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BSI Standards Publication

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

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

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National foreword

This British Standard is the UK implementation of EN ISO 19040-2:2022. It is identical to ISO 19040-2:2018. It supersedes BS ISO 19040-2:2018, which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee EH/3/5, Biological Methods.

A list of organizations represented on this committee can be obtained on request to its committee manager.

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EN ISO 19040-2

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Water quality - Determination of the estrogenic potential of water and waste water - Part 2: Yeast estrogen screen (A-YES, Arxula adeninivorans) (ISO 19040-2:2018)

Qualité de l'eau - Détermination du potentiel oestrogène de l'eau et des eaux résiduaires - Partie 2: Test d'oestrogénicité (A-YES, Arxula adeninivorans) (ISO 19040-2:2018)

Wasserbeschaffenheit - Bestimmung des estrogenen Potentials von Wasser und Abwasser - Teil 2: Hefe-Estrogenscreening (A-YES, Arxula adeninivorans) (ISO 19040-2:2018)

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The text of ISO 19040-2:2018 has been prepared by Technical Committee ISO/TC 147 "Water quality" of the International Organization for Standardization (ISO) and has been taken over as EN ISO 19040-2:2022 by Technical Committee CEN/TC 230 "Water analysis" the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by March 2023, and conflicting national standards shall be withdrawn at the latest by March 2023.

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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*.

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

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

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 adeninivorans*. This reporter gene assay is based on the activation of the human estrogen receptor alpha.

Arxula adeninivorans 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 17β-estradiol equivalents (EEQ). The upper threshold of the dynamic range for this test is between 25 ng/l and 40 ng/l 17β-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.

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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
EC_{50}
concentration of a compound which causes 50 % of an effect

Note 1 to entry: In the sense of this document the EC_{50} 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]

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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, 3.4, 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]

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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 (ERα) 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 adeninivorans* 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.

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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 1 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*(HCl) = 1 mol/l, molecular weight 36,46 g/mol, CAS: 7647-01-0.

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

7.1.3 Ethanol, ≥99,8 %, C_2H_5OH, molecular weight 46,07 g/mol, CAS: 64-17-5.

7.1.4 17*β*-estradiol, ≥98 %, $C_{18}H_{24}O_2$, molecular weight 272,38 g/mol, CAS: 50-28-2.

7.1.5 Maltose monohydrate, >95 %, $C_{12}H_{22}O_{11}{\cdot}H_2O$, 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.

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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 %, $FeCl_3 \cdot 6H_2O$, molecular weight 270,29 g/mol, CAS: 10025-77-1.

7.1.10 Calcium nitrate, >99 %, $Ca(NO_3)_2$, molecular weight 164,09 g/mol, CAS: 10124-37-5.

7.1.11 Calcium D-pantothenate, >98 %, $C_{18}H_{32}CaN_2O_{10}$ molecular weight 238,27 g/mol, CAS: 137-08-6.

7.1.12 Thiamine hydrochloride, >98,5 %, $C_{12}H_{18}Cl_2N_4OS$, molecular weight 337,27 g/mol, CAS: 67-03-8.

7.1.13 Niacin, >99,5 %, $C_6H_5NO_2$, molecular weight 123,11 g/mol, CAS: 59-67-6.

7.1.14 Pyridoxine hydrochloride, >99 %, $C_8H_{11}NO_3 \cdot HCl$, molecular weight 205,64 g/mol, CAS: 58-56-0.

7.1.15 D-(+)-Biotin, ≥98,5 %, $C_{10}H_{16}N_2O_3S$, molecular weight 244,31 g/mol, CAS: 58-85-5.

7.1.16 Inositol, ≥99 %, $C_6H_{12}O_6$, 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 %, $CuSO_4 \cdot 5H_2O$, 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 %, $MnSO_4 \cdot H_2O$, molecular weight 169,02 g/mol, CAS: 10034-96-5.

