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Water quality — Biochemical and physiological measurements on fish —

Part 3: Determination of vitellogenin

Qualité de l'eau — Mesurages biochimiques et physiologiques sur poisson —

Partie 3: Dosage de la vitellogénine

Reference number
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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 23893-3 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

ISO 23893 consists of the following parts, under the general title *Water quality — Biochemical and physiological measurements on fish*:

— *Part 1: Sampling of fish, handling and preservation of samples*

— *Part 2: Determination of ethoxyresorufin*-O-*deethylase (EROD)* [Technical Specification]

— *Part 3: Determination of vitellogenin*

Introduction

Vitellogenin (Vtg) is a large phospholipoglycoprotein produced as the yolk protein precursor in the liver of oviparous vertebrates, such as fish. Vtg is secreted from hepatocytes through the secretory pathway, enters the circulation and is taken up by the growing oocyte. Plasma Vtg concentrations are normally an indication of the maturational status of the female fish, for reviews see References [2] [18]. More than a decade ago, several studies demonstrated that male fish caught in rivers and streams had high concentrations of plasma Vtg (e.g. References [14][23]), caused by chemicals acting like oestrogens present in the environment. Since then, numerous studies have shown the fish Vtg to be a highly responsive biomarker for oestrogenic compounds in both *in vitro* hepatocyte cell cultures, *in vivo* aquaria studies, and field studies, for reviews see References [1][2][10][13][16][20][26]. Hence, Vtg in fish has become an accepted biomarker of xenooestrogenic and antioestrogenic exposure of chemicals, effluents, and discharges, and has been proposed in chemical testing, as well as environmental monitoring programmes, e.g. Reference [13].

However, recent genetic and immunological analyses have demonstrated a general multiplicity of Vtg forms in fish, References [9][10]. The concentrations of circulating Vtg proteins (or Vtg gene transcripts) during oogenesis and their degree of induction by oestrogens appear to vary among species and among different types of Vtg within a species. The kinetics of induction of distinct types of Vtg by oestrogens in fish appears to depend on environmental factors (e.g. water temperature and photoperiod), life history stage, sex, and the concentration and type of oestrogenic compound. Based on these findings, it is important that the Vtg targets in a bioassay for oestrogens in a specific species be demonstrated to be an oestrogen-responsive form, and that the assay be validated with the species in question before embarking on a monitoring programme, Reference [10].

The scientific literature contains a multitude of publications on procedures for determining Vtg in fish samples, using immunoassays. Whereas radioimmunoassays (RIA) were a predominant method in the 1980s and early 1990s, e.g. References [4][29], enzyme-linked immunosorbent assays (ELISAs) are the dominating principle today. Both the sandwich and competitive ELISA principles provide sensitive results without the use of radioactive isotopes, and have been successfully applied to determine Vtg levels in several fish species, e.g. References [3][6][8][12][15][17][19][21][22][24][25][27][28][31][32].

This part of ISO 23893 presents a generalized protocol for both the sandwich and competitive ELISA for use in quantification of Vtg in fish blood plasma samples. The application of standardized methods is strongly advised within monitoring programmes.

Water quality — Biochemical and physiological measurements on fish —

Part 3: Determination of vitellogenin

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably qualified staff.

1 Scope

This part of ISO 23893 specifies a method for measuring vitellogenin (Vtg) concentrations in a fish plasma sample employing an enzyme-linked immunosorbent assay (ELISA) method.

It applies to fish that are sampled in the environment (fresh, estuarine or salt water) and to fish exposed to substances or effluents in a laboratory. The method is quantitative when using Vtg antibodies and a Vtg standard well characterized with the species of choice.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 23893-1, *Water quality — Biochemical and physiological measurements on fish — Part 1: Sampling of fish, handling and preservation of samples*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
limit of detection
LOD
lowest content that can be measured with reasonable statistical certainty

EXAMPLE The LOD is often expressed as the reagent blank value plus three times its standard deviation.

Note 1 to entry: The method LOD is determined by taking the sample dilution factor into the calculation.

3.2
limit of quantification
LOQ
content equal to or greater than the lowest concentration point in the calibration curve

EXAMPLE The LOQ is often expressed as the reagent blank value plus 10 times its standard deviation (Reference [11]).

Note 1 to entry: LOQ is also determined by taking the sample dilution factor into the calculation.

3.3
matrix blank
representative sample that does not contain detectable levels of analyte

Note 1 to entry: For the purposes of this part of ISO 23893, the analyte is vitellogenin.

3.4
selectivity
ability to measure accurately the analyte in the presence of components that can be expected to be present in the matrix

Note 1 to entry: For the purposes of this part of ISO 23893, the analyte is vitellogenin and the matrix is plasma.

Note 2 to entry: Selectivity is demonstrated by using "matrix blanks".

4 Principle

Samples of fish blood plasma are collected essentially as specified in ISO 23893-1; however, with addition of a protease inhibitor (see 7.13 and 9.2). Vitellogenin is determined in the sample by an antibody-based immunoassay, using either of two established variants.

In the first, so-called sandwich ELISA, the blood plasma sample is allowed to react with a capture antibody specific for Vtg (from the same or a closely related species), in a microtitre plate well coated with the antibody. An enzyme-labelled detecting Vtg antibody is then used to produce an antibody "sandwich" that can be detected based on a chromogenic substrate for the enzyme label (e.g. horseradish peroxidase). A secondary enzyme-labelled antibody can also be used to develop the assay, if the detecting antibody is unlabelled. A standard series based on a purified reference material (Vtg protein from the same or closely related species) is used to develop a quantitative relationship between sample signal and standard amount.

