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

Part 1: Yeast estrogen screen (*Saccharomyces cerevisiae*)

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

*Partie 1: Essai d'oestrogénicité sur levures (*Saccharomyces cerevisiae*)*

Reference number
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Foreword

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

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 1:
Yeast estrogen screen (*Saccharomyces cerevisiae*)

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 genetically modified yeast strains *Saccharomyces cerevisiae*. This reporter gene assay is based on the activation of the human estrogen receptor alpha.

This method is applicable to:

— fresh water;

— waste 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 8 ng/l and 15 ng/l 17β-estradiol equivalents (EEQ) based on the results of the international interlaboratory trial (see Annex F). The upper threshold of the dynamic range for this test is between 120 ng/l and 160 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.

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*

ISO 7027, *Water quality — Determination of turbidity*

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 http://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

[SOURCE: ISO 6107-6:2004, 24]

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
induction rate
quotient of the mean signal measured after exposure to a dose of the test sample or with a positive control, and the mean signal measured for the negative control using the same experimental conditions

[SOURCE: ISO 6107-6:2004, 43, modified — “corrected absorbance” replaces “mutant colonies”; “wells” replaces “corresponding plates”, “quotient” replaces “difference”.]

3.8
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]

3.9
limit of quantification
LOQ
lowest value of a determinant that can be determined with an acceptable level of accuracy and precision

[SOURCE: ISO 15839:2003, 3.18]

3.10
lowest ineffective dilution
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
overnight culture
culture started late in the afternoon and incubated overnight to be ready during the following morning for purposes such as the inoculation of a preculture

Note 1 to entry: The procedure for the overnight culture is described in 9.2.

[SOURCE: ISO 6107-6:2004, 54, modified — deleted: "usually about 16 h".]

3.13
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”.]

3.14
reporter gene activity
quantitative activity of a gene attached to the promoter sequence of another gene

3.15
stock culture
culture of a strain of organisms maintained under conditions to preserve original features such as nucleotide sequences

[SOURCE: ISO 6107-6:2004, 87]

3.16
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 Yeast Estrogen Screen (YES) is a reporter gene assay which can be used for the measurement of the activation of the human estrogen receptor alpha (hERα) in the presence of a sample containing compounds which activate the estrogen receptor (ER).

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 on the whole process of the reporter gene expression.

The basic concept of such assays is explained in References [10] and [11]. The hERα is heterologously expressed in the yeast cell under control of a copper dependent 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 which translocates to the nucleus. This activation of the estrogen receptor is measured by the induction of the reporter gene *lacZ* which encodes the enzyme β-galactosidase. The *lacZ* is fused to a promoter containing estrogen responsive elements (ERE) and is thus controlled by the activity of the estrogen receptor. The ER-dimer binds to the promoter and by this activates the expression of the β-galactosidase. Finally, the activity of the β-galactosidase 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 cell density and/or the detection of the reaction product of the reporter enzyme β-Galactosidase (see Clause 10 for further information).

Toxic effects of the test sample may lead to a reduction of viable cells and to a reduction of the measurable signal. Consequently, estrogenic effects of a sample may be masked by acute toxic effects leading to false negative test results (see Clause 10 for further information).

High salinity can cause toxic effects due to the resulting osmotic pressure. The conductivity of the sample is a measure for its salinity. The yeast strain constructed by McDonnell et al. (Reference [10]) tolerates a conductivity of the sample up to 34 000 µS/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.

Due to the high sensitivity of this test avoid any contamination of buffer, media and all reagents used with compounds exhibiting estrogenic activity to avoid false positive test results.

6 Apparatus and materials

For suitable sampling devices see Clause 8. Usual laboratory apparatus and glassware is required. In particular, the following material is needed:

6.1 Incubator shaker, temperature- and time-controlled, 30 °C ± 1 °C and 37 °C ± 1 °C.

6.2 pH meter.

6.3 Steam sterilizer.

6.4 Dry sterilizer.

6.5 Centrifuge, with a rotor for 15 ml and 50 ml tubes up to 2 500 *g* and with a rotor for 96-well plates up to 2 500 *g*.

6.6 Rotary mixer.

6.7 Freezer, at least ≤−18 °C and ≤−70 °C.

6.8 Sterile filter, cellulose acetate, 0,2 µm pore size.

6.9 Inoculation loops.

6.10 Multi-channel multistepper pipette (repeater pipette).

6.11 Multi-channel pipettes, 5 µl to 50 µl and 50 µl to 300 µl.

6.12 Spectrophotometer.

6.13 Transparent sterile polystyrene 96-well plates, for suspension cultures with flat bottom and lid.

6.14 Microplate photometer for 96-well plates, for absorbance measurement at 540 nm ± 20 nm or 580 nm ± 20 nm and at 600 nm ± 20 nm.