7.1.21 Zinc sulfate heptahydrate, >99,5 %, $ZnSO_4 \cdot 7H_2O$, molecular weight 287,56 g/mol, CAS: 7446-20-0.

7.1.22 Sodium molybdate dihydrate, >99,5 %, $Na_2MoO_4 \cdot 2H_2O$, 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 %, $C_6H_5Na_3O_7 \cdot 2H_2O$, molecular weight 294,10 g/mol, CAS: 6132-04-3.

7.1.25 Citric acid, >99,5 %, $C_6H_8O_7$, molecular weight 192,12 g/mol, CAS: 77-92-9.

7.1.26 4-Nitrophenyl phosphate disodium salt hexahydrate (pNPP), $C_6H_4NNa_2O_6P \cdot 6H_2O$, 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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7.1.28 Sea salt with the specifications: chloride (Cl) 19 290 mg/l, sodium 10 780 mg/l, sulfate 2 660 mg/l, potassium 420 mg/l, calcium 400 mg/l, carbonate (bicarbonate) 200 mg/l, strontium 8,8 mg/l, boron 5,6 mg/l, bromide 56 mg/l, iodide 0,24 mg/l, lithium 0,3 mg/l, fluoride 1,0 mg/l, magnesium (Mg) 1 320 mg/l.

7.1.29 Sodium chloride, ≥99 %, NaCl, molecular weight 58,44 g/mol, CAS: 7647-14-5.

7.1.30 Aceton (purity p.a.), C_3H_6O, molecular weight 58,08 g/mol, CAS: 67-64-1.

7.2 Water, grade 3, as defined in ISO 3696; water with conductivity up to 5 µS/cm is acceptable, or ultrapure water.

If sterile water is needed, autoclave or sterilize by filtration (cellulose acetate, 0,2 µm).

Water as specified here is also used for the preparation of dilution water which is used for the stepwise dilution of the test sample.

7.3 Test strain

This test strain is derived from *Blastobotrys adeninivorans* G1214 Syn.: *Arxula adeninivorans* G1214 (*aleu2 aura3::ALEU2*), Reference [1]. This strain displays an uracil auxotrophy. To prevent any formations of antibiotic resistances in environment and to increase acceptance regarding legal requirements the test organism contains no antibiotic resistance markers.

Genetic modifications: Integration of the selection marker *AURA3mm* in the plasmid Xplor2-102-hERα-GAA2(ERE107)-phyK after exchange of the marker *ALEU2* promoter-*ATRP1m*. The selection marker *AURA3mm* was isolated from the plasmid pCR4-AURA3mm-13. The sequences of *E. coli* and Kanamycin resistant marker were eliminated through restriction digestion. Stable integration of Xplor2-102-hERα-GAA2(ERE107)-phyK in *Arxula adeninivorans* genome was achieved by transformation the cassette in uracil auxotroph mutant of *A. adeninivorans* G1214 (*aleu2 aura3::ALEU2*) through recombination with 25S-rDNA.

The yeast cell suspension for determination of the estrogenic potential of aqueous samples is prepared from lyophilized yeast cells. As the *Arxula adeninivorans* cells are lyophilized the test can be conducted under highly standardized conditions and no specific lab equipment for long-term cell cultivation is required.

The yeast cells are available commercially. Store the lyophilized yeast cells between 4 °C and 8 °C and follow the manufacturer's recommendations. After reactivation, the yeast cells can directly be used for the test, precultivation is not needed for testing.

An alternative to commercially available lyophilized yeast cells are self-made lyophilized yeast cells. The preparation procedure is described in Annex B. The yeast strain can be isolated from commercially available lyophilisate.

7.4 Media.

If autoclaving is necessary autoclave for 20 min at 121 °C ± 2 °C. Cover vessels loosely (e.g. with aluminium foil). Never seal air-tight.

7.4.1 17*β*-estradiol (E2) stock solution.

Dissolve 10 mg of 17*β*-estradiol (E2) (7.1.4) in 10 ml ethanol (7.1.3). Store the 17*β*-estradiol (E2) stock solution at ≤–18 °C. Store the stock solution no longer than 18 months.

If available certified 17*β*-estradiol reference standard of equal concentration can be used as stock solution.

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7.4.2 17*β*-estradiol (E2) working solution.