The second variant is the competitive ELISA technique, where sample Vtg competes with purified Vtg coated to the microtitre plate walls for binding to a (labelled or unlabelled) Vtg antibody in solution. Development of assay signal follows the same principle as in the sandwich variant, although the standard series produces an inverse relationship with signal intensity.

Only Vtg antibodies or assays that have been demonstrated to perform according to specified performance criteria with the fish species studied should be used in this protocol.

5 Minimum performance criteria

The criteria listed below should be regarded as the minimal acceptable performance as defined from a user standpoint on the purpose of performing Vtg analysis. Specific performance criteria need to be established for each specific assay to be used in the study based on in-house (within laboratory) performance.

Selectivity:	Matrix blank <LOD (with the necessary dilution factor to avoid matrix effects)
Calibration:	Standard curve working range >10-fold, preferably 50-fold to 100-fold to be practical with the dynamic range found in Vtg concentrations
Recovery:	>50 %

NOTE The characterization of the "matrix effect" is an important challenge in this regard. It can be difficult to ensure that a "matrix blank" sample is really devoid of any Vtg.

6 Test environment

All handling operations of plasma samples and standards including the measurement shall be carried out at a temperature of (4 ± 2) °C or on crushed ice, except where indicated in the test procedure.

7 Reagents

Unless otherwise specified, use only reagents of recognized analytical grade.

7.1 Sulfuric acid, 0,3 mol/l or 1,5 mol/l, stop solution.

7.2 Crushed ice.

7.3 Coating buffer, 50 mmol/l carbonate–bicarbonate, pH 9,6.

7.4 Washing buffer, phosphate-buffered saline (PBS), pH 7,3, containing 0,5 g/l polysorbate 20 detergent.

7.5 Blocking buffer, washing buffer containing 10 g/l bovine serum albumin (BSA).

7.6 Dilution buffer, 10 g/l BSA in PBS.

7.7 Substrate buffer, phosphate–citric acid buffer, pH 5,0.

7.8 Vtg reference sample.[1)]

7.9 Capture antibody, monoclonal or polyclonal anti-Vtg.[1)]

7.10 Detecting antibody, monoclonal or polyclonal anti-Vtg,[1)] unconjugated or conjugated to horseradish peroxidase, HRP. In the alternative where the detecting antibody is not conjugated, the detecting antibody shall be harvested from a different species than the capture antibody.

7.11 Secondary antibody,[2)] antibody to Fc (Fragment crystallizable) part of detecting antibody, conjugated to HRP.

7.12 Peroxidase substrate, tetramethylbenzidine (TMB), or *ortho*-phenylenediamine (OPD) + H_2O_2.

7.13 Protease inhibitor, such as aprotinin.

8 Apparatus

8.1 96-Well microtitre plates, clear, flat-bottomed, absorbing.

8.2 96-Well microtitre plates, clear, flat-bottomed, non-absorbing, for the competitive ELISA variant.

8.3 Microplate sealing film.

8.4 Microplate reader, wavelength 450 nm or 490 nm, depending on substrate used.

8.5 Pipettes, with disposable tips 5 µl to 1 000 µl.

8.6 Multi-channel pipette and reagent reservoir. Alternatively, a stepper pipette with disposable tips (100 µl) can be used.

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1) Vtg reference samples, monoclonal or polyclonal antibodies to fish Vtgs, and complete assay kits (Vtg ELISA kits) are available commercially.

2) Enzyme-labelled secondary antibodies are available commercially.

8.7 Test tubes, 1 ml to 50 ml.

8.8 Microplate washing device. An automatic or manual plate washer is recommended, but a squeeze bottle or a multichannel/stepper pipette can also be used.

8.9 Vortexer.

9 Sampling procedure

9.1 Sampling of fish

Sampling should be carried out in the natural environment by fishing or in a laboratory on fish exposed to substances or effluents as specified in ISO 23893–1. Sample a minimum of 10 fish of the same species and sex and of uniform size from each group to be examined for Vtg concentrations. Do not take samples during the spawning season because the behaviour and the physiological activities of the fish can be modified by sexual activity (unless these aspects are part of the study design).

Taking into account the factors likely to influence Vtg concentrations, the following conditions shall be determined and recorded in the test report:

a) water temperature;

b) date;

c) time of day;

d) a general description of the health condition of each fish (sex, length, body mass, presence of external and internal injuries) — this is usually reported in connection with sampling as described in ISO 23893–1.

Depending on the objectives of the study, ensure that the control fish (from the reference location or laboratory group) are taken from an environment of good ecological quality. Handle the control fish and their samples in the same manner as those from the examined or experimentally treated groups, except for exposure to the substance(s) of concern.

9.2 Sampling of blood plasma

After fishing or on completion of exposure, the fish are killed and the blood sampled one by one on removal from the water, essentially as specified in ISO 23893–1. A volume of approximately 20 µl to 100 µl or more of blood should be sampled from each fish, as soon as possible after it has been killed. The blood sample is taken using a heparinized syringe ideally from the caudal vessels or by cardiac puncture. Blood samples shall be processed directly to produce blood plasma by centrifugation of vials at 3 000 × *g* for 10 min at 4 °C (or 7 000 × *g* for 3 min), and the resulting supernatant (plasma) collected and aliquoted. The plasma sample is immediately transferred to vials which have been coated with a protease inhibitor (e.g. aprotinin at 2 trypsin inhibitor units [TIU]/ml).

