
**Water quality — Determination of
the estrogenic potential of water and
waste water —**

**Part 2:
Yeast estrogen screen (A-YES, *Arxula
adeninivorans*)**

*Qualité de l'eau — Détermination du potentiel oestrogène de l'eau et
des eaux résiduaires —*

*Partie 2: Test d'oestrogénicité (A-YES, *Arxula adeninivorans*)*

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CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Fax: +41 22 749 09 47
Email: copyright@iso.org
Website: www.iso.org

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Contents

	Page
Foreword	v
1 Scope	1
2 Normative references	2
3 Terms and definitions	2
4 Principle	4
5 Interferences	4
6 Apparatus and materials	4
7 Reagents, media and test strains	5
8 Sampling and samples	9
8.1 General.....	9
8.2 Bottles and material for sampling.....	9
8.3 Bottles and material pre-cleaning.....	10
8.4 Sampling procedure.....	10
8.5 Transport of samples.....	10
8.6 Pretreatment of samples.....	10
8.7 Storage of samples.....	11
9 Procedure	11
9.1 Test set up.....	11
9.1.1 Preparation of the reference dilution series.....	11
9.1.2 Reactivation of the yeast.....	11
9.1.3 Negative control.....	12
9.1.4 Blank replicate.....	12
9.1.5 Sample dilution.....	12
9.1.6 Field blank.....	13
9.1.7 Plate set up.....	13
9.1.8 Inoculation of the test plate.....	13
9.2 Measurement.....	14
9.2.1 Measurement of the reporter gene activity.....	14
9.2.2 Measurement of the cell density.....	15
9.3 Calculations.....	15
9.3.1 Background correction.....	15
9.3.2 Calculation of the relative growth.....	16
9.3.3 Calculations for assessment of sample blanks.....	17
9.3.4 Calculation of the reporter gene induction.....	19
10 Validity criteria	22
11 Assessment criteria	23
12 Test report	23
13 Verification	23
Annex A (informative) Plate set up	25
Annex B (informative) Lyophilization of <i>Arxula adenivorans</i> cells	26
Annex C (informative) Scheme of test principle	28
Annex D (informative) Test set up for chemicals and extracts	29
Annex E (informative) Preparation of dilution series	30
Annex F (informative) Performance data	31
Annex G (informative) Statistical assessment	41

Annex H (informative) Calculation of estradiol equivalents	42
Annex I (informative) Alternative test design for EEQ determination	45
Annex J (informative) Measurement of the lowest ineffective dilution (LID) of a waste water — A simplified evaluation for testing of waste water	46
Annex K (informative) Example for statistical evaluation	48
Bibliography	55

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

A list of all parts in the ISO 19040 series can be found on the ISO website.

Voorbeeld
Preview

Water quality — Determination of the estrogenic potential of water and waste water —

Part 2: Yeast estrogen screen (A-YES, *Arxula adenivorans*)

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.

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

1 Scope

This document specifies a method for the determination of the estrogenic potential of water and waste water by means of a reporter gene assay with a genetically modified yeast strain *Arxula adenivorans*. This reporter gene assay is based on the activation of the human estrogen receptor alpha.

Arxula adenivorans is a highly robust and salt- and temperature-tolerant test organism and is especially suitable for the analysis of samples with high salinity (conductivity up to 70 mS/cm). The test organism can be cultivated in medium with sodium chloride content up to 20 %.

This method is applicable to:

- fresh water;
- waste water;
- sea water;
- brackish water;
- aqueous extracts and leachates;
- eluates of sediments (fresh water);
- pore water;
- aqueous solutions of single substances or of chemical mixtures;
- drinking water.

The limit of quantification (LOQ) of this method for the direct analysis of water samples is between 1,5 ng/l and 3 ng/l 17 β -estradiol equivalents (EEQ). The upper threshold of the dynamic range for this test is between 25 ng/l and 40 ng/l 17 β -estradiol equivalents (EEQ). Samples showing estrogenic potencies above this threshold have to be diluted for a valid quantification. Extraction and pre-concentration of water samples can prove necessary, if their estrogenic potential is below the given LOQ.

