of vtg

Serum and mucus from male Zebrafish as well as the assay standard were run in a Western Blot - samples of untreated animals (Serum C; Mucus C) show no visible antibody-binding, whereas the samples (day 7) of treated animals (1000µg/L Bisphenol A) show the vtg characteristic double binding (around 170 kDA), which correspond well with the standard material used in the assay.



Picture 2

Western Blot of treated (1000µg/L Bisphenol A; day 7)) and untreated male Zebrafish using the antibody used in the assay.

Performance data

The performance data of the Cyprinid Vitellogenin ELISA (Table 1) show high sensitivities and very low inter- and intraassay variations.

Performance Characteristics	Results
Standard Range in undiluted samples	0.4 ng/mL - 35 ng/mL
LLOQ	< 0.4 ng/mL
LLD	< 0.1 ng/mL
Intra-Assay CV at 2.1 ng/mL	3.6%
Intra-Assay CV at 17.1 ng/mL	2.6%
Inter-Assay CV at 2.1 ng/mL	6.1%
Inter-Assay CV at 16.9 ng/mL	3.0%

Table 1

Performance characteristics of the TECO® Cyprinid Vitellogenin ELISA

Cyprinids were treated with 1 µg/mL estradiol and showed a clear vtg response on day 4. Dilution linearity studies were done in the elevated samples from day 4. Recoveries were between 92% and 110% with one outlier of 75% at a very low concentration level (Table 2).

	Day 0				Day 4				
Species	Sample	Measured ng/ml	Dilution	Concentration ng/ml	Sample	Measured ng/ml	Dilution	Concentration ng/ml	Dilution recovery (%)
Fathead minnow	1	0,08	1	0,08	16	0,15	10	1,5	
	2	0,09	1	0,09		0,03	100	2,6	n/a
	3	2,20	1	2,20	17	45,07	10	450,7	
	4	0,08	1	0,08		4,44	100	443,5	98
	5	0,20	1	0,20	18	15,65	10	156,5	
	20	0,28	1	0,30		1,49	100	149,2	95
					19	44,12	10	441,1	
						4,48	100	447,5	101
					20.1	2,35	10	23,5	
						0,26	100	25,8	110
	Day 0				Day 4				
Species	Sample	Measured ng/ml	Dilution	Concentration ng/ml	Sample	Measured ng/ml	Dilution	Concentration ng/ml	Dilution recovery (%)
Zebrafish	6	0,01	1	0,01	21	16,44	10	164,4	
	7	0,01	1	0,01		1,77	100	177,4	108
	8	0,01	1	0,01	22	11,58	10	115,8	
	9	0,01	1	0,01		1,23	100	122,6	106
	10	0,01	1	0,01	23	9,56	10	95,6	
						0,98	100	98,1	103
					24	6,21	10	62,1	
						0,64	100	64,2	103
					25	3,56	10	35,6	
						0,35	100	34,6	97
	Day 0				Day 4				
Species	Sample	Measured ng/ml	Dilution	Concentration ng/ml	Sample	Measured ng/ml	Dilution	Concentration ng/ml	Dilution recovery (%)
Goldfish	31	0,51	1	0,51	36	10,19	100	1018,8	
	32	0,08	1	0,08		1,06	1000	1054,9	104
	33	0,16	1	0,16	37	6,41	100	641,1	
	34	0,90	1	0,90		0,99	1000	993,0	92
					38	1,08	100	108,1	
						0,08	1000	80,6	75
					39	11,53	100	1153,4	
						1,14	1000	1141,5	99

Table 2

Treatment effect on mucus vtg and dilution linearity before (day 0) and after (day 4) estradiol treatment (1 µg/l) in cyprinid species

Mean recovery of vtg spiked to mucus samples of untreated cyprinids varied between 103-112% (Table 3).

Species	Sample	Before addition ng/ml	Added ng/ml	Expected ng/ml	Measured ng/ml	Recovery %	Mean %	SD %
Fathead minnow	1	0,1	6,1	6,2	7,2	116	112	3,3
	2	0,1	6,1	6,2	7,0	113		
	3	1,8	6,1	7,9	8,8	111		
	4	0,0	6,1	6,1	6,6	108		
Zebrafish	6	0,0	6,1	6,1	6,8	111	110	4,0
	7	0,0	6,1	6,1	7,0	115		
	8	0,0	6,1	6,1	6,5	107		
	9	0,0	6,1	6,1	6,5	107		
Medaka	11	0,0	6,1	6,1	6,6	108	103	5,7
	12	0,0	6,1	6,1	6,6	108		
	13	0,1	6,1	6,2	6,1	98		
	14	0,1	6,1	6,2	6,1	98		
Goldfish	31	0,3	6,1	6,4	6,4	100	111	11,5
	32	0,0	2,8	2,8	3,3	118		
	33	0,1	2,8	2,9	3,6	124		
	34	0,2	2,8	3,0	3,1	103		

