INTERNATIONAL STANDARD

ISO 19040-2

First edition
2018-08

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*)*

Reference number
ISO 19040-2:2018(E)

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Published in Switzerland

Contents

Page

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.

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).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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.

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.

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]

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]

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.

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.

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.

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 17β-estradiol (E2) stock solution.

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

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

7.4.2 17β-estradiol (E2) working solution.

Dilute 17β-estradiol (E2) stock solution (7.4.1) 1→100 by adding 10 µl of the 17β-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 17β-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:

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.

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.

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 17β-estradiol working solution (7.4.2). Dilute the working solution 1→100 [e.g. by adding 10 µl of the 17β-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).

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.

A recommended set up for chemicals and extracts is shown in Annex D.

NOTE If the conductivity of a series of samples differs not more than 10 % the mean conductivity for these samples can be used for adjustment of the conductivity of the dilution water.

9.1.6 Field blank

Test at least the undiluted field blank (3.6) in four replicates. In this case the blank replicates of the negative control may be used for the field blank also.

If appropriate the field blank may also be analysed like a sample. If so, prepare appropriate dilutions of the field blank as described in 9.1.5. Concerning the blank replicates proceed as described in 9.1.7.

For each sample a representative field blank has to be analysed using equivalent media, cell culture and equipment. If a field blank is representative for a series of samples it may be tested only once.

9.1.7 Plate set up

Use a volume of 400 µl for each well (sample, sample dilutions, dilutions of reference (E2), negative control, field blank, blank replicates).

For each sample, appropriate dilutions should be tested (for waste water samples at least seven dilution levels are recommended). Test at least four replicates of each dilution level on the same 96-well plate. Test for each sample at least two blank replicates on the same 96-well plate. Use the undiluted sample or the highest sample concentration that is analysed in the test for the blank replicates.

Additionally, the sample plate shall contain at least two replicates of the negative control and two replicates for the blank of the negative control.

Test on each sample plate a dose response relationship of the reference 17β-estradiol (7.1.4) in duplicate. Test for the calibration level (reference dilution) 80 ng/l E2 at least two blank replicates.

A recommended plate setup is shown in Annex A.

The inoculation with the reactivated yeast cells has to be done within 2 h after the plate setup.

A recommendation for an alternative test design (plate set up) with only one sample dilution is shown in Annex I.

9.1.8 Inoculation of the test plate

9.1.8.1 Preparation of test sample specific inoculation medium

Prepare for every sample the inoculation medium.

For samples with a conductivity equal or lesser than 8,0 mS/cm use saline yeast minimal medium with maltose (7.4.11) as inoculation medium. If only few dilution levels of a test sample should be analysed and no prior knowledge of the estrogenic activity of the test sample exists it's also possible to use yeast minimal medium with maltose (7.4.10) as inoculation medium.

Adjust the conductivity of the inoculation medium for samples with conductivity greater than 8,0 mS/cm by mixing different volume fractions of yeast minimal medium (7.4.10) and saline yeast minimal medium (7.4.11) as follows: use 0,143 × c(sample) ml of the yeast minimal medium with maltose (7.4.9) and [5 – 0,143 × c(sample)] ml of the saline yeast minimal medium with maltose (7.4.11), where c(sample) denotes the salinity of the sample. Estimate the salinity from the conductivity by a dilution series of NaCl (7.1.30) in water (7.2) or measure the salinity with an appropriate measuring device. Mix the volume fractions of the two media.

For test samples with a conductivity greater or equal 54,2 mS/cm use the yeast minimal medium with maltose only (7.4.10).

EXAMPLE A test sample has a conductivity of 27,9 mS/cm at 20 °C. By means of a dilution series of NaCl in ultrapure water at 20 °C the relation between conductivity and salinity was determined and salinity of the sample was calculated to be 18. The volume of yeast minimal medium with maltose (7.4.9) is 0,143 × 18 = 2,57 ml. The volume of saline yeast minimal medium with maltose (7.4.11) is 5 – 0,143 × 18 = 2,43 ml.

NOTE If the conductivity of a series of samples differs not more than 10 % the mean conductivity for these samples can be used for adjustment of the conductivity of the inoculation medium.

9.1.8.2 Inoculation of wells without test sample

For inoculation of the blank for the negative control and the blank for the reference compound proceed as follows: Pipette 100 µl of the saline yeast minimal medium with maltose (7.4.11) in the respective wells.

For inoculation of the negative control, the reference compound proceed as follows: Fill 4,8 ml of the saline yeast minimal medium with maltose (7.4.10) and 0,25 ml of reactivated yeast (9.1.2) into one tube (e.g. 15 ml tube). Gently sway the tube and pour the yeast minimal medium mix into a pipette trough. Inoculation is performed by pipetting 100 µl of the prepared yeast minimal medium mix in wells of the deep well plate (multi-channel pipette or multi-stepper recommended).

If for samples with conductivity equal or lesser than 8,0 mS/cm, the yeast minimal medium with maltose (7.4.10) is used as inoculation medium, then this same medium shall also be used for the inoculation of wells without test sample. In this case please ensure that only samples with conductivity equal or lesser than 8,0 mS/cm are analysed in parallel with one 96-deep well plate.

9.1.8.3 Inoculation of wells with test sample

Pipette 100 µl of sample specific inoculation medium (9.1.8.1) to the sample blanks.

Mix the remaining volume (4,8 ml) of the sample specific inoculation medium with 0,25 ml of reactivated yeast (9.1.2) in one tube (e.g. 15 ml tube). Gently sway the tube and pour the yeast minimal medium mix into a pipette trough. Inoculate the respective sample and the respective sample dilutions by pipetting 100 µl of the prepared sample specific inoculation medium with yeast in the respective wells of the deep well plate (multi-channel pipette or multi-stepper recommended).

After inoculation of the complete test plate seal the deep well plate with the air permeable foil and centrifuge briefly (max. 200 x *g*, 10 s). Place the deep well plate on the platform of a shaking incubator and incubate the plate at 30 °C till 32°C (±2 °C) for 22 h ± 2 h. The frequency of the shaker depends on the shaker orbit and should in the frequency range given in Table 2.

9.2 Measurement

9.2.1 Measurement of the reporter gene activity

Bring substrate buffer and developer to room temperature before using them. Prepare at least 15 ml of phytase substrate solution (7.4.12).

Put the deep well plate into a suitable centrifuge with a rotor for microtiter plates. Centrifuge the cells for 10 min at 700 x *g*. After centrifugation, transfer 50 µl of the clear supernatant liquid out of each well into a new microtiter plate (multi-channel pipette recommended). Try to avoid bubbles and do not disrupt the yeast pellet. The pipette tips have to be replaced after each step.

Afterwards, add 50 µl of the prepared phytase substrate solution (7.4.12) in each well of the microtiter plate with the clear supernatant (multi-channel pipette recommended). Cover the microtiter plate with a lid and centrifuge it briefly (max. 200 x *g*, 10 s). Transfer the microtiter plate in an incubator for 1 h ± 5 min at 37 °C ± 2 °C.

After 1 h ± 5 min of incubation measure the absorption with a photometer at a wavelength of 405 nm ± 20 nm. Afterwards, fill 100 µl of developer solution in each of the wells (multi-channel pipette recommended) and measure again the absorption with a photometer at a wavelength of 405 nm ± 20 nm.

If the absorption measurements are out of the linear range of the photometer a further dilution may overcome this problem. It's also possible to reduce the incubation time slightly, e.g. from 1 h to 45 min.

9.2.2 Measurement of the cell density

Depending on the growth of the yeast cells in the deep well plate and the linear range of the photometer for microtiter plates a suitable dilution of the cell suspension in a whole volume of 300 µl has to be made. Determination of the quantifiable range of the photometer for microtiter plates can be made with a precise dilution series of yeast cell suspension and assessment of the relation between dilution and cell density.

In the case of sufficient yeast cell growth with a good shaped pellet after centrifugation of the deep well plate fill 290 µl of ultrapure water in every well of a 96-well microtiter plate (multi-channel pipette recommended). Mix the yeast cell suspension in the deep well plate thoroughly by vigorous shaking (a lab mini shaker is recommended). Transfer immediately 10 µl of yeast cell suspension per well of the deep well plate into the microtiter plate (avoid sedimentation of yeast cells, multi-channel pipette recommended). Measure the optical density at 630 nm ± 5 nm or alternatively at 600 nm ± 20 nm. In the case of poor yeast cell growth with a small or diffuse pellet use a lower dilution e.g. 270 µl of ultrapure water and 30 µl of cell suspension.

Use a second microtiter plate and a further dilution if the measurement values of the cell density are out of the linear range of the photometer for microtiter plates.

Calculate the final optical density for the negative control and reference compound by multiplication of the dilution factor with the background corrected optical density at 630 nm. Use the OD 630 value of the blank for the negative control for background correction. Calculate subsequently the mean optical density for the negative control and reference compound.

EXAMPLE Mixing of 30 µl yeast cell suspension and 270 µl ultrapure water corresponds to a dilution factor of 10. For an OD 630 value of 0,641 and a dilution factor of 10 the final OD is 0,641 × 10 = 6,41.

9.3 Calculations

Almost all calculations are based on the measurement of the reporter gene activity and cell density after addition of developer solution. Only for the assessment of artificial substrate cleavage the measurements of the absorbance at 405 nm before and after addition of developer solution are used.

An example for the evaluation of a test according to 9.3. is shown in Annex K.

