SLOVENSKI STANDARD
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Kakovost vode - Biokemijske in fiziološke meritve v ribah - 3. del: Določevanje vitelogenina

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

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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

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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.