Dilute 17*β*-estradiol (E2) stock solution (7.4.1) 1→100 by adding 10 µl of the 17*β*-estradiol (E2) stock solution (7.4.1) to 990 µl ethanol (7.1.3) and mix well. Make a further 1→10 dilution by adding 100 µl of the first dilution to 900 µl ethanol (7.1.3) and mix well. The final concentration is 1 mg/l. The working solution shall be aliquoted in order to avoid thawing and freezing of the working solution. Store the 17*β*-estradiol (E2) working solution at ≤–18 °C. Store the working solution no longer than six months.

NOTE Pipetting of organic solvents requires the usage of adequate calibrated pipettes or other suitable liquid handling equipment.

7.4.3 Maltose solution.

Dissolve 20 g of maltose monohydrate (7.1.5) in 70 ml ultrapure water (7.2). Fill the maltose solution up to 100 ml with ultrapure water (7.2). Autoclave the solution. Store the maltose solution at 2 °C to 8 °C. Store the maltose solution no longer than six months.

7.4.4 Salt solution for yeast minimal medium.

Dissolve 3,7 g of $NaNO_3$ (7.1.6), 8,4 g KH_2PO_4 (7.1.7) and 1 g of $MgSO_4$ (7.1.8) in 70 ml ultrapure water (7.2). Fill up to 100 ml with ultrapure water (7.2). Autoclave the solution. Store the salt solution for yeast minimal medium at 2 °C to 8 °C. Store the salt solution no longer than six months.

7.4.5 Salt solution for saline yeast minimal medium.

Dissolve 28 g of NaCl (7.1.29), 3,7 g of $NaNO_3$ (7.1.6), 8,4 g KH_2PO_4 (7.1.7) and 1 g of $MgSO_4$ (7.1.8) in 70 ml ultrapure water (7.2). Fill up to 100 ml with ultrapure water (7.2). For the analysis of sea water samples and brackish water samples the 28 g of NaCl may be replaced by 28 g of a salt composition similar to the salt composition of sea water. Autoclave the solution. Store the salt solution for saline yeast minimal medium at 2 °C to 8 °C no longer than six months.

7.4.6 Micronutrient solution.

Weigh the following chemicals separately:

0,05 g H_3BO_3 (7.1.17), 0,01 g $CuSO_4{\cdot}5H_2O$ (7.1.18), 0,01 g KI (7.1.19), 0,04 g $MnSO_4{\cdot}H_2O$ (7.1.20), 0,04 g $ZnSO_4{\cdot}7H_2O$ (7.1.21), 0,02 g $Na_2MoO_4{\cdot}2H_2O$ (7.1.22), 0,01 g $CoCl_2$ (7.1.23).

Merge the chemicals and dissolve them in 100 ml ultrapure water (7.2). Autoclave the solution. Precipitation after autoclaving has no influence on quality. Shake the solution before using it. Store the micronutrient solution at 2 °C to 8 °C. Store the micronutrient solution no longer than 12 months.

7.4.7 $FeCl_3$ solution.

Weigh 0,2 g of Fe(III)Cl_3 hexahydrate (7.1.9) and dissolve it in 20 ml ultrapure water (7.2). Sterilize the $FeCl_3$ solution by filtration (0,2 µm). Store the $FeCl_3$ solution at 2 °C to 8 °C. Store the $FeCl_3$ solution no longer than six months.

7.4.8 $Ca(NO_3)_2$ solution.

Weigh 10 g of $Ca(NO_3)_2$ (7.1.10) and dissolve it in 10 ml of ultrapure water (7.2). Sterilize the $Ca(NO_3)_2$ solution by filtration (0,2 µm). Store the $Ca(NO_3)_2$ solution at 2 °C to 8 °C. Store the $Ca(NO_3)_2$ solution no longer than six months.

7.4.9 Vitamin mix.

Weigh the following chemicals separately:

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0,2 g Calcium-D-panthothenate (7.1.11), 0,2 g thiamin hydrochloride (7.1.12), 0,05 g niacin (7.1.13), 0,02 g biotin (7.1.15), 0,2 g pyridoxin hydrochloride (7.1.14), 2 g inositol (7.1.16).