9.3.1 Background correction

Calculate the background corrected absorbance $A_{BC,405}$ for every replicate j of test i (sample dilutions SD, reference dilutions RD, negative control NC) according to Formula (1):

$$A_{BC,405}(i,j) = A_{405}(i,j) - \overline{B_{405}}(NC) \quad (1)$$

where

$A_{BC,405}(i,j)$ is the background corrected absorbance at 405 nm for replicate j of test i;

$A_{405}(i,j)$ is the absorbance at 405 nm for replicate j of test i;

$\overline{B_{405}}(NC)$ is the (robust) mean of the absorbance at 405 nm for blank replicates of the negative control;

$\overline{B_{405}}(RD)$ is the (robust) mean of the absorbance at 405 nm for blank replicates of the reference.

Calculate the background corrected absorbance $A_{BC,630}$ for every replicate j of test i (sample dilutions SD, reference dilutions RD, negative control NC) according to Formula (2):

$$A_{BC,630}(i,j) = A_{630}(i,j) - \overline{B_{630}}(NC) \quad (2)$$

where

$A_{BC,630}(i,j)$ is the background corrected absorbance at 630 nm for replicate j of test i;

$A_{630}(i,j)$ is the absorbance at 630 nm for replicate j of test i;

$\overline{B_{630}}(NC)$ is the (robust) mean of the absorbance at 630 nm for blank replicates of the negative control;

$\overline{B_{630}}(RD)$ is the (robust) mean of the absorbance at 630 nm for blank replicates of the reference.

If no significant differences between $\overline{B_{405}}(NC)$ and $\overline{B_{405}}(RD)$, and $\overline{B_{630}}(NC)$ and $\overline{B_{630}}(RD)$, respectively, exist, the absorbance for the blanks of the negative control and the blanks for the reference can be used instead of the results of the negative control alone. Significance can be determined using variance analysis (significance level 1 %). Additionally, $\overline{B_{405}}(RD)$ and $\overline{B_{630}}(RD)$ should not deviate more than 30 % from $\overline{B_{405}}(NC)$ and $\overline{B_{630}}(NC)$, respectively.

9.3.2 Calculation of the relative growth

The relative growth $G(i, j)$ for every replicate j of test i (sample dilutions SD, reference dilutions RD) is calculated in order to assess yeast growths effects. The relative growth $G(i, j)$ is calculated according to Formula (3):

$$G(i,j) = \frac{A_{BC,630}(i,j)}{\overline{A_{BC,630}}(NC)} \quad (3)$$

where

$G(i,j)$ is the relative growth for replicate j of test i;

$A_{BC,630}(i,j)$ is the background corrected absorbance at 630 nm for replicate j of test i;

$\overline{A_{BC,630}}(NC)$ is the (robust) mean of the background corrected absorbance at 630 nm for replicates of the negative control;

$\overline{A_{BC,630}}(RD)$ is the (robust) mean of the background corrected absorbance at 630 nm for replicates of all reference dilutions.

If no significant differences between $\overline{A_{BC,630}}(NC)$ and $\overline{A_{BC,630}}(RD)$ exist, the absorbance for the negative control and the reference can be used instead of the results of the negative control alone. Significance can be determined using variance analysis (significance level 1 %). Additionally, the dilution specific mean corrected absorbance at 630 nm for the reference $\left[\overline{A_{BC,630}}(RD)\right]$ should not deviate more than 30 % from the mean corrected absorbance at 630 nm for the negative control $\left[\overline{A_{BC,630}}(NC)\right]$.

For each test *i* the relative standard deviation of the background corrected absorbance at 630 nm of replicates $\left[A_{BC,630}(i,j)\right]$ should be ≤15 %. Otherwise, technical problems during test performance may have occurred. The results for the relative standard deviation of $A_{BC,630}(i,j)$ have to be included in the test report.

Furthermore, the relative growth $\bar{G}(i)$ for every test *i* (sample dilutions SD, reference dilutions RD) is calculated according to Formula (4):

$$\bar{G}(i) = \text{mean}\left[G(i,j)\right] \tag{4}$$

where

$G(i,j)$ is the relative growth for replicate *j* of test *i*;

$\bar{G}(i)$ is the relative growth for test *i*.

For the calculation of the mean a robust statistical approach is applicable.

9.3.3 Calculations for assessment of sample blanks

9.3.3.1 Calculations for assessment of sample coloration and artificial cleavage of substrate

Sample coloration

Calculate the relative absorbance A_D for sample S according to Formula (5):

$$A_D(S) = \frac{\overline{B_{405}}(S)}{\overline{B_{405}}(NC)} \tag{5}$$

where

A_D is the relative absorbance for sample S;

$\overline{B_{405}}(S)$ is the (robust) mean of the absorbance at 405 nm for blank replicates of sample S;

$\overline{B_{405}}(NC)$ is the (robust) mean of the absorbance at 405 nm for blank replicates of the negative control.

If $A_D(S)$ >1,5 the sample has a coloration similar to the product of the reporter enzyme and/or an artificial cleavage of the substrate takes place. If so, compute the correction value A_E for each sample dilution *i* of sample S according to Formula (6):

$$A_E(i) = \frac{\left[\overline{B_{405}}(S) - \overline{B_{405}}(NC)\right] \times P(i)}{0,8} \tag{6}$$

where

$A_E(i)$ is the correction value for sample dilution *i* of sample S;

$P(i)$ is the portion of sample for sample dilution *i*.

If $A_D(S) \leq 1{,}5$ the correction values $A_E(i)$ are set to 0.

EXAMPLE 250 µl sample, 150 µl dilution water and 100 µl inoculation medium gives a total volume of 500 µl. The relating portion of sample for this dilution *i* is *P(i)* = 250/500 = 0,5.

If no significant differences between $\overline{B_{405}}(NC)$ and $\overline{B_{405}}(RD)$ exist, the absorbance for the negative control and the reference can be used instead of the results of the negative control alone. Significance can be determined using variance analysis (significance level 1 %). Additionally, $\overline{B_{405}}(RD)$ should not deviate more than 30 % from $\overline{B_{405}}(NC)$.

Artificial cleavage of substrate

Based on the measurements of the absorbance at 405 nm of sample blanks, negative control blanks and reference blanks before and after addition of developer solution (9.2.1) it is possible to determine if the sample coloration may partially be caused by artificial substrate cleavage.

Calculate the difference D_{SC} for every replicate *j* of test *i* [sample blank SB, blank for negative control (NB), blank for the reference (RB)] according to Formula (7):

$$D_{SC}(i,j) = B_{405}(i,j) - B_{405,before}(i,j) \quad (7)$$

where

$D_{SC}(i,j)$ is the difference for replicate *j* of test *i*;

$B_{405}(i,j)$ is the absorbance at 405 nm for replicate *j* of test *i* after addition of developer solution;

$B_{405,before}(i,j)$ is the absorbance at 405 nm for replicate *j* of test *i* before addition of developer solution.

Furthermore, calculate the mean difference D_{SC} for every test *i* [sample blank (SB), blank for negative control (NB), blank for the reference (RB)] according to Formula (8):

$$\overline{D_{SC}}(i) = \text{mean}\left[D_{SC}(i,j)\right] \quad (8)$$

Artificial cleavage of substrate takes place if the mean difference for sample blanks $\left[\overline{D_{SC}}(SB)\right]$ minus the mean difference for negative control blanks $\left[\overline{D_{SC}}(NB)\right]$ amounts not more than 15 % of the mean corrected absorbance at 405 nm of the negative control $\left[\overline{A_C}(NC)\right]$ [see Formulae (13) and (14), respectively].

As alternative, use the parameter *a* instead of the corrected absorbance at 405 nm of the negative control [*a* is the bottom curve point of the four-parameter model which describes the dose response relationship between the reference compound and the corrected absorbance (see Annex H for additional information)].

If no significant differences between $D_{SC}(NB)$ [differences for negative control blanks (NB)] and $D_{SC}(RB)$ [differences for reference blanks (RB)] exist, the mean difference $\overline{D_{SC}}(NB,RB)$ for negative

control blanks (NB) and reference blanks (RB) can be used instead of the results for negative control blanks $\left[\overline{D_{SC}}(\mathrm{NB})\right]$ alone. Significance can be determined using variance analysis (significance level 1 %).

It has to be included in the test report if the sample shows coloration similar to the product of the reporter enzyme and/or artificial cleavage of the substrate takes place. However, it should be noted that these effects are considered and corrected by the described calculation procedures.

9.3.3.2 Calculations for assessment of sample turbidity

Calculate the relative absorbance A_F for sample S according to Formula (9):

$$A_F(\mathrm{S}) = \frac{\overline{B_{630}}(\mathrm{S})}{\overline{B_{630}}(\mathrm{NC})} \tag{9}$$

where

A_F is the relative absorbance for sample S;

$\overline{B_{630}}(\mathrm{S})$ is the (robust) mean of the absorbance at 630 nm for blank replicates of sample S;

$\overline{B_{630}}(\mathrm{NC})$ is the (robust) mean of the absorbance at 630 nm for blank replicates of the negative control.

If no significant differences between $\overline{B_{630}}(\mathrm{NC})$ and $\overline{B_{630}}(\mathrm{RD})$ exist, the absorbance for the negative control and the reference can be used instead of the results of the negative control alone. Significance can be determined using variance analysis (significance level 1 %). Additionally, $\overline{B_{630}}(\mathrm{RD})$ should not deviate more than 30 % from $\overline{B_{630}}(\mathrm{NC})$.