An international interlaboratory trial for the validation of this document has been carried out. The results are summarized in [Annex F](#).

NOTE Extraction and pre-concentration of water samples can prove necessary.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

blank replicate

additional replicate that contains no test organism, but is treated in the same way as the other replicates of a sample

[SOURCE: ISO 10872:2010, 3.5]

3.2

culture medium

nutrients presented in a form and phase (liquid or solidified) which support microbiological growth

3.3

dilution level

D

denominator of the dilution coefficient (using the numerator 1) of a mixture of water or waste water with dilution water as integral number

Note 1 to entry: For undiluted water or waste water, this coefficient per definition is 1→1. The corresponding and smallest possible value of *D* is 1. In this document, the arrow indicates the transition from initial total volume to final total volume.

[SOURCE: ISO 6107-6:2004, 28]

3.4

dilution water

water added to the test sample to prepare a series of defined dilutions

[SOURCE: ISO 20079:2005, 3.7]

3.5

50 % effect concentration

EC₅₀

concentration of a compound which causes 50 % of an effect

Note 1 to entry: In the sense of this document the EC₅₀ is the concentration of a compound which induces 50 % of the maximal reporter gene activity which can be achieved by this compound.

3.6

field blank

container prepared in the laboratory, using reagent water or other blank matrix, and sent with the sampling personnel for exposure to the sampling environment to verify possible contamination during sampling

[SOURCE: ISO 11074:2015, 4.5.3]

3.7**growth rate**

proportional rate of increase in cell density

[SOURCE: ISO 10253:2006, 3.2]

3.8**induction rate**

quotient of the mean value of wells with enhanced reporter gene activity measured on the plates treated with a dose of the test sample, and the mean value of the corresponding wells treated with the negative control using the same strain under identical conditions

Note 1 to entry: Instead of the negative control, the estimated parameter A of the four-parameter model, which describes the dose response relationship between reference compound and the induction rate, can be used.

[SOURCE: ISO 6107-6:2004, 43, modified — "wells with enhanced reporter gene activity measured" replaces "mutant colonies"; "corresponding wells" replaces "corresponding plates"; "quotient" replaces "difference".]

3.9**inoculum**

fraction of a culture of microorganisms used to start a new culture, or an exponentially growing preculture, in fresh medium

[SOURCE: ISO 6107-6:2004, 44]

3.10**lowest ineffective dilution value****LID**

lowest dilution within a test batch which does not show any effect, i.e. no statistically significant increase in the reporter gene activity compared with the negative control

[SOURCE: ISO 11350:2012, 7.4, modified — "increase in the reporter gene activity" replaces "increase in the number of revertant wells".]

3.11**negative control**

dilution water without test sample

[SOURCE: ISO 6107-6:2004, 51]

3.12**reference compound**

compound with one or more property values that are sufficiently reproducible and well established to enable the calibration of the measurement method

[SOURCE: ISO 7405:2008, 3.6, modified — "compound" replaces "material"; "the calibration of the measurement method" replaces "use of the material or substance for the calibration of an apparatus, the assessment of a measurement method or for the assignment of values to materials".]

3.13**reporter gene activity**

quantitative activity of a gene attached to the promoter sequence of another gene

3.14**test sample**

undiluted, diluted or otherwise prepared portion of a sample to be tested, after completion of all preparation steps such as centrifugation, filtration, homogenization, pH adjustment and determination of ionic strength

[SOURCE: ISO 6107-6:2004, 92]

4 Principle

The A-YES (*Arxula* Yeast Estrogen Screen) is a reporter gene assay which can be used for the measurement of the activation of the human estrogen receptor alpha (ER α) in the presence of a sample containing compounds which cause estrogenic effects. By this means the assay detects the estrogenic activity of the whole sample in its actual state as an integral measure including possible additive, synergistic and antagonistic mixture-effects.