Merge and dissolve the vitamins in 50 ml ultrapure water (7.2). Sterilize the vitamin mix by filtration (0,2 µm). Store the vitamin mix at 2 °C to 8 °C no longer than six months.

7.4.10 Yeast minimal medium with maltose.

Pipette 1 ml of $FeCl_3$ solution (7.4.7), 1 ml of $Ca(NO_3)_2$ solution (7.4.8), 1 ml of micronutrient solution (7.4.6) and 0,5 ml vitamin mix (7.4.9) to 96,5 ml of the salt solution (7.4.4). Add 100 ml maltose solution (7.4.3).

The yeast minimal medium with maltose is five-fold concentrated. Store the yeast minimal medium at 2 °C to 8 °C. Store the yeast minimal medium no longer than six months.

7.4.11 Saline yeast minimal medium with maltose.

Pipette 1 ml of $FeCl_3$ solution (7.4.7), 1 ml of $Ca(NO_3)_2$ solution (7.4.8), 1 ml of micronutrient solution (7.4.6) and 0,5 ml vitamin mix (7.4.9) to 96,5 ml of the salt solution for saline yeast minimal medium (7.4.5). Add 100 ml maltose solution (7.4.3).

The saline yeast minimal medium with maltose is five-fold concentrated. Store the saline yeast minimal medium at 2 °C to 8 °C. Store the saline yeast minimal medium no longer than six months.

7.4.12 Substrate buffer.

Weigh 10,35 g of trisodiumcitrate dihydrate (7.1.24) and 12,45 g of citric acid (7.1.25) separately. Dissolve every reagent in 60 ml of ultrapure water (7.2). Merge the two solutions and fill up to 200 ml with ultrapure water (7.2). Autoclave the substrate buffer and cool down before using it. Store the substrate buffer at 2 °C to 8 °C. Store the substrate buffer no longer than six months.

7.4.13 Phytase substrate solution.

Weigh at least 15 mg of 4-Nitrophenyl phosphate disodium salt hexahydrate (7.1.26) and dissolve it in equivalent volume substrate buffer. A volume of 15 ml phytase substrate solution is sufficient for two test plates. Prepare phytase substrate solution always fresh and use within 2 h.

7.4.14 Developer.

Dissolve 24 g of solid sodium hydroxide (7.1.27) in 200 ml ultrapure water (7.2). Autoclave the sodium hydroxide solution and cool down before using it. Store the developer at room temperature. Store the developer solution no longer than six months.

8 Sampling and samples

8.1 General

This document describes specific requirements for the sampling with respect to the determination of estrogenic activity in water samples. For general information about sampling consider ISO 5667-16.

8.2 Bottles and material for sampling

Use clean glass bottles (borosilicate glass) with polytetrafluoroethylene (PTFE)-lined caps. To avoid photo-degradation of compounds of interest, use amber glass bottles. If transparent glass bottles are used, wrap the bottles in aluminium foil or store them in a dark container.

Alternatively, bottles made from aluminium or stainless steel (both uncoated) may be used. Assess that a material different from borosilicate glass does not affect results.

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8.3 Bottles and material pre-cleaning

After the routine cleaning procedure, additionally clean the bottles and the caps as follows: rinse the clean bottles and the caps three times with a minimum amount of acetone (7.1.30). Let the residual acetone evaporate (e.g. drying oven). Close the bottles immediately after drying.

Rinse all glassware, spatulas etc. getting in contact with the sample three times with a minimum amount of acetone (7.1.30). Let the residual acetone evaporate.

8.4 Sampling procedure

Use disposable nitrile-gloves during sampling. Do not use any hand-cream prior to sampling and avoid skin contact with the sample. Use material from glass, PTFE, aluminium or stainless steel only.

Fill the bottles completely. Consider possible expansion of the sample due to a change of temperature. If the samples are to be frozen as part of their preservation, the bottles shall not be completely filled. This is in order to prevent breakage which may arise from expansion of ice during the freezing and thawing process.