Alternative whole body homogenate (WBH) procedure (see note): Homogenize the liver or whole body in cold sample buffer (1 + 2 mass + volume; PBS, 10 g/l BSA with aprotinin at 2 TIU/ml) until the tissue is finely processed, e.g. using a glass hand-held homogenizer, volume 7 ml.

NOTE When using small fish species (e.g. medaka, zebrafish), or larvae or fry from larger fish, it can be impossible to obtain a sufficient volume of plasma to determine Vtg. In these cases, a liver or WBH can be prepared and used instead (see References [9][10]).

9.3 Storage of blood plasma samples

If the Vtg determination cannot take place on the day of sampling, the plasma samples shall be frozen immediately to below −70 °C, e.g. by using liquid nitrogen or dry ice. The samples can thereafter be stored for up to 12 months in liquid nitrogen or at a temperature below −70 °C.

If measurements of Vtg are to be initiated on the day of sampling, then the preparatory step shall be started within 1 h and the plasma samples shall be stored at or below 4 °C.

10 Analytical procedure

10.1 Preparation of the samples

Vtg is an unstable molecule, and all sample and standard dilutions should be prepared and kept on ice. Prior to conducting the determination, a dilution series of the samples need to be made. After thawing of samples and the Vtg standard on ice, at least three different dilutions of each sample and a twofold serial dilution of the standard in blocking/dilution buffer are prepared. The level of dilution of the samples should range from approximately 1→50 to 1→500 000, and the serial dilution of the standard should include 9 to 11 dilution steps.

10.2 Determination of vitellogenin

10.2.1 Calibration

Carry out a calibration using a purified Vtg reference sample. The reference sample is used to prepare a calibration curve against which the unknown samples are quantified. Vtg is an unstable molecule, and all sample and standard dilutions should be prepared and kept on ice. Reconstituted Vtg cannot be frozen and reused quantitatively at a later date. A dilution series (e.g. 2 ng/ml to 1 000 ng/ml) prepared from freshly reconstituted Vtg standard should be run in every assay.

10.2.2 Assay procedure 1 — sandwich ELISA

The described assay procedures 1 and 2 reflect the general principles of the ELISA method. According to the species and the origin of antibodies or standard used, the experimental conditions can vary, with special regard to incubation time, buffer composition, and dilution of samples and standard.

In the sandwich ELISA method,the wells of the microplates are precoated 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. The whole procedure takes 2 d to complete.

This procedure does not apply to kits. If commercially available kits are used, follow the manufacturer's instructions.

10.2.2.1 Precoating of absorbing plates

Unless plates precoated with appropriate Vtg antibody are purchased from a vendor, precoating shall be carried out prior to the day of the assay.

Dilute capture antibody in coating buffer to 10 µg/ml (12 ml required per plate).

Add 100 µl of this to all wells of all plates to be used.

Seal the plates with microplate sealing film to prevent evaporation and incubate at 4 °C overnight.

After the overnight incubation, wash the wells three times with 200 µl washing buffer per well.

Block non-specific binding-sites by adding 200 µl of dilution buffer to each well and leave for 1 h at 4 °C.

Empty the wells and place the plate upside down on tissue paper.

10.2.2.2 Incubation with standard and diluted samples

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 plate layout in Figure 1.

Add 100 µl dilution buffer to each of the two non-specific binding (NSB) wells. NSB wells should be included on every plate. These wells are used to determine non-specific binding (unspecific background signal).

Add in duplicate 100 µl of each Vtg standard dilution (S1 to S11).

Add in duplicate 100 µl of each sample dilution (P1 to P36).

Seal the plates and incubate at a room temperature of 20 °C to 25 °C for 1,5 h.

Ensure equal incubation times when running more than one plate, as the addition of standards and samples often take several minutes. If there is a substantial time difference between plates, they can be synchronized at this point: after 1,5 h 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.

	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	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

Key

NSB non-specific binding wells S1 to S11 standards 1 to 11 P1 to P36 samples

Figure 1 — Recommended plate layout for sandwich ELISA protocol

10.2.2.3 Incubation with detecting antibody

Dilute the detecting antibody to appropriate dilution in 12 ml dilution buffer for each plate to be used in the assay run.

Wash the plates three times with 300 µl washing buffer per well.

Add 100 µl of the diluted detecting antibody to all wells.

Seal the plates and incubate at a room temperature of 20 °C to 25 °C for 0,5 h.

10.2.2.4 Development

Wash the plates five times with 300 µl washing buffer per well.

Add 100 µl room temperated TMB substrate solution to all wells (TMB is light sensitive, and once prepared should be kept in the dark).

Incubate in the dark (cover the plates, e.g. with aluminium foil) at a room temperature of 20 °C to 25 °C for 20 min.

Stop the reaction by adding 100 µl 0,3 mol/l H_2SO_4 to all wells.

Read the absorbance at 450 nm with a microplate reader.

10.2.3 Assay procedure 2 — competitive ELISA

In the competitive ELISA, both plate coating and sample incubation shall be performed overnight. The whole procedure therefore takes 2 d to complete.

10.2.3.1 Precoating of plates

See suggested plate layout in Figure 2.

Prepare a solution of reference Vtg (or Vtg purified for coating purpose only) by diluting stock Vtg to 100 ng/ml in coating buffer. Approximately 10 ml should be prepared for each plate to be used in the assay.