If the relative absorbance A_F for sample S is >3 the sample might be turbid. One reason may be the relatively high microbial load of the sample. On the other hand there may be particles present in the sample. In this case, (sterile) filtration of the respective sample may prove useful. Anyway, this has to be included in the test report.

9.3.4 Calculation of the reporter gene induction

Calculate the corrected absorbance A_C for every replicate j of test i (sample dilutions SD, reference dilutions RD, negative control NC).

Calculation of the corrected absorbance for sample dilutions

Calculate the corrected absorbance A_C for every replicate j of sample dilution i and sample S according to the following procedure:

Determine the significance (significance level 1 %) of growth effects across all sample dilutions i for sample S using the background corrected absorbance at 630 nm $A_{BC,630}(i,j)$ for sample S. Significance of growth effects can be determined using variance analysis.

Furthermore calculate the mean relative growth $\overline{\overline{G}}$ for sample S according to Formula (10):

$$\overline{\overline{G}}(\mathrm{S}) = \mathrm{mean}\left[\overline{G}(i)\right] \tag{10}$$

where

$\bar{\bar{G}}(\mathrm{S})$ is the mean relative growth for sample S;

$\bar{G}(i)$ is the mean relative growth for sample dilution *i* of sample S.

For the calculation of the mean a robust statistical approach is applicable.

Perform a growth correction if:

1) there are significant growth effects between sample dilutions of sample S and if the relative growth $\bar{G}(i)$ for the dilution level with the lowest amount of sample is ≥ 0,85;

   OR

2) there are no significant growth effects between sample dilutions of sample S, but the mean relative growth $\bar{\bar{G}}(\mathrm{S})$ is statistically significant > 1.

Perform the growth correction by calculation of the corrected absorbance A_C for every replicate *j* of sample dilution *i* and sample S according to Formula (11):

$$A_C(i,j) = \frac{A_{BC,405}(i,j) - A_E(i) - \overline{A_{BC,405}}(\mathrm{NC})}{\bar{G}(i)} + \overline{A_{BC,405}}(\mathrm{NC}) + \overline{B_{405}}(\mathrm{NC}) \quad (11)$$

If no growth correction is necessary, calculate the corrected absorbance A_C for every replicate *j* of sample dilution *i* and sample S according to Formula (12):

$$A_C(i,j) = A_{BC,405}(i,j) - A_E(i) + \overline{B_{405}}(\mathrm{NC}) \quad (12)$$

where

$A_C(i,j)$ is the corrected absorbance for replicate *j* of sample dilution *i* of sample S;

$A_{BC,405}(i,j)$ is the background corrected absorbance at 405 nm for replicate *j* of sample dilution *i* of sample S;

$\overline{A_{BC,405}}(\mathrm{NC})$ is the mean background corrected absorbance at 405 nm of the negative control. As alternative, use the background corrected parameter a instead, i.e. $a - \overline{B_{405}}(\mathrm{NC})$ [a is the bottom curve point of the four-parameter model which describes the dose response relationship between the reference compound and the corrected absorbance (see Annex H for additional information)];

$\overline{B_{405}}(\mathrm{NC})$ is the (robust) mean of the absorbance at 405 nm for blank replicates of the negative control;

$A_E(i)$ is the correction value for sample dilution *i* of sample S;

$\bar{G}(i)$ is the relative growth for sample dilution *i* of sample S.

Calculation of the corrected absorbance for dilutions of the reference compound and negative control

Calculate the corrected absorbance A_C for every replicate *j* of reference dilution *i* according to the following procedure:

Determine the significance (significance level 1 %) of growth effects across all reference dilutions *i* using the background corrected absorbance at 630 nm $A_{BC,405}(i,j)$. Significance of growth effects can be determined using variance analysis.

Perform a growth correction if there are significant growth effects between reference dilutions and if the relative growth for the reference dilution 1 ng/l is ≥ 0,85. Calculate the corrected absorbance A_C for every replicate j of reference dilution i according to Formula (13).

$$A_C(i,j) = \frac{A_{BC,405}(i,j) - \overline{A_{BC,405}}(NC)}{\bar{G}(i)} + \overline{A_{BC,405}}(NC) + \overline{B_{405}}(NC) \quad (13)$$

If no growth correction is necessary, calculate the corrected absorbance A_C for every replicate j of reference dilution i according to Formula (14):

$$A_C(i,j) = A_{BC,405}(i,j) + \overline{B_{405}}(NC) \quad (14)$$

where

$A_C(i,j)$ is the corrected absorbance for replicate j of reference dilution i;

$A_{BC,405}(i,j)$ is the background corrected absorbance at 405 nm for replicate j of reference dilution i;

$\overline{A_{BC,405}}(NC)$ is the background corrected absorbance at 405 nm for the negative control;

$\overline{B_{405}}(NC)$ is the (robust) mean of the absorbance at 405 nm for blank replicates of the negative control;

$\bar{G}(i)$ is the relative growth for reference dilution i.

Estimated growth correction factors can be used instead of $\bar{G}(i)$ in order to reduce the impact of imprecise growth measurements.

Calculate the corrected absorbance A_C for every replicate j of the negative control according to Formula (14).

Calculation of the mean corrected absorbance

Calculate subsequently the mean corrected absorbance $\bar{A}_C(i)$ for every test i (sample dilutions SD, reference dilutions RD, negative control NC) using the corrected absorbance $A_C(i,j)$ for every replicate j of test i. For the calculation of the mean a robust statistical approach is applicable.

Calculation of induction rates

Calculate the induction rate I for test i (sample dilutions SD, reference dilutions RD, negative control NC) according to Formula (15).

$$I(i) = \frac{\bar{A}_C(i)}{\bar{A}_C(NC)} \quad (15)$$

where

$I(i)$ is the induction rate for test *i*;

$\bar{A}_C(i)$ is the (robust) mean of the corrected absorbance of all replicates of test *i*;

$\bar{A}_C(NC)$ is the (robust) mean of the corrected absorbance of all replicates of the negative control. As alternative, use the parameter *a* instead (*a* is the bottom curve point of the four-parameter model which describes the dose response relationship between the reference compound and the induction rate [see Annex H for additional information)].

For the construction of the dose-response curve use the corrected absorbance of all replicates of the negative control and the dilution series of the reference compound $\left[A_C(i,j)\right]$.

Use the mean corrected absorbance $\bar{A}_C(i)$ for the calculation of estradiol equivalents (see Annex H for additional information). Use the mean corrected absorbance $\bar{A}_C(i)$ or the induction rates $I(i)$ for the calculation of the lowest ineffective dilution (see Annex J for additional information).

10 Validity criteria

The overall test is valid if:

a) the EC_{50} of the 17β-estradiol dose response relationship is ≥7 and ≤35 ng/l (see Annex H for additional information) (use an appropriate statistical method for calculation of the EC_{50} (References [16],[17],[18]));

b) the critical concentration, which corresponds to the concentration above an estrogenic effect is measurable (see Annex G for additional information), is ≤8 ng/l 17β-estradiol (use an appropriate statistical method for calculation of the dose response curve and the calculation of the critical concentration for the induction rate 1,18 (References [16],[17],[18]));

c) the ratio d / a is ≥4 and ≤20 (see Annex H for additional information) (use an appropriate statistical method for calculation of bottom curve point (a) and the curve plateau (d) of the dose response curve (References [16],[17],[18]));

d) the ratio between the mean corrected absorbance at 405 nm for the highest concentration of the reference standard (80 ng/l 17β-estradiol) and the curve plateau (d) of the dose response curve is ≥0,75 (use an appropriate statistical method for calculation of the curve plateau (d) of the dose response curve (References [16],[17],[18]));

e) the mean relative standard deviation (RSD) of the corrected absorbance at 405 nm of replicates for the negative control and the dilution series of the reference compound is ≤12 % (see Annex H for additional information) (use an appropriate statistical method for calculation of the dose response curve (References[16],[17],[18]));

f) the mean OD for the cell density of the negative control and the dilution series of the reference compound is ≥1 after 22 h incubation.

A dilution level of a sample is valid if:

g) the relative growth of yeast cells in this dilution is ≥0,3 and ≤3,5;

h) the relative standard deviation of the corrected absorbance at 405 nm of replicates is ≤15 %.

If criterion (h) is not met and more than 3 replicates are available an outlier test (Grubbs test) can be performed. If afterwards criterion (h) is again not met then exclude all replicates from further statistical evaluation. Alternatively a statistically robust calculation of this standard deviation can be applied.

Usually the fulfilment of criterion (a) and (b) can be achieved by precise adjustment of the shaker frequency.

11 Assessment criteria

The test sample is assessed to mediate an estrogenic activity, if an increase of reporter gene activation is measured in terms of the induction rate, which exceeds the critical limit 1,18 [see Annex G for a description of the determination of the critical limit (CL)]. Additionally, the estrogenic activity of the field blank, which is also measured in terms of the induction rate, shall not exceed the critical limit 1,18.

If the estrogenic activity of the field blank exceeds the induction rate 1,18 the critical limit is set to the induction rate of the field blank. In this case, the test sample is assessed to mediate an estrogenic activity if the induction rate of the sample exceeds the induction rate of the field blank.