The human estrogen receptor α is constitutively expressed in the yeast cell under control of a *TEF1* promoter. The estrogen receptor belongs to the family of nuclear hormone receptors. If agonists of the estrogen receptor enter the yeast cell, they bind to the estrogen receptor protein and thus induce its conformational change. As a consequence two receptor proteins form a receptor dimer. This activation of the estrogen receptor is measured by the induction of the reporter gene *phyK* which encodes the enzyme phytase. The *phyK* is fused to an estrogen dependent promoter which contains estrogen responsive elements (*ERE*). The ER-dimer binds to the promoter and by this activates the expression and secretion of the phytase. Finally, the activity of the phytase as a measure for the estrogenic potential of the sample is determined using an appropriate substrate which is cleaved to a coloured reaction product. The reaction product can be measured photometrically. See [Annex C](#) for a scheme of the test principle.

5 Interferences

Coloured or turbid samples might interfere with the photometric detection of the cell density and/or the detection of the cleaved substrate of the reporter enzyme phytase (see [Clause 10](#) for further information).

Effects of the sample matrix may lead to a reduction or increase of viable cells and to a reduction or increase of the measurable signal. Estrogenic effects of a sample may be masked by matrix effects leading to false negative or false positive test results.

High salinity can cause toxic effects due to the resulting osmotic pressure. The conductivity of a sample is a measure for its salinity. The *Arxula adenivorans* yeast tolerates a conductivity of the sample up to 20 % sodium chloride which meets a conductivity of 180 mS/cm.

Bacterial growth in the test wells is assessed by the blank replicate ([3.1](#)). See [Clause 10](#) for further information.

If filtered samples are tested in order to remove bacteria from the sample solid particles are separated from the sample also. Thus, substances with estrogenic activity which are adsorbed on particles might not be detected.

For detailed information about appropriate sampling material that does not influence the test result see [Clause 8](#).

6 Apparatus and materials

For suitable sampling devices see [Clause 8](#). Use usual laboratory apparatus and glassware if required. In particular, the following material is needed:

6.1 Temperature- and time-controlled incubator shaker, shaker orbit at least 3 mm, 30 °C to 37 °C with an accuracy of ± 1 °C.

If the shaker has no incubation function use a lab shaker with a shaker orbit of at least 3 mm in combination with an incubator ([6.17](#)).

6.2 Lab mini shaker.

6.3 Multi-parameter measurement device for pH and conductivity or separate devices for each parameter.

6.4 Steam sterilizer.

6.5 Centrifuge, with a rotor for 96-well plates up to 1 000 *g* and a rotor for 2 ml reaction tubes.

6.6 Sterile filters, cellulose acetat, 0,2 µm pore size.

6.7 Single-channel pipettes, nominal volume 10 µl up to 10 000 µl.

6.8 Multi-channel pipettes, nominal volume 100 µl and 300 µl.

6.9 Transparent polystyrene 96-well plates with flat bottom (F-profile, 300 µl) and lid.

6.10 96-deep well plates with at least 1 ml volume with round bottom and square wells.

6.11 Microplate photometer for 96-well plates, for absorbance measurement for wavelength 405 nm ± 20 nm and 630 nm ± 5 nm or alternatively 600 nm ± 20 nm.

6.12 Air-permeable adhesive foil for deep well plates.

6.13 Reaction tubes, 2 ml.

6.14 Test tubes, 15 ml and 50 ml.

6.15 Multi-channel pipette trough

6.16 Balance, minimum load 10 mg, d = 0,1 mg.

6.17 Incubator, 30 °C to 37 °C with an accuracy of ±1 °C. For the purpose of using the incubator in combination with a shaker a cooled incubator is required.

7 Reagents, media and test strains

7.1 Reagents

As far as possible, use “reagent grade“-chemicals.

7.1.1 Hydrochloric acid solution, $c(\text{HCl}) = 1 \text{ mol/l}$, molecular weight 36,46 g/mol, CAS: 7647-01-0.

7.1.2 Sodium hydroxide solution, $c(\text{NaOH}) = 1 \text{ mol/l}$, molecular weight 40,00 g/mol, CAS: 1310-73-2.

7.1.3 Ethanol, ≥99,8 %, $\text{C}_2\text{H}_5\text{OH}$, molecular weight 46,07 g/mol, CAS: 64-17-5.

7.1.4 17β-estradiol, ≥98 %, $\text{C}_{18}\text{H}_{24}\text{O}_2$, molecular weight 272,38 g/mol, CAS: 50-28-2.