Add 100 µl of this coating solution to each well of an absorbing microtitre plate.[3)] Leave two wells for addition of 100 µl coating buffer alone to serve as NSB wells on each plate.

Seal the coating plates with a microtitre plate sealing film to prevent evaporation, and incubate overnight at 4 °C.

10.2.3.2 Preparation of samples and standard

Dilute samples and standard (reference Vtg) in blocking buffer to concentrations appropriate to be measured in the assay.

Add 60 µl of each dilution in duplicate to the wells of a non-coated, non-absorbing microtitre plate.

Add 60 µl blocking buffer only to two wells for NSB, and two wells for maximum binding control.

Add 60 µl of an appropriate dilution of the detecting antibody in blocking buffer to all wells on the plate.

Mix plates gently on a plate shaker, cover with a sealing film, and incubate overnight at 4 °C.

10.2.3.3 Blocking of plates

After overnight incubation, wash the coating plates three times with washing buffer.

Without allowing the plates to dry out, add 150 µl of blocking buffer to each well and reseal the plates. Incubate the plates at 37 °C for 30 min.

Wash the coating plates again three times in washing buffer.

10.2.3.4 Sample incubation

After overnight incubation at 4 °C, incubate the sample plates for 30 min at 37 °C.

Transfer 100 µl of samples and standards to the emptied wells of the blocked coating plates. Ensure that NSB samples are transferred to the correct corresponding wells of the coating plates.

Reseal and incubate the plates at 37 °C for 1 h.

Wash the plates three times with washing buffer.

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3) Nunc Maxisorp is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

If unlabelled detecting antibody is used, add 125 µl of an appropriate dilution of labelled secondary antibody (e.g. goat anti-rabbit IgG-HRP) to each well.

Reseal the plates and incubate at 37 °C for 2 h.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	NSB	Max.	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	P23	P24	P25	P26	P27	P28	P29	P30	P31	P32	P33	P34
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

Key

NSB non-specific binding wells

Max maximum binding wells

S1 to S12 standards 1 to 12

P1 to P34 samples

Figure 2 — Recommended plate layout for competitive ELISA protocol

10.2.3.5 Development

Wash plates three times with washing buffer.

Add 125 µl substrate buffer containing OPD and H_2O_2, prepared immediately prior to use, to each well. Substrate buffer is light sensitive and once prepared shall be kept in the dark (alternatively, add 125 µl TMB substrate solution, which is a non-carcinogenic alternative to OPD).

Incubate the plates at room temperature in the dark until colour has developed (generally between 5 min and 15 min).

Stop the colour reaction by adding 30 µl 1,5mol/l H_2SO_4 to each well.

Read the absorbance of each well on a plate reader using a wavelength of 490 nm (450 nm for TMB).

10.2.4 Calculation of results — sandwich ELISA

10.2.4.1 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. The mean NSB value should not be higher than 0,2 absorbance units.

10.2.4.2 Preparation of the standard curve

Calculate the mean of the NSB-corrected absorbance values for each set of standard duplicates.

The absorbance units of the highest standard should be higher than 2,0.

The concentration difference should not be more than 15 % between two duplicate wells for any standard value.

Plot absorbance values against the Vtg concentration. Perform a regression analysis, using for example log-log (Figure A.1), linear (Figure A.2) or four -parameter (Figure A.3) transformation of the data.

Determine the working range of the standard curve. Data points with NSB-corrected absorbance values lower than 0,010 should not be included.

10.2.4.3 Calculation of Vtg concentration in the samples

Calculate the mean of the NSB-corrected absorbance values for each set of sample duplicates.

Calculate the Vtg concentration in the diluted sample using the equation for the adjusted standard curve determined above.

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.

The concentration difference should not be more than 15 % between two duplicate wells for a given sample. If so, the sample should be reassayed.

Only sample dilutions with absorbance values that fall within the standard curve working range should be used (see example in the following).

If all dilutions of a sample give absorbance values outside the working range, the sample should be reassayed at different dilutions.

If more than one dilution of a sample falls 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 dilutions is the most reliable. Samples having absorbance values close to the ends or plateaus of the standard curve should be used with care, as these parts of the standard curve are less reliable. Preferably, such samples should be reassayed with more dilutions.

10.2.5 Calculation of results — competitive ELISA

10.2.5.1 Calculation of relative maximum binding

Calculate the mean of all duplicate (or triplicate) wells for both samples, standards, NSB and maximum binding wells.

Then calculate the relative maximum binding using Formula (1):

$$\frac{B}{B_0} = \frac{\overline{A}_{\text{sample}} - \overline{A}_{\text{NSB}}}{\overline{A}_{B_0} - \overline{A}_{\text{NSB}}} \times 100\,\% \qquad (1)$$

where

$\overline{A}$ indicates mean optical density (absorbance) for the particular instance;

NSB is the non-specific binding;