12 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this document, i.e. ISO 19040-2:2018);

b) identity of the test sample (origin and date of sampling, pH value, conductivity);

c) reference compound (chemical name, source, batch number or comparable data, if available);

d) storage of sample and preparation of test sample (storage conditions (if not tested directly), adjustment of pH value, centrifugation (including *g* and time), filtration (including filter material and pore size) and other manipulations);

e) test strain (strain, date of arrival in the laboratory, storage conditions);

f) testing environment (address of testing laboratory, date of test, method of counting);

g) results for validity criteria and fulfilment (see Clause 10);

h) results for plausibility criteria (turbidity and microbial load of the sample (see 9.3.3.2), sample coloration and/or artificial substrate cleavage (see 9.3.3.1), for each dilution level of a sample the relative standard deviation of the background corrected absorbance at 630 nm of replicates $A_{\mathrm{BC},\,630}(i,j)$ (see 9.3.2);

i) test results (induction rates I and corrected absorbance A_{C} for each tested sample, qualitative assessment of the estrogenic activity of each tested sample (yes/no), if required EEQ and LID for each tested sample), indication of toxic effects (if any), other observations (e.g. precipitation, contamination). If 17β-estradiol equivalent (EEQ) values for the sample or sample dilutions are calculated then additional reporting as described in Annex H is recommended. If LID values are calculated then additional reporting is recommended as described in Annex J.

13 Verification

Before the method described in this standard is routinely used in the performing laboratory, it shall first demonstrate its competence to perform the method. The subsequent verification protocol describes the design of the verification and the performance characteristics used to assess the competence of the performing laboratory.

The verification protocol requires performing the method at least two times independently. In these tests both the reference compound and appropriate test samples should be analysed. Analysis of test samples is not required but highly recommended.

Performance evaluation in terms of the reference compound is made based on the criteria (a) to (f) according to Clause 10 of this document. As soon as the criteria (a) to (f) are fulfilled by the laboratory, the competence to perform the validated method is proven.

Performance evaluation in terms of the sample type is made based on the criteria (g) and (h) according to Clause 10 of this document. If the criterion (g) is fulfilled the method performance for the sample is assessed using criteria (h).

A lab specific quality control chart should be used to evaluate the stability and vitality of the lyophilized yeast cells by means of the criteria (a) to (f) according to Clause 10 of this document. The quality control chart enables the laboratory to monitor the criteria for long-term periods and set specific decision limits for using the lyophilized yeast cells for test purposes.

For quality control charts either a spreadsheet based software or a validated software (e.g. mqVAL) for advanced requirements of quality assurance may be used.

Annex A
(informative)

Plate set up

A.1 General

This annex shows a scheme of a plate set up for the analysis of two samples with eight successive dilution levels (See Table A.1).

A.2 Scheme of plate set up

Table A.1 — Plate set up with two samples with eight successive dilution levels in four replicates

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank S1	*NC*	S1 (dilution level 16)				*NC*	S2 (dilution level 16)				FB S1
B	Blank S1	E2, *CL* 1	S1 (dilution level 12)				E2, *CL* 1	S2 (dilution level 12)				
C	Blank S2	E2, *CL* 2	S1 (dilution level 8)				E2, *CL* 2	S2 (dilution level 8)				
D	Blank S2	E2, *CL* 3	S1 (dilution level 6)				E2, *CL* 3	S2 (dilution level 6)				
E	Blank *NC*	E2, *CL* 4	S1 (dilution level 4)				E2, *CL* 4	S2 (dilution level 4)				FB S2
F	Blank *NC*	E2, *CL* 5	S1 (dilution level 3)				E2, *CL* 5	S2 (dilution level 3)				
G	Blank R_{CL7}	E2, *CL* 6	S1 (dilution level 2)				E2, *CL* 6	S2 (dilution level 2)				
H	Blank R_{CL7}	E2, *CL* 7	S1 (dilution level 1)				E2, *CL* 7	S2 (dilution level 1)				

NC negative control
S1 sample 1
S2 sample 2
E2 17β-estradiol
CL calibration level (reference dilution)
Blank S1 blank for sample 1
Blank S2 blank for sample 2
Blank R_{CL7} blank for the reference compound, calibration level (reference dilution) 7
FB S1 field blank for sample 1
FB S2 field blank for sample 2

Annex B
(informative)

Lyophilization of *Arxula adeninivorans* cells

B.1 General

Annex B describes the preparation of lyophilized *Arxula adeninivorans* cells.

B.2 Reagents

B.2.1 Glucose, ≥99,5 %, $C_6H_{12}O_6$, molecular weight 180,16 g/mol, CAS: 50-99-7.

B.2.2 Sorbitol, ≥98 % for biochemistry, $C_6H_{14}O_6$, molecular weight 182,18 g/mol, CAS: 50-70-4.

B.2.3 di-Potassium hydrogen phosphate, >99 %, K_2HPO_4, molecular weight 174,18 g/mol, CAS: 7758-11-4.

B.2.4 Agar, Kobe I

B.2.5 L-Lysin, ≥98,5 %, Ph. Eur. for biochemistry, $C_6H_{14}N_2O_2$, molecular weight 146,19 g/mol, CAS: 56-87-1.

B.2.6 Milk powder blotting grade, low-fat, CAS: 68514-61-4.

B.2.7 Sucrose, ≥99,5 %, p.a., $C_{12}H_{22}O_{11}$, molecular weight 342,30 g/mol, CAS: 57-50-1.

B.3 Media

B.3.1 Glucose solution

Dissolve 20 g of glucose (B.2.1) in 70 ml of ultrapure water (7.2). Fill the glucose solution up to 100 ml with ultrapure water (7.2). Autoclave the solution.

B.3.2 Sorbitol solution

Dissolve 20 g of sorbitol (B.2.2) in 70 ml of ultrapure water (7.2). Fill the sorbitol solution up to 100 ml with ultrapure water (7.2). Autoclave the solution.

B.3.3 PBS buffer

Weigh 2,25 g NaCl (7.1.29), 0,435 g K_2HPO_4 (B.2.3) and 0,34 g KH_2PO_4 (7.1.7) and dissolve the reagents in 250 ml ultrapure water (7.2). Autoclave PBS.

B.3.4 Cryo medium

Dissolve 45 g of sorbitol (B.2.2) and 100 g of sucrose (B.2.7) in 600 ml of ultrapure water (7.2). Add 100 g of milk powder (B.2.6) and adjust the pH to 7,5 with 1 M NaOH (7.1.2) and fill up to 1 000 ml with ultrapure water (7.2). Sterilize the medium four times for 30 min in a stream vessel.

B.3.5 Lysin solution

Dissolve 1 g of lysin (B.2.5) in 100 ml of ultrapure water (7.2). Filter the lysin solution through a sterile filter (0,2 µm, cellulose acetate or cellulose mixed ester) in a sterile glass bottle with screw cap.

B.3.6 Yeast minimal medium (YMM) with glucose

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 glucose solution (B.3.1) to the prepared mix. The yeast minimal medium with glucose is five-fold concentrated.

B.3.7 Yeast minimal medium (YMM) with sorbitol

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 sorbitol solution (B.3.2) to the prepared mix. The yeast minimal medium with sorbitol is five-fold concentrated.

B.4 Cultivation of *Arxula adeninivorans* on solid agar

Dissolve 3 g of Agar (B.2.4) in 100 ml of ultrapure water (7.2) and heat the agar to the boiling point. Stir the solution. Autoclave the Agar. Cool the Agar down to approximately 60 °C.

Dilute 40 ml of yeast minimal medium with glucose (B.3.6) with 60 ml of ultrapure water (7.2) and warm up. Merge the warmed-up yeast minimal medium with glucose with the agar and mix the solution. Pour approximately 20 ml of the yeast minimal medium agar in sterile Petri-Dishes. Spread *Arxula adeninivorans* cells on yeast minimal medium agar plate. Incubate the yeast minimal medium agar plate at 30 °C for 72 h.

B.5 Cultivation of starter and main culture

Inoculate 10 ml of one-fold concentrated yeast minimal medium with glucose (B.3.6) with 10^7 cells of *Arxula adeninivorans* from an agar plate and cultivate the starter culture at 30 °C for 48 h on a shaker.

Transfer 5 ml from the pre-culture to 500 ml one-fold concentrated yeast minimal medium with sorbitol (B.3.7). Cultivate the main culture in Erlenmeyer flasks at 30 °C for 48 h on a shaker.

B.6 Preparing of lyophilized *Arxula adeninivorans* cells

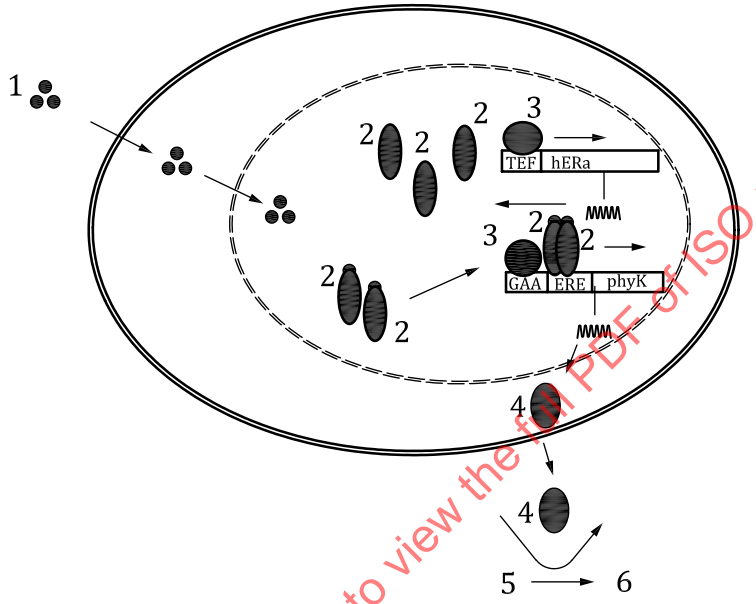
Transfer the cell suspension from the main culture to centrifuge containers (determine weight of container) and centrifuge the culture at 4 °C for 10 min at 2 000 g. Discard the supernatant. Wash cell pellet in 30 ml of PBS and transfer it to a 50 ml tube. Centrifuge the tube at 2 000 g for 15 min at 4 °C. Discard the supernatant. Determine weight of cell pellet.