Do not stabilize the samples with chemicals.

Either cool down the samples to 2 °C to 8 °C or freeze the samples at ≤–18 °C.

8.5 Transport of samples

Deliver the samples to the laboratory as soon as possible after sampling.

During transport keep the sample container frost- and break-proof, protected from exposure to light, temperature increase and external contamination.

Cooling or freezing procedures shall be applied to the samples in order to increase the time period available for transport and storage. Cooling should commence as soon as possible after sampling for instance in cool boxes with ice, frozen gel packs, or cooling elements. A cooling device in the transport vehicle is also suitable. A cooling temperature during transport of 2 °C to 8 °C has been found suitable. The suggested cooling temperature applies to the surrounding of the sample (e.g. inside the cooling box) and not for the sample itself.

If the sample is frozen, avoid thawing of the sample (e.g. transport on dry ice). If dry ice is chosen for transport, the bottles should be wrapped in paper or in air bubble film to avoid direct contact with the dry ice.

8.6 Pretreatment of samples

Preferably analyse the samples non-filtered immediately after sampling because of the possible loss of particle associated estrogenic activity. The decision about a sample filtration (e.g. cellulose acetate, 0,45 µm pore size) is to be taken by the performing laboratory according to the application and based on the experience with the sample type under investigation. Report in any case if a filtered or non-filtered sample was tested. Further information about possible impacts of filtration on samples with estrogenic activity is given in References [13] and [14].

Adjust the sample to a pH of 7,2 ± 0,2 using either HCl (7.1.1) or NaOH solution (7.1.2). Select the acid or alkali concentrations such that the added volumes are as small as possible. Avoid over-titration. Dilute HCl (7.1.1) or NaOH solution (7.1.2) if necessary. The adjustment of the sample pH might affect the sample. Report all visible changes caused by the adjustment of the pH value (See ISO 5667-16).

Measure the conductivity of the sample.

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8.7 Storage of samples

Test the samples immediately after sampling. If this is not possible, keep water samples at 2 °C to 8 °C (<7 d) or below −18 °C (up to two months). For multiple testing divide larger samples in advance into appropriate portions, since thawed samples can only be used on the same day. Avoid thawing and freezing of samples more than once before analysis. Thaw the sample in the dark at a maximal temperature of 25 °C (e.g. water bath) or between 2 °C and 8 °C over night. Do not use a microwave to thaw samples.

Storage of the sample may impact the estrogenic activity of the sample. Possible changes are sample depending. Specify the duration and conditions of sample storage based on experience with the specific sample type. Further information about possible impacts of storage on samples with estrogenic activity is given in References [15] and [16].

9 Procedure

9.1 Test set up

9.1.1 Preparation of the reference dilution series

Thaw the 17*β*-estradiol working solution (7.4.2). Dilute the working solution 1→100 [e.g. by adding 10 µl of the 17*β*-estradiol working solution (7.4.2) to 990 µl ultrapure water (7.2)]. Make a further 1→10 dilution [e.g. by adding 100 µl of the first dilution to 900 µl ultrapure water (7.2)]. The final concentration is 1 µg/l.

Dilution series of the E2-reference: Prepare the dilutions of E2 for the dose response curve in amber glass vials with a cap which is lined with polytetrafluoroethylene (PTFE). Table 1 summarizes the preparation of the E2 dilution series for the dose response curve. Use ultrapure water (7.2) for the dilution series. For the dose response curve, seven E2 concentrations are used (1 ng/l, 2 ng/l, 4 ng/l, 8 ng/l, 20 ng/l, 40 ng/l, 80 ng/l E2). After addition of the yeast cells to the test well the final E2 concentrations are 0,8 ng/l, 1,6 ng/l, 3,2 ng/l, 6,4 ng/l, 16 ng/l, 32 ng/l, 64 ng/l E2.

The total volumes of the E2 dilutions shown in Table 1 are sufficient for one test plate with two replicates per dilution level (400 µl per replicate).