Table 3

Recovery of vtg spiked to mucus samples of untreated cyprinids

Vtg Measurements using TECO® Cyprinide Vitellogenin ELISA

Vtg levels were measured in homogenate of head and tail, serum and mucus in untreated Zebrafish using the TECO® Cyprinid Vitellogenin ELISA. The vtg concentration level for the homogenates and serum are in the range of or above µg/ml, while the vtg concentration in mucus is in the range of ng/ml (Fig.4). All samples show a clear discrimination between male and female fish.

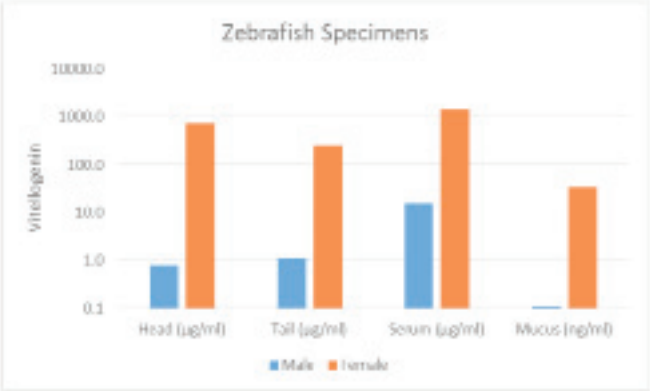


Figure 4

Vtg in different tissues, serum and mucus of a male and a female zebrafish (TECO® Cyprinid Vitellogenin ELISA). Tissue extracts were obtained according to OECD guidelines.

Exposure to estrogens and xenoestrogens will induce a vtg response in male fish. In Fig.5 the response of four different Cyprinids to an exposure of 500 ng/L estradiol at day 4 is shown.

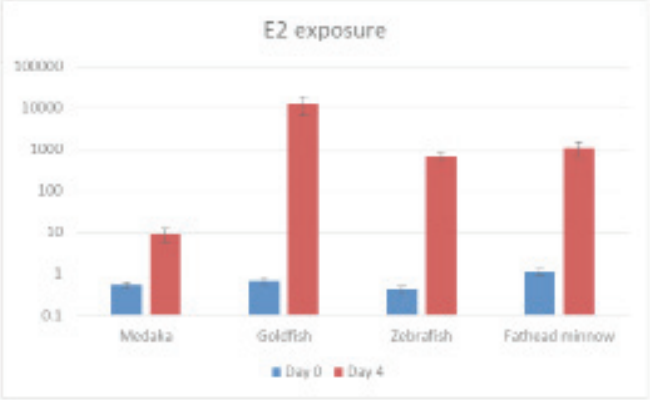


Figure 5

Estrogen sensitivity (Exposure to 500 ng/L) in Cyprinids; Mucus sampling at day 0 and day 4 (TECO® Cyprinid Vitellogenin ELISA).

Exposure of male Zebrafish, Fathead Minnow and Goldfish to bisphenol A at different concentrations, resulted in a vtg rise both in serum and mucus (Fig.6, Fig.7; Fig.8)

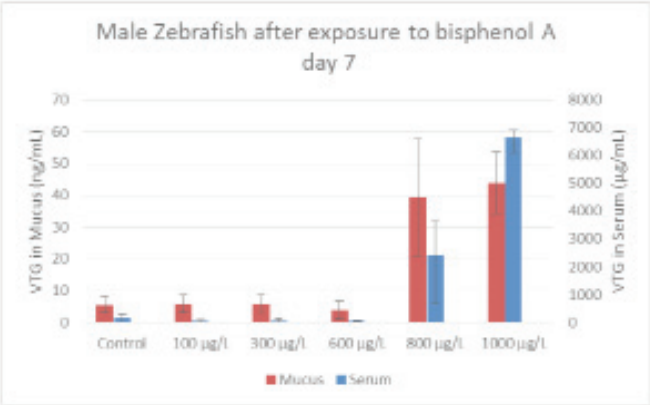


Figure 6

Vtg response in serum and mucus after exposure to BPA in male Zebrafish at day 7 (TECO® Cyprinid Vitellogenin ELISA).