Add 10 ml lysin solution (B.3.5) to 100 ml of cryo medium and resuspend the pellet (use 5 ml for 1 g bio moist mass). Cool the mixture down at 4 °C for 10 min. Aliquote 200 µl of cool cells to lyophilization vials and cool down to −80 °C. Cool down lyophilization plant, insert vials with frozen cells on storage space and start the program with the following lyophilization parameters: Ice Condenser: −80 °C; Main Drying: Pressure: 0,05 mbar; Temperature Storage space 10 °C; Safety pressure 0,1 mbar; 6 h to 12 h process.

Annex C
(informative)

Scheme of test principle

Annex C illustrates the underlying test principle of the *Arxula adeninivorans* yeast estrogen screen (See Figure C.1).



Key

1 endocrine disruptor
2 human estrogen receptor alpha
3 polymerase
4 phytase
5 4-Nitrophenylphosphate
6 4-Nitrophenol + phosphate

Figure C.1 — Scheme of the test principle in *Arxula adeninivorans*

The (xeno)estrogen (Estrogen Disruptor) diffuses into *Arxula adeninivorans* cell. The human estrogen receptor (hERα) is heterologously expressed [the respective gene is under the control of a constitutive promoter (TEF)]. The estrogen receptor construct is integrated in the *Arxula adeninivorans* genome. The Estrogen Disruptor binds to the estrogen receptor and induces a dimerization of hERα. Due to receptor binding and dimerization a nuclear localization signal is exposed and the dimer is imported in the cell nucleus. The receptor dimer binds to and activates a promoter that contains an estrogen responding element (ERE). The reporter construct is integrated in the *Arxula adeninivorans* genome. The expression of the reporter gene is proportional to the activation of the hERα by the Estrogen Disruptor. The reporter Phytase is secreted to extracellular matrix and cleaves 4-Nitrophenyl phosphate into 4-Nitrophenol and phosphate. 4-Nitrophenol can be measured photometrical.

Annex D
(informative)

Test set up for chemicals and extracts

D.1 General

For the analysis of certain matrices such as surface water or drinking water the samples need to be concentrated by extraction procedures. Annex D gives information about possible extraction procedures and the changes in the procedure for testing extracts and pure compounds.

D.2 Extraction of water samples

Water sample extracts can also be tested with the following adaptations in the procedure. Extract water samples using liquid-liquid extraction (LLE) or solid phase extraction (SPE); both methods generally show high (>90 %) recoveries for known (xeno)estrogens. For LLE, extract the water three times with ethyl acetate, using approximately 10 % of the sample size as extraction solvent volume. For SPE, extract with suitable columns, e.g. Oasis HLB or C18, and elute with methanol or ethyl acetate. Evaporate the organic solvent and dissolve the final extracts in ultrapure water.

D.3 Test with diluted organic solutions or extracts

The procedure is identical to the description in Clause 9. Use only saline yeast minimal medium with maltose (7.4.11) for inoculation.

Use 400 µl of the aqueous extract for each well and add 100 µl of the prepared cell suspension (inoculation medium) to the negative control, the sample dilutions and the dilution series of the reference compound. For the preparation of the field blank use the sample procedure and materials as for the preparation of a sample extract or the preparation of an organic solution of a chemical.

Annex E
(informative)

Preparation of dilution series

Table E.1 describes the preparation of dilution series for the testing of water samples.

Table E.1 — Preparation of dilution series

Dilution level *D*	Pre-dilution of the sample with dilution water	Dilution of the sample by addition of the test strain in culture medium	Final dilution of sample (1→x) with dilution water and cell suspension	Final percentage of the sample in the test
1	1→1 No pre-dilution. The sample is used as it is.	1→1,25 400 µl sample + 100 µl test strain in culture medium	1→1 (per definition) For undiluted water or waste water, the dilution coefficient per definition is 1→1 even if the sample is diluted by the addition of the test strain in culture medium.	80 %
2	1→1,6 e.g. 1 ml sample + 0,6 ml dilution water	1→1,25	1→2	50 %
3	1→2,4 e.g. 1 ml sample + 1,4 ml dilution water	1→1,25	1→3	33,3 %
4	1→3,2 e.g. 1 ml sample + 2,2 ml dilution water	1→1,25	1→4	25 %
6	1→4,8 e.g. 1 ml sample + 3,8 ml dilution water	1→1,25	1→6	16,7 %
8	1→ 6,4 e.g. 1 ml sample + 5,4 ml dilution water	1→1,25	1→8	12,5 %
12	1→ 9,6 e.g. 1 ml sample + 8,6 ml dilution water	1→1,25	1→12	8,3 %

Annex F
(informative)

Performance data

F.1 Validation study

An interlaboratory trial based on the procedure described in this document was carried out in fall 2015 for the generation of validation data. To assess the performance of the ISO 19040-2 method within the framework of the interlaboratory trial, the statistical analysis was conducted on the basis of ISO 5725-2.

F.1.1 Samples and participating laboratories

Fourteen laboratories participated in the interlaboratory trial for the validation of this standard, whereby thirteen laboratories reported valid results. Each participating laboratory tested nine water samples in the scope of the method (Table F.1 and Table F.2). The A-YES method is applicable for surface water, waste water, drinking water, sea water, brackish water, aqueous extracts and eluates, pore water, eluates from sediments, aqueous solutions of compounds and compounds mixtures as well as organic extracts of aqueous samples.

Table F.1 — Sample information for obligate samples

Sample	Description
1	Effluent, WWTP Koblenz
2	Effluent, WWTP Koblenz, spiked with 10 ng/l EE2
3	Influent, WWTP Koblenz, (centrifuged)
4	Surface water (Rhine)
5	Surface water (Rhine), spiked with 15 ng/l EE2
6	Field Blank (deionized water)
WWTP	Waste water treatment plant
EE2	17α-ethinylestradiol

Table F.2 — Sample information for ISO 19040-2 specific samples

Sample	Description
7	Influent, Hospital-WWTP, Gelsenkirchen
8	Mixture of single substances (39 µg/l Bisphenol Z, 48 µg/l Bisphenol S, 242 µg/l Bisphenol A)
9	Saline water (Baltic Sea), spiked with 1,4 ng/l E2 and 8 ng/l EE2
WWTP	Waste water treatment plant
E2	17β-estradiol
EE2	17α-ethinylestradiol

F.1.2 Results for lowest ineffective dilution (LID) and 17β-estradiol equivalent concentrations (EEQ)

Table F.3 summarizes the performance characteristics for the LID values. In case of large differences between subsequent dilution levels the LID is highly affected by dilution levels. By linear interpolation of the induction rate between subsequent dilution levels the theoretic dilution level can be derived at

which the induction rate achieves 1,18. This dilution level is denoted the "interpolated LID". Table F.4 summarizes the performance characteristics for the interpolated LID values.

Table F.5 summarizes the performance characteristics for the 17β-estradiol equivalents (EEQ).

The performance characteristics are based on the test results, which are fulfilling the validity criteria described in Clause 10.

Table F.3 — Summary of performance characteristics for LID values obtained with the *Arxula* Yeast Estrogen Screen (A-YES)

	Sample								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Number of laboratories	13	13	13	13	13	13	13	13	13
Number of measurements	28	28	28	29	28	28	29	29	29
Invalid measurements	6	4	5	5	5	6	8	5	5
% Invalid measurements	21,4	14,3	17,9	17,2	17,9	21,4	27,6	17,2	17,2
Number of measurements LID > 12	0	0	18 (20)	0	2 (5)	0	15 (21)	"> 8": 0	"> 8": 2
Number of measurements LID = 1	20	0	0	24	0	22	0	0	0
Outliers excluded (single measurements)	0	0	—	0	0	0	—	0	0
Outliers excluded (means)	0	0	—	0	0	0	—	0	0
Number of laboratories for statistics	13	13	3	12	12	13	4	12	12
Number of measurements for statistics	22	24	5	24	21	22	6	24	22
Laboratory mean - n									
L01	1 - 1	8,0 - 1	—	1 - 1	—	1 - 1	—	—	—
L02	1 - 1	6,0 - 3	18,0 - 2 >12,0 - 1	1 - 3	16,0 - 3	1 - 1	18,0 - 2 >12,0 - 1	4,0 - 3	8,0 - 2
L03	1 - 2	7,0 - 2	>12,0 - 2	1 - 2	12,0 - 1 >12,0 - 1	1 - 2	>12,0 - 2	4,0 - 2	6,0 - 2
L04	1 - 2	10,0 - 2	12,0 - 1 >12,0 - 1	1 - 2	12,0 - 2	1 – 2	24,0 - 1	4,0 - 1	6,0 - 2
L05	1 - 1	4,0 - 1	>12,0 - 1	—	8,0 - 1	1 - 1	>12,0 - 1	4,0 - 2	4,0 - 2
L06	1 - 1	12,0 - 1	>12,0 - 1	1 - 2	12,0 - 1	1 - 2	24,0 - 1 >12,0 - 1	4,0 - 2	4,0 - 2
L07	1 - 2	12,0 - 2	>12,0 - 2	1 - 2	12,0 - 2	1 - 2	>12,0 - 2	6,0 - 2	8,0 - 1 > 8,0 - 1
L08	1,3 - 3	6,7 - 3	>12,0 - 3	1 - 2	10,7 - 3	1 - 2	>12,0 - 3	4,0 - 3	6,7 - 3
L09	1 - 1	12,0 - 1	>12,0 - 2	1 - 2	12,0 - 1 > 12,0 - 1	1 - 2	>12,0 - 2	4,0 - 2	8,0 - 1 > 8,0 - 1
L10	2,5 - 2	5,0 - 2	>12,0 - 2	1 - 2	7,0 - 2	1 - 2	>12,0 - 1	4,0 - 1	8,0 - 1
L11	1 - 2	6,0 - 2	>12,0 - 1	1 - 2	12,0 - 2	1 - 2	>12,0 - 1	4,0 - 2	8,0 - 2
L12	1 - 2	5,0 - 2	>12,0 - 2	1 - 2	10,0 - 2	1 - 2	>12,0 - 1	4,0 - 2	6,0 - 2
L13	1 - 2	6,0 - 2	18,0 - 2	1 - 2	8,0 - 1	1 - 1	18,0 - 2	4,0 - 2	6,0 - 2