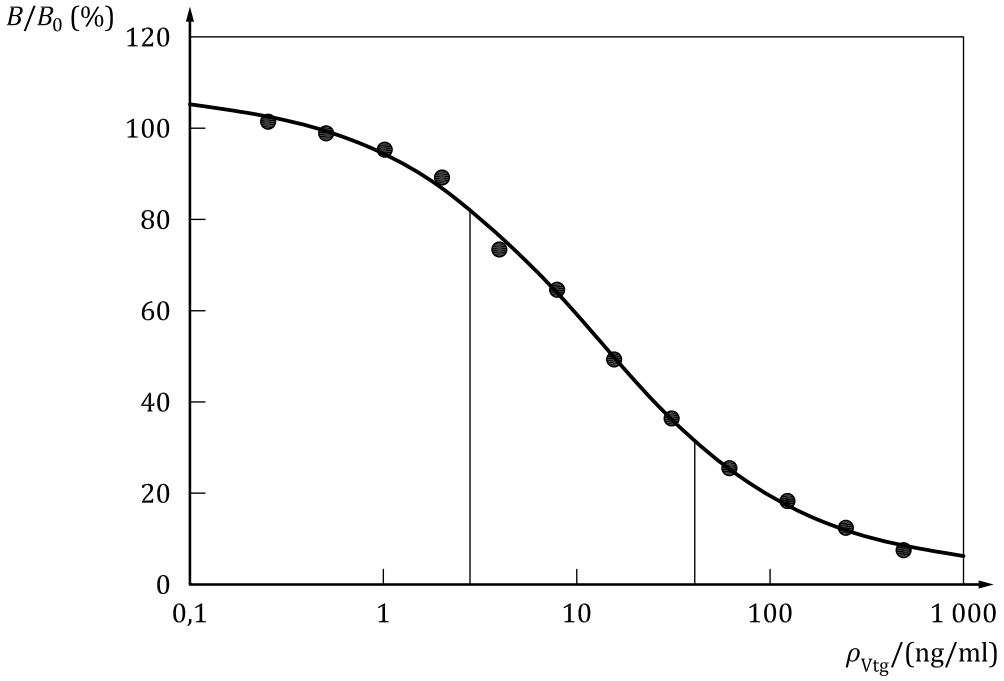
B_0 is the maximum binding.

10.2.5.2 Preparation of the standard curve

Prepare the standard curve by plotting the values of B/B_0 obtained for each of the standards against Vtg concentration (see Figure 3).

10.2.5.3 Calculation of Vtg concentrations in the samples

Using the standard curve and appropriate statistical software,[4)] the Vtg concentration in each plasma sample can be calculated. Select the dilution of sample giving values closest to 50 % binding, and within the 30 % to 80 % B/B_0 working range of the assay.



Key

B/B_0 relative maximum binding

ρ_{Vtg} Vtg concentration

The vertical lines indicate the approximate working range of the assay (30 % to 80 % binding, approximately 2 ng/l to 40 ng/l Vtg). Using the appropriate dilution, the B/B_0 for each sample should fall within this range, on the linear part of the standard curve.

Figure 3 — Typical competitive Vtg ELISA standard curve

11 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this part of ISO 23893 (ISO 23893-3:2013);

b) sampling date and time, water temperature, information about the fishing station (natural environment) or the batch of fish (laboratory), number of fish sampled (as specified in ISO 23893-1;

c) for each fish: length, body mass, sex, summary diagnosis of the health condition of the fish (as specified in ISO 23893-1;

d) for each fish, the Vtg concentration calculated according to 10.2.4.3 or 10.2.5;

e) summary tables or bar plots of Vtg concentrations, ρ_{Vtg}, in the different groups of fish ($\bar{\rho}_{Vtg} \pm s$, where s is standard deviation), indicating statistical differences calculated by appropriate methods;

f) results from internal standards (positive and negative controls, CRMs, if available);

4) Appropriate statistical software is commercially available.

g) all operating details not specified in this part of ISO 23893, or considered as optional, together with details of any incidents which may have influenced the results.

Annex A
(informative)

Examples of results: Fathead minnow sandwich ELISA

A.1 General

See Table A.1 and Figures A.1 to A.3, source: Reference [33].

A.2 Single-lab validation — Fathead minnow Vtg sandwich ELISA

A single-lab (in-house) validation was performed based on international guidelines, see References [5] [11]. Vtg concentrations were measured in sample blanks (dilution buffer, plasma or WBHs) as well as in buffer, plasma or WBH spiked with Vtg levels of 0,5 ng/ml, 2,5 ng/ml, and 12,5 ng/ml. The samples were aliquoted and frozen at −80 °C, and on five successive days, three aliquots of each sample were thawed and analysed independently (n = 15 independent analyses). All analyses were performed in triplicate (three ELISA wells per standard or sample dilution). In addition,, some freshly spiked samples were analysed (selectivity, recovery).