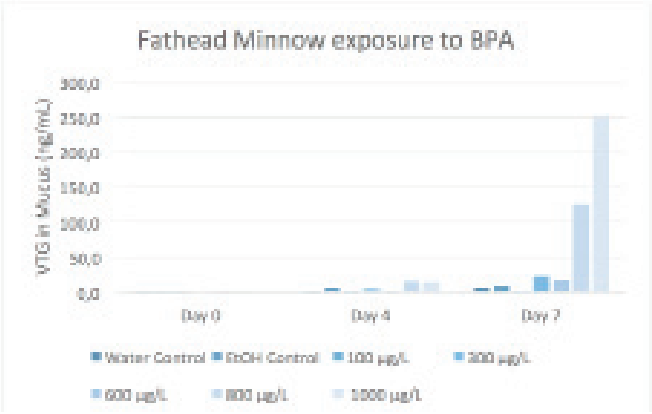


Figure 7

Vtg response in mucus after exposure to BPA in Fathead Minnow

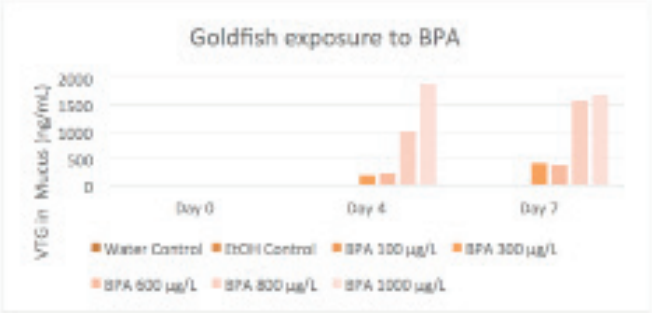


Figure 8

Vtg response in mucus after exposure to BPA in Goldfish

The data demonstrate, that the vtg patterns are similar in wbh, serum and mucus. The TECO® Cyprinid Vitellogenin ELISA's sensitivity allows to discriminate female from male Zebrafish using vtg in mucus. Also the expected vtg rise after exposure to estrogens and xenoestrogens is demonstrated in serum and mucus samples of different fish species.

In order to show the variation of vtg determination in mucus under routine conditions, mucosa sampling was performed using one swab for each body side at day 0, 4, 7 from Goldfish under BPA exposure. The samples were measured independently in two different laboratories.
The results from the different sampling side on the fish and the different laboratories are nearly identical (Fig.9).

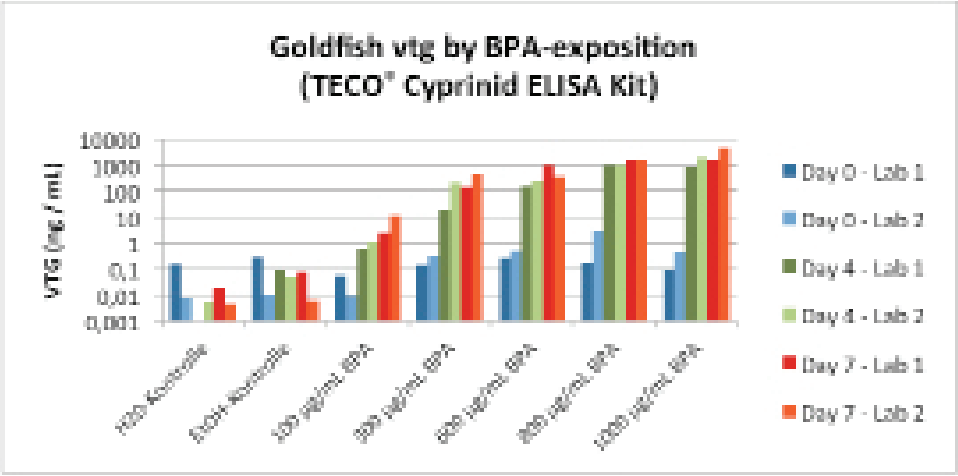
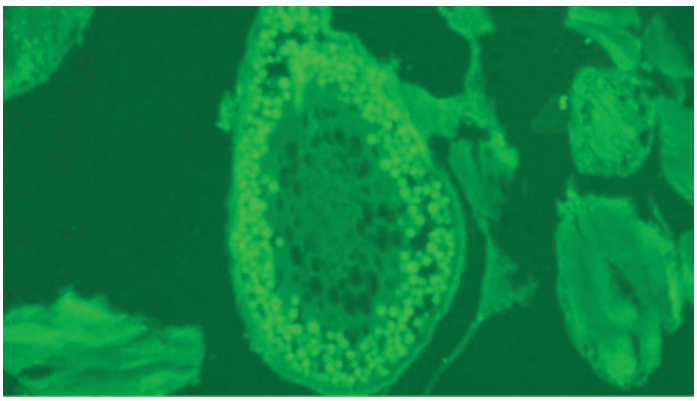


Figure 9

Vtg values from Goldfish measured for samples of the same individuals in the Gobio and TECOmedical laboratories (each lab measuring the swapping sample of a different side of each specimen), with 5 specimens per test concentration

TECO®Perch (Perciformes) Vitellogenin ELISA

The Perch Vitellogenin ELISA is a sensitive enzyme immune-sorbent assay for the quantitative determination of vtg in Bluegill (Lepomis macrochirus) and European Perch (Perca fluviatilis).
Validated sample types are serum and epidermal mucus.
The antibody used in this ELISA was also used in an immune histological staining of vtg in an oocyte (see Picture 3).