Table F.3 *(continued)*

min-max	1–2,5	4,0–12,0	≥12	1	7,0–16,0	1	≥12	4,0–6,0	4,0–8,0
factor max/min	2,5	3,0	—	1	2,3	1	—	1,5	2,0
	Sample								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Mean	1,2	7,3	>12,0	1,0	11,3	1,0	>12,0	4,2	6,4
95 % CI		5,9–8,8	—	—	9,9–12,8	—	—	3,8–4,5	5,5–7,2
95 % PI (based on Ln-trans, data)	—	1,8–12,9 (3,5–13,8)			9,7–13,0 (7,8–15,2)			3,8–4,5 (3,9–4,4)	5,4–7,3 (5,1–7,1)
99 % PI (based on Ln-trans, data)		0,0–15,1 (2,6–18,2)			9,1–13,6 (6,8–17,4)			3,6–4,7 (3,8–4,5)	5,1–7,7 (4,8–7,6)
s_r (repeatability)	0,8	1,2		0,0	3,6	0,0		0,8	2,1
s_L (interlab variability)	0,0	2,4		0,0	0,0	0,0		0,0	0,0
s_R (reproducibility)	0,8	2,7		0,0	3,6	0,0		0,8	2,1
s_r % (based on Ln-trans. data)	64,1 (37,7)	16,8 (17,7)		0,0 (0,0)	31,8 (24,8)	0,0 (0,0)		19,6 (14,1)	32,5 (35,8)
s_L % (based on Ln-trans. data)	0,0 (0,0)	33,3 (30,4)		0,0 (0,0)	0,0 (13,7)	0,0 (0,0)		0,0 (0,0)	0,0 (0,0)
s_R % (based on Ln-trans. data)	64,1 (37,7)	37,3 (35,2)		0,0 (0,0)	31,8 (28,3)	0,0 (0,0)		19,6 (14,1)	32,5 (35,8)
s_R/s_r	1,0	2,2		—	1,0	—		1,0	1,0

s_r repeatability standard deviation
s_R reproducibility standard deviation
s_L laboratory standard deviation

Table F.4 — Summary of performance characteristics for interpolated LID values obtained with the *Arxula* Yeast Estrogen Screen (A-YES)

	Sample								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Number of laboratories	13	13	13	13	13	13	13	13	13
Number of measurements	28	28	28	29	28	28	29	29	29
Invalid measurements	6	4	5	5	5	6	8	5	5
% Invalid measurements	21,4	14,3	17,9	17,2	20,7	21,4	27,6	17,2	17,2
Number of measurements LID > 12	0	0	20	0	3	0	18	“>8”: 0	“>8”: 2
Number of measurements LID = 1	20	0	0	24	0	22	0	0	0
Outliers excluded (single measurements)	—	0	0	—	0	—	0	0	0

Table F.4 *(continued)*

Outliers excluded (means)	—	0	0	—	0	—	0	0	0
Number of laboratories for statistics	13	13	12	12	12	13	12	12	12
Number of measurements for statistics	22	24	23	24	23	22	20	24	24
Laboratory mean - n									
L01	1 - 1	6,4 - 1	—	1 - 1	—	1 - 1	—	—	—
L02	1 - 1	4,9 - 3	15,8 - 3	1 - 3	13,4 - 3	1 - 1	16,4 - 3	3,2 - 3	6,6 - 2
L03	1 - 2	6,2 - 2	25,4 - 2	1 - 2	12,0 - 2	1 - 2	20,5 - 2	3,4 - 2	4,9 - 2
L04	1 - 2	7,9 - 2	16,0 - 2	1 - 2	10,3 - 2	1 - 2	12,1 - 1	3,4 - 1	4,9 - 2
L05	1 - 1	4,0 - 1	18,5 - 1	--	6,4 - 1	1 - 1	12,3 - 1	2,6 - 2	3,3 - 2
L06	1 - 1	10,1 - 1	18,7 - 1	1 - 2	10,6 - 1	1 - 2	17,6 - 2	3,9 - 2	3,6 - 2
L07	1 - 2	8,7 - 2	21,4 - 2	1 - 2	10,1 - 2	1 - 2	18,1 - 2	3,6 - 2	9,8 - 2
L08	1,1 - 3	5,6 - 3	22,4 - 3	1 - 2	8,9 - 3	1 - 2	21,9 - 3	3,1 - 3	5,1 - 3
L09	1 - 1	8,1 - 1	21,8 - 2	1 - 2	12,5 - 2	1 - 2	13,6 - 2	3,6 - 2	9,4 - 2
L10	2,2 - 2	4,6 - 2	18,7 - 2	1 - 2	6,3 - 2	1 - 2	21,7 - 1	2,8 - 1	5,0 - 1
L11	1 - 2	4,8 - 2	30,8 - 1	1 - 2	8,3 - 2	1 - 2	13,5 - 1	2,9 - 2	5,4 - 2
L12	1 - 2	4,1 - 2	17,0 - 2	1 - 2	7,6 - 2	1 - 2	18,3 - 1	3,4 - 2	4,3 - 2
L13	1 - 2	4,7 - 2	13,5 - 2	1 - 2	7,2 - 1	1 - 1	13,6 - 2	3,0 - 2	3,8 - 2
min-max	1 - 2,2	4,0 - 10,1	13,5 - 30,8	1	6,3 - 13,4	1	12,1 - 21,9	2,6 - 3,9	3,3 - 9,7
factor max/min	2,2	2,5	2,3	1	2,1	1	1,8	1,5	3,0
	Sample								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Mean	1,1	5,9	19,6	1,0	9,8	1,0	17,3	3,2	5,5
95 % CI	—	4,9 - 6,9	17,1 - 22,0	—	8,4 - 11,2	—	15,4 - 19,2	3,0 - 3,4	4,3 - 6,7
95 % PI (based on Ln-trans. data)		2,0–9,8 (3,1–10,5)	11,7–27,4 (13,2–27,1)		6,4–13,1 (6,1–14,4)		11,6–23,0 (12,0–23,7)	2,7–3,8 (2,7–3,8)	2,3–8,7 (2,9–8,8)
99 % PI (based on Ln-trans. data)		0,4–11,4 (2,4–13,4)	8,5–30,6 (11,4–31,4)		5,1–14,5 (5,1–17,2)		9,2–25,4 (10,4–27,3)	2,5–4,0 (2,6–4,0)	0,9–10,0 (2,3–11,0)
s_r (repeatability)	0,6	0,6	3,8	0,0	2,8	0,0	3,1	0,4	2,3
s_L (interlab variability)	0,0	1,7	3,3	0,0	1,4	0,0	2,4	0,2	1,3
s_R (reproducibility)	0,6	1,8	5,1	0,0	3,1	0,0	4,0	0,5	2,6
s_r % (based on Ln-trans. data)	51,1 (30,3)	9,5 (10,5)	19,4 (23,3)	0,0 (0,0)	28,6 (22,6)	0,0 (0,0)	18,2 (18,6)	12,7 (13,0)	41,6 (31,4)
s_L % (based on Ln-trans. data)	0,0 (0,0)	29,2 (26,9)	17,1 (15,0)	0,0 (0,0)	13,8 (18,2)	0,0 (0,0)	14,0 (14,4)	6,7 (6,5)	24,3 (23,4)
s_R % (based on Ln-trans. data)	51,1 (30,3)	30,7 (28,9)	25,9 (27,7)	0,0 (0,0)	31,7 (29,0)	0,0 (0,0)	22,9 (23,5)	14,4 (14,5)	48,2 (39,1)
s_R/s_r	1,0	3,2	1,3	—	1,1	—	1,3	1,1	1,2

Table F.4 *(continued)*

s_r	repeatability standard deviation
s_R	reproducibility standard deviation
s_L	laboratory standard deviation

Table F.5 — Summary of performance characteristics for 17β-estradiol equivalents obtained with the *Arxula* Yeast Estrogen Screen (A-YES)