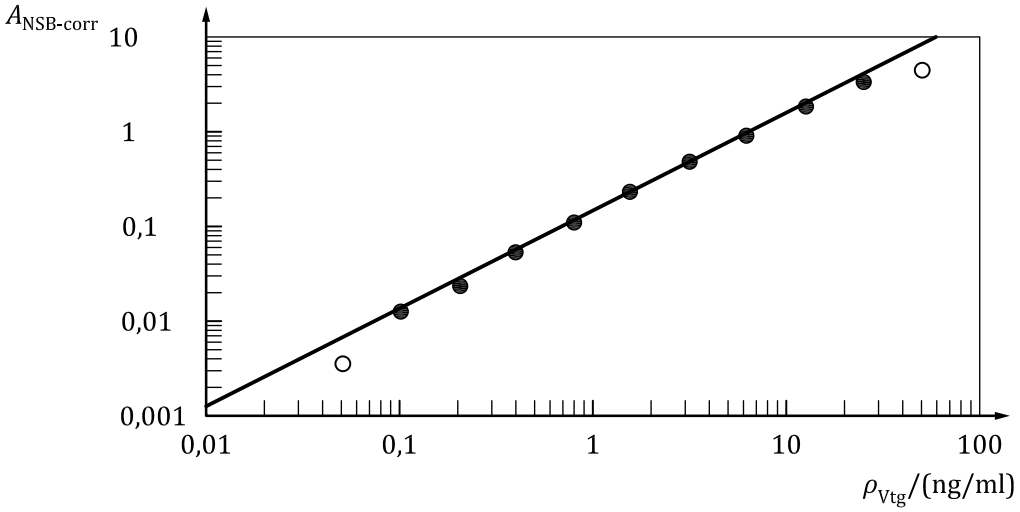
Selectivity (matrix effect):	No response at minimum dilution = 1→50 (plasma), 1→100 (WBH)
Calibration and working range:	Linear range 0,1 ng/ml to 25 ng/ml; 250-fold (n = 15 standard curves)
Within-day precision: coefficient of variation of repeatability, $C_{V,r}$:	6,0 %
Between-day precision: coefficient of variation of repeatability, $C_{V,r}$:	7,7 %
Accuracy (recovery):	75 % (samples frozen and thawed once); 79 % to 106 % (freshly spiked samples)
Limit of detection (LOD), limit of quantification (LOQ):	LOD 1,0 ng/ml (plasma), 4,0 ng/ml (WBH); LOQ 4,5 ng/ml (plasma), 11,0 ng/ml (WBH)
Within-day precision: coefficient of variation of repeatability, $C_{V,r}$:	4,5 %
Between-day precision: coefficient of variation of repeatability, $C_{V,r}$:	9,9 %
Ruggedness:	Elevated buffer and incubation temperature have strongest effects on quantification of Vtg
Comparison with existing methods:	Biosense Carp[a] Vtg ELISA kit: R^2 > 0,99; Competitor FHM[a] Vtg ELISA kit: R^2 > 0,99

NOTE R^2 is the correlation coefficient of a standard curve obtained by linear regression analysis.

[a] Product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Table A.1 — Fathead minnow Vtg sandwich ELISA standard curve: absorbance readings and NSB-corrected absorbance results

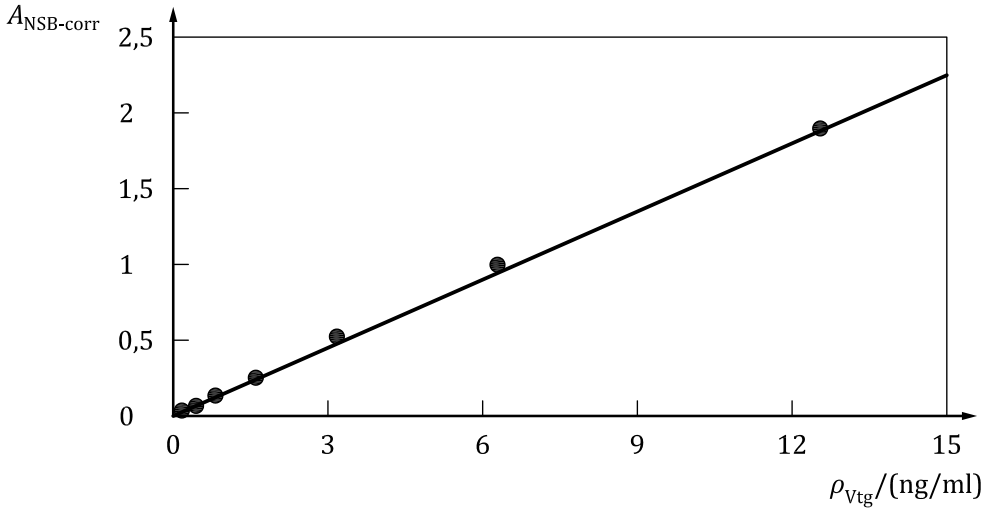
Fathead minnow Vtg standard Vtg concentration ng/ml	**Absorbance at** 450 nm	**NSB-corrected absorbance**[a]
50	4,589	4,535
25	3,453	3,399
12,5	1,939	1,885
6,25	1,029	0,975
3,13	0,553	0,498
1,56	0,298	0,244
0,78	0,169	0,115
0,39	0,109	0,055
0,20	0,080	0,026
0,10	0,066	0,012
0,05	0,059	0,005
[a] Mean NSB absorbance value: 0,054		



Key

$A_{NSB-corr}$ NSB-corrected absorbance ρ_{Vtg} Vtg concentration ○ omitted data point