Picture 3

Oocyte of L.macrochirus: Immune histological staining of vtg

Performance data

The performance data of the TECO® Perch Vitellogenin ELISA show high sensitivities and very low inter- and intraassay variations.

The performance data of the are summarized below (table 4).

Performance Characteristics	Results
Standard Range in undiluted samples	1 ng - 80 ng/mL
LLOQ	<1 ng/mL
LLD	< 0.5 ng/mL
Intra-Assay CV at 1.3 ng/mL	4.6%
Intra-Assay CV at 2.9 ng/mL	5.2%
Intra-Assay CV at 11.9 ng/mL	2.6%
Intra-Assay CV at 16.2 ng/mL	1.1%
Inter-Assay CV at 7.1 ng/mL	7.3%
Inter-Assay CV at 34.9 ng/mL	6.2%

Table 4

Performance characteristics of the TECO® Perch Vitellogenin ELISA

The dilution recovery and spiking recovery varied between 86% and 102% (table 5) and 97% and 120% respectively (table 6).

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 5

Dilution Linearity (pooled mucus plus standard vtg)

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 6

Recovery (pooled mucus plus standard vtg)

Vtg Measurements using TECO® Perch Vitellogenin ELISA

The vtg increasing effect of estradiol and estrogen exposure in serum and mucus are shown in Figure 10 and Figure 11.

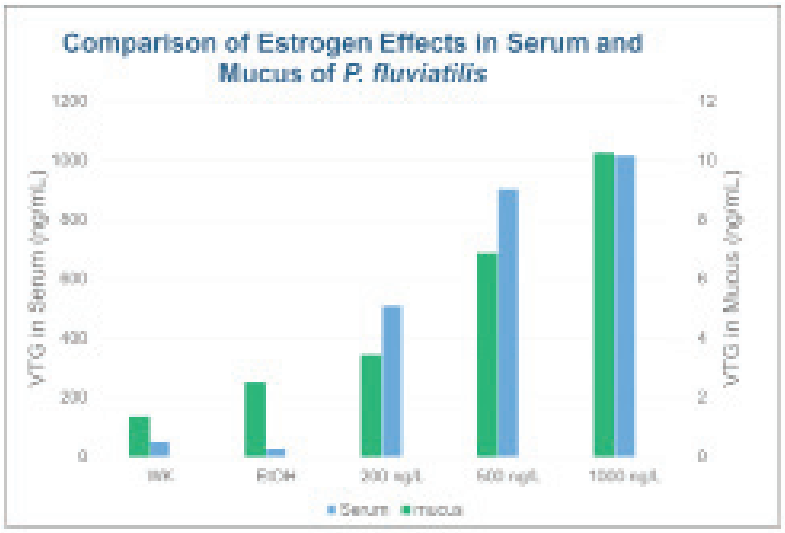


Figure 10

Dose-depending induction of vtg in serum and mucosa by exposure of P.fluviatilis to different concentrations of 17-ß estradiol after six days.