	Sample								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Number of laboratories	13	13	13	13	13	13	13	13	13
Number of measurements	28	28	28	29	28	28	29	29	29
Invalid measurements	6	3	3	4	5	5	9	5	5
% Invalid measurements	21,4	10,7	10,7	13,8	17,9	17,9	31,0	17,2	17,2
Number of measurements < LOD	17	1	0	22	0	23	0	1	0
Outliers excluded (single measurements)	—	0	0	—	0	—	0	—	0
Outliers excluded (means)	—	0	0	—	0	—	0	1 (L05)	0
Number of laboratories for statistics	3	13	12	13	12	13	12	11	12
Number of measurements for statistics	5	24	25	3	23	0	20	21	24
Laboratory mean [ng/l E2] - n									
L01	<LOD	8,2 - 1	—	<LOD	—	<LOD	—	—	—
L02	<LOD	6,6 - 3	13,0 - 3	<LOD	20,0 - 3	<LOD	21,9 - 3	<LOD 5,2 - 2	10,0 - 2
L03	<LOD	12,8 - 2	18,2 - 2	<LOD	25,0 - 2	<LOD	27,4 - 2	8,2 - 2	11,3 - 2
L04	<LOD	10,7 - 2	11,9 - 2	<LOD	16,4 - 1	<LOD	17,0 - 1	6,3 - 1	8,9 - 2
L05	<LOD	7,4 - 1	15,3 - 1	<LOD	14,6 - 1	<LOD	20,9 - 1	5,5 - 2	6,9 - 2
L06	2,0 - 1	<LOD 6,3 - 1	14,0 - 2	<LOD	17,7 - 2	<LOD	19,7 - 2	7,0 - 2	7,9 - 2
L07	<LOD	12,2 - 2	21,7 - 2	<LOD	19,5 - 2	<LOD	23,1 - 1	6,7 - 2	12,0 - 2
L08	2,1 - 3	8,3 - 3	11,8 - 3	<LOD 1,8 - 1	14,5 - 3	<LOD	19,7 - 3	5,1 - 3	8,7 - 3
L09	<LOD	12,7 - 1	17,1 - 2	<LOD 1,9 - 1	19,9 - 2	<LOD	20,9 - 2	7,2 - 2	11,9 - 2
L10	<LOD 2,9 - 1	9,0 - 2	12,2 - 2	<LOD	14,4 - 2	<LOD	19,3 - 1	4,9 - 1	7,2 - 1
L11	<LOD	7,6 - 2	14,5 - 2	<LOD 1,0 - 1	14,9 - 2	<LOD	17,6 - 1	6,0 - 2	8,4 - 2
L12	<LOD	7,0 - 2	9,8 - 2	<LOD	13,7 - 2	<LOD	16,8 - 1	6,4 - 2	7,1 - 2
L13	<LOD	6,5 - 2	9,6 - 2	<LOD	15,6 - 1	<LOD	16,2 - 2	6,4 - 2	7,2 - 2

Table F.5 *(continued)*

min-max	—	6,3–12,8	9,6–21,7	—	13,7–25,0	—	16,2–27,4	4,9–8,2	6,9–12,0
factor max/min	—	2,0	2,3	—	1,8	—	1,7	1,7	1,7
	Sample								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Mean		8,8	13,9		17,4		20,4	6,3	9,0
95 % CI		7,5–10,1	11,9–15,9		15,4–19,4		18,6–22,2	5,7–6,9	8,0–10,1
95 % PI (based on Ln-trans. data)		3,8–13,8 (5,0–14,4)	6,7–21,1 (8,3–21,8)		12,0–22,8 (12,5–22,9)		13,7–27,1 (14,7–27,6)	4,1–8,5 (4,4–8,8)	5,8–12,3 (6,3–12,3)
99 % PI (based on Ln-trans. data)		1,8–15,7 (4,0–17,9)	3,8–24,0 (6,8–26,6)		9,8–25,0 (11,1–26,0)		10,9–29,9 (12,9–31,4)	3,2–9,4 (3,8–10,2)	4,5–13,6 (5,4–14,1)
s_r (repeatability)		1,1	2,4		3,7		1,7	0,5	1,8
s_L (interlab variability)	—	2,2	3,1	—	2,2	—	2,9	0,9	1,4
s_R (reproducibility)		2,5	3,9		4,3		3,4	1,1	2,2
s_r % (based on Ln-trans. data)		12,9 (14,2)	17,0 (17,4)		21,4 (18,6)		8,6 (9,0)	7,5 (7,5)	19,6 (20,6)
s_L % (based on Ln-trans. data)		24,9 (23,3)	22,3 (20,9)		12,8 (12,7)		14,3 (13,6)	14,8 (14,8)	15,2 (14,2)
s_R % (based on Ln-trans. data)		28,0 (27,3)	28,0 (27,1)		24,9 (22,5)		16,7 (16,3)	16,6 (16,6)	24,8 (25,0)
s_R/s_r		2,2	1,6		1,2		1,9	2,2	1,3

s_r repeatability standard deviation
s_R reproducibility standard deviation
s_L laboratory standard deviation

F.1.3 Trueness evaluation

A trueness evaluation was performed for the samples S2, S5, S8 and S9. For each laboratory, the assigned EEQ values for the samples S2 and S5 were calculated as sum of the measured EEQ value of the unspiked sample (S1 and S4 respectively) and the expected EEQ value of the spiking. For the calculation of the expected EEQ a relative equivalent potency of 1,2 was used for 17α-ethinylestradiol. Hence, the assigned EEQ for sample 2 is the sum of the EEQ of sample 1 and the expected EEQ of 12 ng/l. The assigned EEQ for sample 5 is the sum of the EEQ of sample 4 and the expected EEQ of 18 ng/l.

As sample 8 is a mixture of bisphenol Z, S and A the assigned EEQ was calculated by means of the relative equivalent potencies for these three chemicals. For sample 8 the assigned EEQ is 8,9 ng/l. The assigned EEQ for sample 9 was calculated according to the same principle. For sample 9 the assigned EEQ is 11,0 ng/l.

Table F.6 summarizes for each laboratory the assigned and real (measured) EEQ, the sample specific mean standard uncertainty (associated with the method and laboratory bias) and the mean relative bias with 95 % confidence interval as well as the respective results across samples.

Table F.6 — Evaluation of trueness for samples S2, S5, S8 and S9

Lab	Sample 2			Sample 5			Sample 8			Sample 9		
	EEQ assigned [ng/l] = EEQ P1 + 12	EEQ [ng/l] real	**Diff real - nom**	EEQ assigned [ng/l] = EEQ P4 + 18	EEQ [ng/l] real	**Diff real - nom**	EEQ assigned [ng/l]	EEQ [ng/l] real	Diff real - nom	EEQ assigned [ng/l]	EEQ [ng/l] real	**Diff real - nom**
L01	12,0	8,2	−3,8 (−32 %)	18,0	—	—	8,9	—	—	11,00	—	
L02	12,0	6,6	−5,4 (−45 %)	18,0	20,0	2,0 (11 %)	8,9	5,2	−3,7 (−42 %)	11,00	10,03	−1,0 (−9 %)
L03	12,0	12,8	0,8 (7 %)	18,0	25,0	7,0 (39 %)	8,9	8,2	−0,7 (−7 %)	11,00	11,25	0,3 (2 %)
L04	12,0	10,7	−1,3 (−11 %)	18,0	16,4	−1,6 (−9 %)	8,9	6,3	−2,6 (−29 %)	11,00	8,94	−2,1 (−19 %)
L05	12,0	7,4	−4,6 (−38 %)	18,0	14,6	−3,4 (−19 %)	8,9	5,5	−3,4 (−39 %)	11,00	6,89	−4,1 (−37 %)
L06	14,0	6,3	−7,7 (−55 %)	18,0	17,7	−0,3 (−1 %)	8,9	7,0	−1,9 (−22 %)	11,00	7,95	−3,1 (−28 %)
L07	12,0	12,2	0,2 (2 %)	18,0	19,5	1,5 (8 %)	8,9	6,7	−2,2 (−25 %)	11,00	11,96	1,0 (9 %)
L08	14,1	8,3	−5,8 (−41 %)	19,8	14,5	−5,4 (−27 %)	8,9	5,1	−3,8 (−42 %)	11,00	8,73	−2,3 (−21 %)
L09	12,0	12,7	0,7 (5 %)	19,9	19,9	0,0 (0 %)	8,9	7,2	−1,7 (−19 %)	11,00	11,93	0,9 (8 %)
L10	14,9	9,0	−5,9 (−40 %)	18,0	14,4	−3,6 (−20 %)	8,9	4,9	−4,0 (−45 %)	11,00	7,19	−3,8 (−35 %)
L11	12,0	7,6	−4,4 (−37 %)	18,0	14,9	−3,1 (−17 %)	8,9	6,0	−2,9 (−33 %)	11,00	8,38	−2,6 (−24 %)
L12	12,0	7,0	−5,0 (−42 %)	18,0	13,7	−4,3 (−24 %)	8,9	6,4	−2,5 (−29 %)	11,00	7,10	−3,9 (−35 %)
L13	12,0	6,5	−5,5 (−46 %)	18,0	15,6	−2,4 (−13 %)	8,9	6,4	−2,5 (−28 %)	11,00	7,22	−3,8 (−34 %)

[a] Standard uncertainty associated with the method and laboratory bias.

[b] This CI refers only to the two samples S2 and S5 and cannot be generalized to other samples.