7.1.5 Maltose monohydrate, >95 %, $\text{C}_{12}\text{H}_{22}\text{O}_{11} \cdot \text{H}_2\text{O}$, molecular weight 360,32 g/mol, CAS: 6363-53-7.

7.1.6 Sodium nitrate, >99 %, NaNO_3 , molecular weight 84,98 g/mol, CAS: 7631-99-4.

7.1.7 Potassium dihydrogen phosphate, ≥ 99 %, KH_2PO_4 , molecular weight 136,09 g/mol, CAS: 7778-77-0.

7.1.8 Magnesium sulfate pure, MgSO_4 , molecular weight 120,37 g/mol (water free), CAS: 7487-88-9.

7.1.9 Iron(III) chloride hexahydrate, > 97 %, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, molecular weight 270,29 g/mol, CAS: 10025-77-1.

7.1.10 Calcium nitrate, > 99 %, $\text{Ca}(\text{NO}_3)_2$, molecular weight 164,09 g/mol, CAS: 10124-37-5.

7.1.11 Calcium D-pantothenate, > 98 %, $\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$ molecular weight 238,27 g/mol, CAS: 137-08-6.

7.1.12 Thiamine hydrochloride, $> 98,5$ %, $\text{C}_{12}\text{H}_{18}\text{Cl}_2\text{N}_4\text{OS}$, molecular weight 337,27 g/mol, CAS: 67-03-8.

7.1.13 Niacin, $> 99,5$ %, $\text{C}_6\text{H}_5\text{NO}_2$, molecular weight 123,11 g/mol, CAS: 59-67-6.

7.1.14 Pyridoxine hydrochloride, > 99 %, $\text{C}_8\text{H}_{11}\text{NO}_3 \cdot \text{HCl}$, molecular weight 205,64 g/mol, CAS: 58-56-0.

7.1.15 D-(+)-Biotin, $\geq 98,5$ %, $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$, molecular weight 244,31 g/mol, CAS: 58-85-5.

7.1.16 Inositol, ≥ 99 %, $\text{C}_6\text{H}_{12}\text{O}_6$, molecular weight 180,16 g/mol, CAS: 87-89-8.

7.1.17 Boric acid, $> 99,8$ %, H_3BO_3 , molecular weight 61,83 g/mol, CAS: 10043-35-3.

7.1.18 Copper(II) sulfate pentahydrate, $> 99,5$ %, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, molecular weight 249,68 g/mol, CAS: 7758-99-8.

7.1.19 Potassium iodide, > 99 %, KI , molecular weight 166,00 g/mol, CAS: 7681-11-0.

7.1.20 Manganese sulfate monohydrate, > 99 %, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, molecular weight 169,02 g/mol, CAS: 10034-96-5.

7.1.21 Zinc sulfate heptahydrate, $> 99,5$ %, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, molecular weight 287,56 g/mol, CAS: 7446-20-0.

7.1.22 Sodium molybdate dihydrate, $> 99,5$ %, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, molecular weight 241,95 g/mol, CAS: 10102-40-6.

7.1.23 Cobalt(II) chloride for synthesis, CoCl_2 , molecular weight 129,84 g/mol, CAS: 7646-79-9.

7.1.24 Trisodium citrate dihydrate, > 99 %, $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$, molecular weight 294,10 g/mol, CAS: 6132-04-3.

7.1.25 Citric acid, $> 99,5$ %, $\text{C}_6\text{H}_8\text{O}_7$, molecular weight 192,12 g/mol, CAS: 77-92-9.

7.1.26 4-Nitrophenyl phosphate disodium salt hexahydrate (pNPP), $\text{C}_6\text{H}_4\text{NNa}_2\text{O}_6\text{P} \cdot 6\text{H}_2\text{O}$, molecular weight 371,14 g/mol, CAS: 333338-18-4.

7.1.27 Sodium hydroxide, ≥ 99 %, NaOH , molecular weight 40,00 g/mol, CAS: 1310-73-2.

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