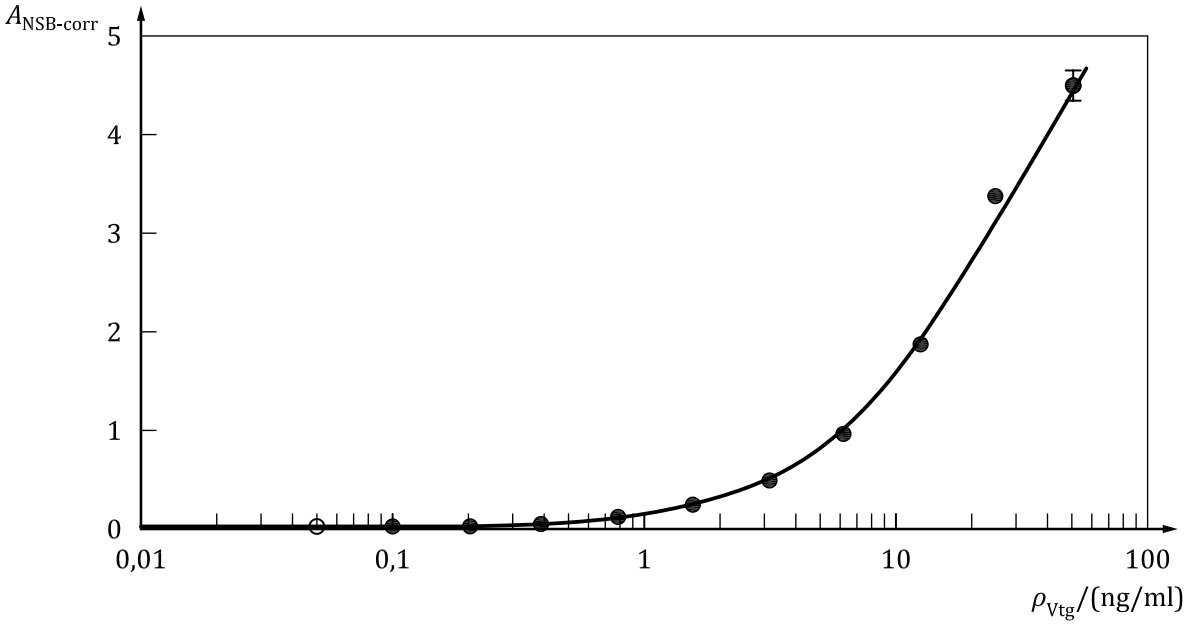
Figure A.1 — Log–log curve fit of fathead minnow Vtg standard curve in sandwich ELISA



Key

$A_{NSB\text{-}corr}$ NSB-corrected absorbance ρ_{Vtg} Vtg concentration

Figure A.2 — Linear curve fit of fathead minnow Vtg standard curve in sandwich ELISA



Key

$A_{NSB\text{-}corr}$ NSB-corrected absorbance ρ_{Vtg} Vtg concentration ○ omitted data point

Figure A.3 — Four-parameter curve fit of fathead minnow Vtg standard curve in sandwich ELISA

A.3 Inter-laboratory comparison — Fathead minnow Vtg sandwich ELISA

A.3.1 General

Inter-laboratory comparison (ring testing) of this type of analysis with multiple laboratories requires a complicated set-up including aquaria exposures with large numbers of fish to obtain sufficient sample volumes of different analyte concentrations for the laboratories involved. In addition, sample handling and shipping need to be performed on dry ice or liquid nitrogen to avoid repeated freeze–thaw cycles. For these reasons, it has not been possible to obtain full-scale inter-laboratory validation data for this method.

An inter-laboratory comparison was performed with four participating laboratories, see References [5] [6]. On two different days, a set of 18 spiked and unspiked samples (dilution buffer, plasma and WBHs, see A.2), as well as naturally incurred plasma and WBH samples, were analysed.

The performance parameters investigated were applicability, precision (repeatability and reproducibility), and accuracy (recovery).

A.3.2 Between-day precision: coefficient of variation of repeatability

$C_{V,r}$: Coefficient of variation of repeatability between results obtained for a given sample on the two different days. *Average* $C_{V,r}$: Average $C_{V,r}$ for all samples analysed by one laboratory *Overall* $C_{V,r}$: Average $C_{V,r}$ for all samples and laboratories. Table A.2 (from Reference [5]) shows that the overall $C_{V,r}$ between results obtained on two different days varied between 7,7 % and 37,4 % for the different laboratories. The overall $C_{V,r}$ for all four laboratories was 16,4 %.

Table A.2 — Between-day precision (coefficient of variation of repeatability) results from four laboratories and measured Vtg levels

Code	Laboratory1				Laboratory2				Laboratory3				Laboratory4			
	Day 1 ng/ml	**Day 2** ng/ml	**Average** ng/ml	$C_{V,r}$ %	**Day 1** ng/ml	**Day 2** ng/ml	**Average** ng/ml	$C_{V,r}$ %	**Day 1** ng/ml	**Day 2** ng/ml	**Average** ng/ml	$C_{V,r}$ %	**Day 1** ng/ml	**Day 2** ng/ml	**Average** ng/ml	$C_{V,r}$ %
PJ	<w.r.	<w.r.	—	—	<w.r.	<w.r.	—	—	<w.r.	<w.r.	—	—	<w.r.	<w.r.	—	—
PQ	0,4	0,4	0,4	2,1	0,3	0,3	0,3	1,8	0,4	0,4	0,4	6,4	0,5	n.d.[b]	0,5	—
PB	2,0	2,0	2,0	1,9	1,4	1,6	1,5	10,0	2,1	2,1	2,1	0,8	n.d.	1,6	1,6	—
PS	10,0	10,5	10,3	3,1	8,5	10,4	9,4	14,5	13,0	12,5	12,7	2,8	8,1	10,1	9,1	15,7
WJ	<w.r.	<w.r.	—	—	<w.r.	<w.r.	—	—	<w.r.	<w.r.	—	—	1,0	<w.r.	1,0	—
WQ	0,3	0,3	0,3	13,3	0,2	0,3	0,2	17,7	<w.r.	<w.r.	—	—	<w.r.	0.2	0,2	—
WB	1,5	1,4	1,5	5,4	1,2	1,2	1,2	0,1	1,0	1,2	1,1	13,6	1,7	n.d.	1,7	—
WS	7,7	7,6	7,7	1,3	7,4	8,0	7,7	5,4	6,1	6,7	6,4	6,8	7,1	5,5	6,3	17,9
BJ	<w.r.	<w.r.	—	—	<w.r.	<w.r.	—	—	<w.r.	<w.r.	—	—	<w.r.	<w.r.	—	—
BQ	0,4	0,4	0,4	1,9	0,3	0,3	0,3	17,5	0,4	0,4	0,4	11,8	n.d.	<w.r.	—	—
BB	2,0	1,9	2,0	5,2	1,8	1,8	1,8	1,7	2,2	1,9	2,1	8,2	<w.r.	n.d.	—	—
BS	10,5	10,9	10,7	2,6	10,4	11,2	10,8	4,9	12,1	11,9	12,0	1,5	5,2	11,0	8,1	50,9
PM	952	1 180	1 066	15 2	1 124	919	1 021	14,2	1 574	1 192	1 383	19,6	2 289	849	1 569	64,9
PA	38 642	54 454	46 548	24,0	40 192	37 687	38 939	4,6	57 282	56 422	56 852	1,1	n.d.	n.d.	—	—
PR	3 940 983	4 883 251	4 412 117	15,1	4 084 024	4 374 596	4 229 310	4,9	5 273 682	5 116 503	5 195 092	2,1	3 935 890	>w.r.	3 935 890	—
WM	7,92	<w.r.	7,92	—	<w.r.	<w.r.	—	—	<w.r.	<w.r.	—	—	n.d.	<w.r.	—	—
WA	27,0	22,4	24,7	13,3	26,3	13,1	19,7	47,1	<w.r.	<w.r.	—	—	n.d.	<w.r.	—	—
WR	469214	516990	493 102	6,9	477 607	728 601	603 104	29,4	618 288	478 045	548 166	18,1	n.d.	409 530	409 530	—
Average $C_{V,r}$				**7,9**				**12,4**				**7,7**				**37,4**
Overall $C_{V,r}$ **for all laboratories:**																**16,4**

