INTERNATIONAL STANDARD

ISO 23893-3

First edition
2013-04-01

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

iTeh STANDARD PREVIEW
(standards.iteh.ai)

ISO 23893-3:2013
https://standards.iteh.ai/catalog/standards/sist/892154d3-9f09-43fa-bbfa-63099ae98903/iso-23893-3-2013

Reference number
ISO 23893-3:2013(E)

© ISO 2013

iTeh STANDARD PREVIEW
(standards.iteh.ai)

ISO 23893-3:2013
https://standards.iteh.ai/catalog/standards/sist/892154d3-9f09-43fa-bbfa-63099ae98903/iso-23893-3-2013

COPYRIGHT PROTECTED DOCUMENT

© ISO 2013

All rights reserved. Unless otherwise specified, no part of this publication may be reproduced or utilized otherwise in any form or by any means, electronic or mechanical, including photocopying, or posting on the internet or an intranet, without prior written permission. Permission can be requested from either ISO at the address below or ISO's member body in the country of the requester.

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

Contents

Page

Foreword ..... **iv**
Introduction ..... **v**
1 Scope **1**
2 Normative references **1**
3 Terms and definitions **1**
4 Principle **2**
5 Minimum performance criteria **2**
6 Test environment **2**
7 Reagents **3**
8 Apparatus **3**
9 Sampling procedure **4**
9.1 Sampling of fish 4
9.2 Sampling of blood plasma 4
9.3 Storage of blood plasma samples 5
10 Analytical procedure **5**
10.1 Preparation of the samples 5
10.2 Determination of vitellogenin 5
11 Test report **10**
Annex A (informative) **Examples of results: Fathead minnow sandwich ELISA** **12**
Bibliography **19**

iTeh STANDARD PREVIEW
(standards.iteh.ai)
ISO 23893-3:2013
https://standards.iteh.ai/catalog/standards/sist/892154d3-9f09-43fa-bbfa-63099ae98903/iso-23893-3-2013

© ISO 2013 – All rights reserved

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*:

iTeh STANDARD PREVIEW
(standards.iteh.ai)

— *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*

ISO 23893-3:2013
https://standards.iteh.ai/catalog/standards/sist/892154d3-9f09-43fa-bbfa-63099ae98903/iso-23893-3-2013

 © ISO 2013 – All rights reserved

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].

iTeh STANDARD PREVIEW
(standards.iteh.ai)
ISO 23893-3:2013

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.

© ISO 2013 – All rights reserved

iTeh STANDARD PREVIEW
(standards.iteh.ai)

ISO 23893-3:2013
https://standards.iteh.ai/catalog/standards/sist/892154d3-9f09-43fa-bbfa-63099ae98903/iso-23893-3-2013

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.

iTeh STANDARD PREVIEW
(standards.iteh.ai)
ISO 23893-3:2013
https://standards.iteh.ai/catalog/standards/sist/892154d3-9f09-43fa-bbfa-63099ae98903/iso-23893-3-2013

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.

© ISO 2013 – All rights reserved

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.

iTeh STANDARD PREVIEW
(standards.iteh.ai)
ISO 23893-3:2013

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.

 © ISO 2013 – All rights reserved

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)]

iTeh STANDARD PREVIEW

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.

(standards.iteh.ai)

ISO 23893-3:2013
https://standards.iteh.ai/catalog/standards/sist/89215487-9169-451a-8bfa-63099ae98903/iso-23893-3-2013

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.

---

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.

© ISO 2013 – All rights reserved

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;

iTeh STANDARD PREVIEW
(standards.iteh.ai)

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.

ISO 23893-3:2013
https://standards.iteh.ai/catalog/standards/sist/892154d3-9f09-43fa-bbfa-62d0ae8d12a3/iso-23893-3-2013

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]).

 © ISO 2013 – All rights reserved

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

iTeh STANDARD PREVIEW
(standards.iteh.ai)

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.

ISO 23893-3:2013
https://standards.iteh.ai/catalog/standards/sist/89219fa2-9409-43fa-bbfa-63099ae98903/iso-23893-3-2013

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.

© ISO 2013 – All rights reserved