Table 1 — Preparation of E2 dilution series for the dose response curve

E2 starting solution	Volume E2 starting solution	Volume of water	c(E2) final solution	Total volume
1 µg/l	0,56 ml	6,44 ml	80 ng/l	7 ml
80 ng/l	2,5 ml	2,5 ml	40 ng/l	5 ml
40 ng/l	2,5 ml	2,5 ml	20 ng/l	5 ml
20 ng/l	2,0 ml	3,0 ml	8 ng/l	5 ml
8 ng/l	2,5 ml	2,5 ml	4 ng/l	5 ml
4 ng/l	2,5 ml	2,5 ml	2 ng/l	5 ml
2 ng/l	2,5 ml	2,5 ml	1 ng/l	5 ml

9.1.2 Reactivation of the yeast

For one 96-well test plate one vial with lyophilized yeast is required.

Bring the yeast minimal medium with maltose (7.4.10) to room temperature before using it. Dilute the yeast minimal medium with maltose (7.4.10) 1→ 5 by adding 8 ml of ultrapure water (7.2) to 2 ml of the yeast minimal medium with maltose (7.4.10).

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Add to the vial with the lyophilized yeast 2 ml of the diluted yeast minimal medium with maltose and mix it well until the pellet is completely solved (e.g. use a lab mini shaker). Transfer the yeast in a closable reaction tube and centrifuge for approx. 20 s at 3 000 *g*. After that carefully remove and discard the supernatant liquid and transfer it into a collecting vessel.

Add 1 ml of the diluted yeast minimal medium with maltose to the pellet and resuspend it (carefully up and down pipetting is recommended). Afterwards, centrifuge the reaction tube for approx. 20 s at 3 000 *g*. After that carefully remove and discard the supernatant liquid and transfer it into a collecting vessel.

Add 1 ml of the diluted yeast minimal medium maltose to the pellet and resuspend it (carefully up and down pipetting is recommended). Afterwards, centrifuge the reaction tube for approx. 20 s at 3 000 *g*. After that carefully remove and discard the supernatant liquid and transfer it into a collecting vessel.

Add 1 ml of the diluted yeast minimal medium with maltose to the pellet and resuspend it (carefully up and down pipetting is recommended). Fix the reaction tube with the yeast in a horizontal position on a shaker platform and incubate for approx. 1 h at 30 °C ± 2 °C. The frequency of the shaker depends on the orbit and should be 50 % of the frequency range given in Table 2.

Table 2 — Shaker frequency subject to the shaker orbit

Orbit mm	Frequency rpm
3 to 4,5	750 to 1 000
15 to 20	450 to 480
25	370 to 390

Lab shakers with the same shaker orbit from different manufactures may have different orbit vibration characteristics. Evaluation of the optimum shaker frequency may thus be necessary.

9.1.3 Negative control

Use ultrapure water (7.2) as negative control. Each 96-well plate shall contain at least two replicates of the negative control. Use a volume of 400 µl per replicate.

9.1.4 Blank replicate

For each sample add at least two blank replicates (3.1), 400 µl each, on the same 96-well plate. Use the undiluted sample for the blank replicates. If it proves necessary, that the sample has to be diluted for the blank replicates (e.g. in case of very high microbial load) this has to be taken into account in Formula (6) (correction of sample coloration and/or artificial substrate cleavage). If so, replace the denominator in Formula (6) by the portion of sample in the sample blanks.

For the negative control and the E2-reference two blank replicates for each are sufficient. For the blank of the reference 17β-estradiol (7.1.4) the calibration level (reference dilution) 80 ng/l is used.

9.1.5 Sample dilution

Homogenize the sample before use by shaking. Prepare successive dilutions of the test sample with the test sample specific dilution water (see Annex E for a dilution series).

For samples with conductivity equal or less than 8,0 mS/cm use ultrapure water (7.2) as dilution water.

For samples with conductivity greater than 8,0 mS/cm the conductivity of the dilution water needs to be adjusted with NaCl (7.1.30) to an equal value. In case of sea water samples or brackish water samples NaCl may be replaced by a salt composition similar to the salt composition of sea water, e.g. by sea salts (7.1.28).

Test at least four replicates for each dilution level.

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