>w.r.: absorbance level above working range of standard curve. <w.r.: absorbance level below working range of standard curve. n.d.: not determined due to error or too high variance.

A.3.3 Between-laboratory precision: coefficient of variation of reproducibility

$C_{V,R}$: Coefficient of variation of reproducibility between results obtained by different laboratories for one sample (average of 2 days). *Overall* $C_{V,R}$: Average $C_{V,R}$ for all laboratories and samples. Table A.3 (from Reference [5]) shows that the $C_{V,R}$ between results obtained in four different laboratories varied between 7,4 % and 37,6 % for the different samples. The overall $C_{V,R}$ for all samples was 18,6 %.

Table A.3 — Between-laboratory precision (coefficient of variation of reproducibility) results from four laboratories and measured Vtg levels

Code	Average day 1 to 2, laboratory 1 ng/ml	Average day 1 to 2, laboratory 2 ng/ml	Average day 1 to 2, laboratory 3 ng/ml	Average day 1 to 2, laboratory 4 ng/ml	Average laboratory 1 to 4 ng/ml	$C_{V,R}$ laboratory1 to 4 %
PJ	—[a]	—	—	—	—	—
PQ	0,4	0,3	0,4	0,5	0,4	18,5
PB	2,0	1,5	2,1	1,6	1,8	16,7
PS	10,3	9,4	12,7	9,1	10,4	16,3
WJ	—	—	—	—	1,0	—
WQ	0,3	0,2	—	—	0,3	15,0
WB	1,5	1,2	1,1	1,7	1,3	17,7
WS	7,7	7,7	6,4	6,3	7,0	12,3
BJ	—	—	—	—	—	—
BQ	0,4	0,3	0,4	—	0,4	15,6
BB	2,0	1,8	2,1	—	1,9	7,4
BS	10,7	10,8	12,0	8,1	10,4	21,0
PM	1 066	1 021	1 383	1 569	1 260	37,6
PA	46 548	38 939	56 852	—	47 446	20,0
PR	4 412 117	4 229 310	5 195 092	3 935 890	4 515 561	12,6
WM	7,9	—	—	—	7,9	—
WA	24,7	19,7	—	—	22,2	28,7
WR	493 102	603 104	548 166	409 530	528 325	20,6
Overall $C_{V,R}$						**18,6**

—: Data not obtainable for those samples (see Table A.2).

A.3.4 Recovery and bias

Recovery was determined using the formula:

$$\frac{\rho_1 - \rho_2}{\rho_3} \times 100$$

and bias by the formula

$$\frac{\rho_3 - (\rho_1 - \rho_2)}{\rho_3} \times 100$$

where

ρ_1 is the concentration measured in the spiked sample;

ρ_2 is the concentration measured in the unspiked sample;

ρ_3 is the theoretical concentration.

The results in Table A.4 (see Reference [5]) show that the recovery and bias varied somewhat with both sample type and spike concentration, with an overall recovery of 69,4 %.

Table A.4 — Measured Vtg levels (average of four laboratories), recovery, and bias from spiked sample analysis

Sample type	Code	Theoretic concentration ng/ml	Average laboratory 1 to 4 ng/ml	Recovery laboratory 1 to 4 %
Plasma	PJ	0	—	—
	PQ	0,5	0,4	74,8
	PB	2,5	1,8	73,3
	PS	12,5	10,4	83,0
WBH	WJ	0	1,0	—
	WQ	0,5	0,3	51,7
	WB	2,5	1,3	52,3
	WS	12,5	7,0	56,1
Buffer	BJ	0	—	—
	BQ	0,5	0,4	72,7
	BB	2,5	1,9	77,7
	BS	12,5	10,4	83,2
Overall Recovery				**69,4**
Bias				**–30,6**
—: Data unobtainable for those samples (see Table A.2).				

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