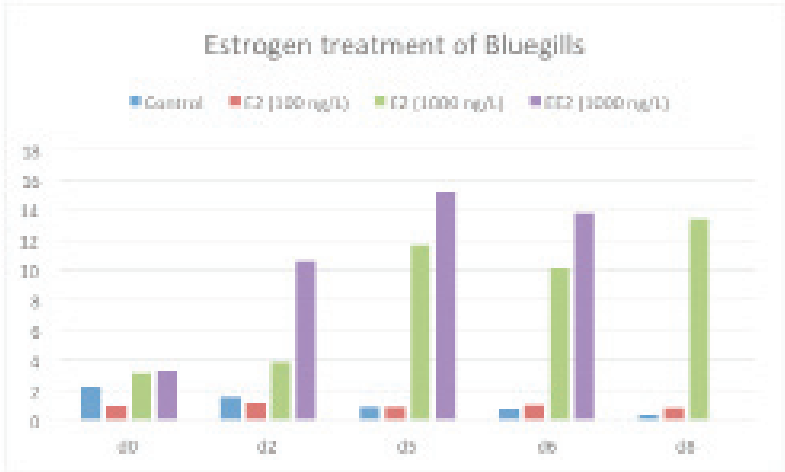


Figure 11

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

Three groups of five fish (L. macrochirus) were exposed for 28 days under semi-static conditions to 0, 600 and 1000 ng/L bisphenol A (BPA). Mucosa sampling was 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. Since the fish were adapted to exposure conditions according to OECD guidelines initial vtg values are considered to be unaffected by pre- exposure rearing conditions (Figure 12). The results from the different sampling side on the fish and the different laboratories are nearly identical.
Vtg values in the epidermal mucosa of samples increased from 0.2 to 11 ng/mL, with highest values obtained in day 7 samples for both concentrations, pointing to the acclimatization (i.e. of the estrogen receptors in epidermal cells) to waterborne BPA.

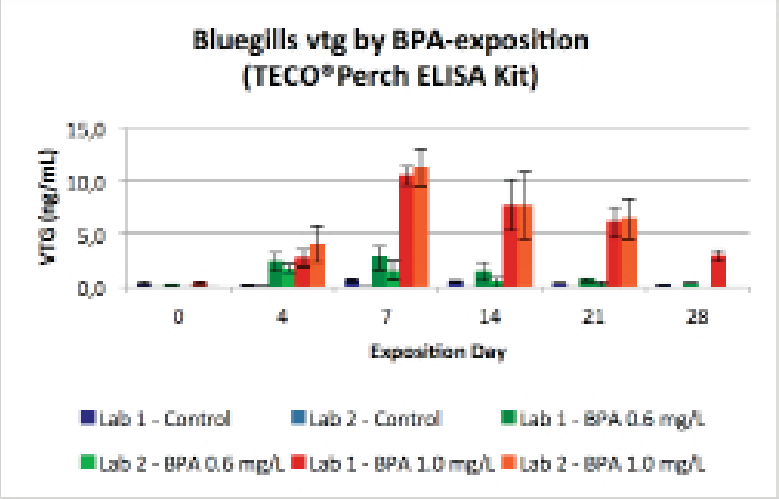


Figure 12

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

Estradiol induced VG concentrations in Perch epidermal mucosa are about one order of magnitude lower than in blood, but they are comparable in terms of dose dependency and time course.

Summary

The TECO® Perch and Cyprinid Vitellogenin ELISA tests cover a broad genetical range of fish species and are the first ELISA tests validated in wbh, serum and mucus. Wbh is a very difficult to standardize, a labor-and cost- intensive method; collecting blood samples from fish need experiences, but is a less labor-intensive method. Both sample types are invasive, destructive methods and routinely used in vtg endpoint determination during testing for endocrine disruptors. The TECO Cyprinid Vitellogenin ELISA is very sensitive and allows even in non-treated Zebrafish the vtg determination in serum and mucus. Labor and cost intensive vtg determination in wbh is thererfore not needed any more using this ELISA kit.
As the vtg concentrations in epidermal mucosa are about one order of magnitude lower than in blood, the accurate determination of vtg in mucus requires very sensitive test systems like the TECO® Perch and Cyprinid Vitellogenin ELISA. In addition to that the TECO® Mucus Collection Set is necessary for sampling using validated swabs and contains a specially designed extraction buffer, which allow a better sample stability compared to blood and homogenate.
Taking mucosa samples from fish is a non-invasive, non-destructive method - fish even do not need to be anaesthetized. Therefore sampling is a simple, fast and highly standardized procedure which can be easily performed under field conditions. In addition mucosa is a strictly defined matrix without protease contamination caused by non-target tissues or lymphatic fluid.
The vtg results in different sample material of various non-exposed and exposed (estrogens, xenoestrogens) fish are similar and therefore "sample type"-independend.

The TECO® Perch and Cyprinid Vitellogenin ELISA provide similar results in vtg determination independently from the sample type (wbh, serum and epidermal mucus) - due to the non-invasive, non-destructive character mucosa samples should be the sample type of choice from practical, but also from ethical point of view.

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TECO® Mucus Collection Set

Cat. No. TE1034 CE
Tests 42 breakable collection Swabs, sample tubes, tube rack and extraction Buffer

TECO® Perch (Perciformes) Vitellogenin ELISA

Cat. No. TE1035 CE
Tests 96 Wells For Mucus and Serum

TECO® Cyprinid Vitellogenin ELISA

Cat. No. TE1037 CE
Tests 96 Wells For Mucus, Serum and Whole Body Homogenate

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