Table F.6 *(continued)*

Lab	Sample 2			Sample 5			Sample 8			Sample 9		
	EEQ assigned [ng/l] = EEQ P1 + 12	EEQ [ng/l] real	**Diff real - nom**	EEQ assigned [ng/l] = EEQ P4 + 18	EEQ [ng/l] real	**Diff real - nom**	EEQ assigned [ng/l]	EEQ [ng/l] real	Diff real - nom	EEQ assigned [ng/l]	EEQ [ng/l] real	**Diff real - nom**
Mean standard uncertainty[a]	35,2 %			19,1 %			31,7 %			24,8 %		
Mean standard uncertainty[a] across samples S2 and S5	28,6 %											
Mean relative bias	−28,6 %			−6,1 %			−29,9 %			−18,5 %		
95 % CI	−40,2 % - −17,0 %			−16,8 % - +4,6 %			−36,1 % - −23,7 %			−28,3 % - −8,8 %		
Mean relative bias across samples S2 and S5	−17,8 %											
95 % CI[b]	−39,9 % - +4,3 %											

[a] Standard uncertainty associated with the method and laboratory bias.

[b] This CI refers only to the two samples S2 and S5 and cannot be generalized to other samples.

F.2 Sensitivity for selected compounds

The estrogenic potential of a compound is frequently expressed as a relative potency to the reference compound 17β-estradiol. The relative potency (%) is calculated as follows:

$$P_R = \frac{EC_{50}(R)}{EC_{50}(C)} \times 100 \quad \text{(F.1)}$$

where

P_R is the relative potency of a compound C relative to a reference compound R (e.g. 17β-estradiol) in percent, %;

$EC_{50}(R)$ is the EC_{50} of the reference compound 17β-estradiol;

$EC_{50}(C)$ is the EC_{50} of the compound C.

Table F.7 shows the relative potencies to 17β-estradiol for selected compounds (%). The repeatability of the relative potencies is on average 52 %. Variability of results reflects usage of different lots of yeast and required reagents and media as well as different test conditions (days, operators and equipment).

Table F.7 — Summary of relative potencies to 17β-estradiol for selected compounds

Compound	Relative potency to 17β-estradiol (%)
1,3,5(10)-Estratrien-3,16α,17β-triol-3-methylether	0,003 17
1,3,5(10)-Estratrien-3,16α,17β-triol-6-one	0,001 87
1,3,5(10)-Estratrien-3,17α-diol-6-one	0,133
1,3,5(10)-Estratrien-3,17β-diol-17-cyclopentylpropionate	2,30
1,3,5(10)-Estratrien-3,17β-diol-6-one	7,29
1,3,5(10)-Estratrien-3-ol-17-oneacetate	110
17α-estradiol	3,46
17β-estradiol (E2)	100
17α-ethinylestradiol (EE2)	120
17α-ethinylestradiol 3-cyclopentyl ether	0,016 2
17β-estradiol 17-valerate	2,15
4-Hydroxytamoxifen	0,036 9
4-n-Nonylphenol	0,001 06
4-Nonylphenol technical grade	0,000 348
8-Prenylnaringenim	2,31
Bis(2-ethylhexyl)phthalate (DEHP)	0,000 394
Bisphenol A (BPA)	0,001 68
Bisphenol C (BPC)	0,003 26
Bisphenol E (BPE)	0,000 907
Bisphenol F (BPF)	0,000 646
Bisphenol G (BPG)	0
Bisphenol S (BPS)	0
Bisphenol Z (BPZ)	0,012 3
Chlordecon	0,000 171
Chlorotrianisene	0,001 68
Corticosteron	0

Table F.7 *(continued)*

Compound	Relative potency to 17β-estradiol (%)
Coumestrol	0,944
Cyclofenil	0,212
Dienestrol	406
Diclofol	0
Dicyclohexyl phthalate (DCHP)	0
Diethylstilbestrol (DES)	573
Dihydrotestosteron (DHT)	0,000 771
Estriol (E3)	2,00
Estron (E1)	22,0
Ethinylestradiol 3-methyl ether	0,477
Fenarimol	0
Hexestrol	213
Indirubin	0
Medroxyprogesterone acetate	0
Mirex	0
MPP dihydrochloride (1,3-bis(4-hydroxyphenyl)-4-methyl-5-{4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride	0,001 33
Progesteron	0
Propylparaben	0,000 555
Pyriproxifen	0
Simazin	0
Tamoxifen	0,004 50
α-Zearalanol	3,39
β-estradiol hemihydrate	235

Annex G
(informative)

Statistical assessment

Annex G describes the determination of the critical limit (CL) which is the threshold value above a sample or a sample dilution is assessed to mediate estrogenic activity.

Sample dilutions with a significant estrogenic activity are determined by the threshold value of 1,18 for the induction rate which is obtained as follows:

Long-term average of standard error of an experienced laboratory was 0,004 5 at a value of 0,250 for the absorbance at 405 nm for the negative control. The decision limit for the corrected absorbance at 405 nm is 0,250 + 0,004 5 × 10 = 0,295 and the corresponding threshold 0,295/0,250 = 1,18.

Annex H
(informative)

Calculation of estradiol equivalents

H.1 General

This annex describes a method for modelling the dose response relationship between the reference compound and the effect measure of the assay (e.g. induction rates or corrected absorbance). Based on the estimated parameters of the dose response relationship the estrogenic potential of a sample or sample dilution can be determined by inserting the respective effect measure into the inverse of the calibration function. The resulting estrogenic potential of a sample is expressed in terms of 17β-estradiol equivalents (EEQs), which means that the estrogenic activity of the sample or sample dilution is equivalent to the estrogenic activity of an equally concentrated 17β-estradiol solution.

H.2 Modelling of dose response relationship

A dose response relationship of the reference compound 17β-estradiol is measured in parallel to each test. Use these dose response relationships to calculate the estradiol equivalents of a sample or sample dilutions which were tested on the same 96-well plate.

Fit the dose response curve of the reference compound 17β-estradiol in terms of the effect measure [corrected absorbance (9.3)] versus test concentration with a suitable mathematical method for a sigmoid dose response relationship, e.g. the following four-parametric logistic function [Formula (H.1)]:

$$f(x) = y = \frac{a-d}{1+\left(\frac{x}{\mathrm{EC}_{50}}\right)^{b}} + d \qquad \text{(H.1)}$$

where

y is the calculated effect measure (e.g. corrected absorbance) at concentration x;

x is the compound concentration which activates the test system to effect measure y;

a is the mean value of y without estrogenic effects (bottom curve point);

d is the mean value of y with the maximal activation of the test (curve plateau);

EC_{50} is the mean effect concentration at which the estrogenic effect attains half of its maximum (EC_{50} – 50 % effect concentration (3.5) describing the curve point of inflection);

b is proportional to the slope of the function at EC_{50}.

Due to different test methods different measures for assessment of the quality of the curve fitting can be used. The quality of the curve fitting can be assessed with the following measure:

RSD (residual standard deviation) ≤12 %.

The residual standard deviation is the square root of the mean squared residuals:

$$\sqrt{\mathrm{mean}\left(\mathrm{residual}_1^2, \ldots, \mathrm{residual}_N^2\right)}$$

$$\text{residual}_i = y_i - \hat{y}_i \text{ with } i = 1,\ldots,N$$

where

y_i is the calculated (measured) effect measure (e.g. corrected absorbance) at concentration x_i;

$\hat{y}_i$ is the estimated effect measure at concentration x_i.

If the quality of the curve fitting is not acceptable use weighting to account for different variances or chose other mathematical models, e.g. a five parametric logistic function. If no acceptable curve fitting is possible the calculation of estradiol equivalents cannot be done with the present data.

H.3 Calculation of estradiol equivalents for test samples

Calculate the estradiol equivalents of a sample or a sample dilution by inserting the respective effect measure (e.g. induction rate or corrected absorbance) into the inverse function with the parameters which were calculated for the reference compound 17β-estradiol. Formula (H.2) shows the inverse function of Formula (H.1):

$$x_S = \left[\frac{y_S - a}{(d - y_S)}\right]^{\left(\frac{1}{b}\right)} \times EC_{50} \quad \text{(H.2)}$$

where

y_S is the mean of the calculated effect measure (e.g. induction rate or corrected absorbance) of the sample or sample dilution;

x_S is the concentration of the sample or sample dilution expressed as 17β-estradiol equivalents in the concentration dimension that was used for the calculation of the parameters of the curve fitting;

d is the mean value of y with the maximal activation of the test (curve plateau derived from the dose response relationship is the of the reference by the curve fitting);

a is the mean value of y without estrogenic effects (bottom curve point derived from the dose response relationship of the reference by the curve fitting);

EC_{50} is the mean effect concentration at which the estrogenic effect attains half of its maximum (EC_{50} – 50 % effect concentration (3.5) describing the curve point of inflection derived from the dose response relationship of the reference by the curve fitting);

b is proportional to the slope of the function at EC_{50}; b is derived from the dose response relationship of the reference by the curve fitting.

Use only data points in the dynamic range of the test for the calculation of estradiol equivalents.

H.4 Reporting of estradiol equivalents for test samples

Report individually the EEQ value (e.g. ng/l) for each sample dilution, which is suitable for the calculation of 17β-estradiol equivalents (see above).

Further minimal information about bio-equivalent estimates according to Reference [19] has to be reported.

— Concentration of the sample e.g. by solid phase extraction (e.g. 1 000-fold for a solid phase extraction of 1 l water to 1 ml organic solvent).