6.15 Clean bench.

6.16 Petri dishes, diameter approximately 94 mm, height approximately 16 mm.

6.17 Cryogenic vials, sterile, 1 ml, 10 ml.

6.18 Disposable nitrile gloves.

6.19 Air-permeable sealing membranes for 96-well plates.

7 Reagents, media and test strain

7.1 Reagents

As far as possible, use "reagent grade" chemicals.

7.1.1 Yeast nitrogen base without amino acids[1].

7.1.2 α-D-Glucose, anhydrous, $C_6H_{12}O_6$, molecular weight 180,15 g/mol, CAS: 50-99-7.

7.1.3 Adenine, $C_5H_5N_5$, molecular weight 135,13 g/mol, CAS: 73-24-5.

7.1.4 L-arginine, $C_6H_{14}N_4O_2$, molecular weight 174,20 g/mol, CAS: 74-79-3.

7.1.5 L-aspartic acid, $C_4H_7NO_4$, molecular weight 133,10 g/mol, CAS: 56-84-8.

7.1.6 L-glutamic acid monosodium salt hydrate, $C_5H_8NNaO_4{\cdot}H_2O$, molecular weight (anhydrous) 169,11 g/mol, CAS: 142-47-2 (anhydrous basis).

7.1.7 L-histidine-HCl, $C_6H_9N_3O_2{\cdot}HCl{\cdot}H_2O$, molecular weight 209,6 g/mol, CAS: 5934-29-2.

7.1.8 L-isoleucine, $C_6H_{13}NO_2$, molecular weight 131,17 g/mol, CAS: 73-32-5.

7.1.9 L-leucine, $C_6H_{13}NO_2$, molecular weight 131,17 g/mol, CAS: 61-90-5.

7.1.10 L-lysine-HCl, $C_6H_{14}N_2O_2{\cdot}HCl$, molecular weight 182,65 g/mol, CAS: 657-27-2.

7.1.11 L-methionine, $C_5H_{11}NO_2S$, molecular weight 149,21 g/mol, CAS: 63-68-3.

7.1.12 L-phenylalanine, $C_9H_{11}NO_2$, molecular weight 165,19 g/mol, CAS: 63-91-2.

7.1.13 L-serine, $C_3H_7NO_3$, molecular weight 105,09 g/mol, CAS: 56-45-1.

7.1.14 L-threonine, $C_4H_9NO_3$, molecular weight 119,12 g/mol, CAS: 72-19-5.

7.1.15 L-tyrosine, $C_9H_{11}NO_3$, molecular weight 181,19 g/mol, CAS: 60-18-4.

7.1.16 L-valine, $C_5H_{11}NO_2$, molecular weight 117,15 g/mol, CAS: 72-18-4.

7.1.17 Copper(II) sulfate pentahydrate, $CuSO_4{\cdot}5H_2O$, molecular weight 249,69 g/mol, CAS: 7758-99-8.

7.1.18 Ampicillin sodium salt, $C_{16}H_{18}N_3NaO_4S$, molecular weight 371,39 g/mol, CAS: 69-52-3.

7.1.19 Streptomycin sulfate salt, $C_{21}H_{39}N_7O_{12}{\cdot}1{,}5H_2SO_4$, molecular weight 728,69 g/mol, CAS: 3810-74-0.

7.1.20 Agar for microbiology, $(C_{12}H_{18}O_9)n$, CAS: 9002-18-0.

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1) Yeast nitrogen base without amino acids contains a nitrogen source such as ammonium sulfate, vitamins and trace elements which are required for growth of yeast cells. Yeast nitrogen base without amino acids is used for the selection of yeast strains depending on requirements for carbon sources and amino acids.

7.1.21 Hydrochloric acid solution, 1 M (HCl), molecular weight 36,46 g/mol, CAS: 7647-01-0.

7.1.22 Sodium hydroxide, NaOH, molecular weight 40,00 g/mol, CAS: 1310-73-2.

7.1.23 Ethanol, ≥99,8 %, CH_3CH_2OH, molecular weight 46,07 g/mol, CAS: 64-17-5.

7.1.24 Glycerol for molecular biology, ≥99 %, $HOCH_2CH(OH)CH_2OH$, molecular weight 92,09 g/mol, CAS: 56-81-5.

7.1.25 17*β*-Estradiol, ≥98 %, $C_{18}H_{24}O_2$, molecular weight 272,38 g/mol, CAS: 50-28-2.

7.1.26 Disodium hydrogen phosphate dihydrate, $Na_2HPO_4{\cdot}2H_2O$, molecular weight 177,99 g/mol, CAS: 10028-24-7.

7.1.27 Sodiumdihydrogen phosphate monohydrate, $NaH_2PO_4{\cdot}H_2O$, molecular weight 137,99 g/mol, CAS: 10049-21-5.

7.1.28 Potassium chloride, KCl, molecular weight 74,55 g/mol, CAS: 7447-40-7.

7.1.29 Magnesium sulfate heptahydrate, $MgSO_4{\cdot}7H_2O$, molecular weight 246,47 g/mol, CAS: 10034-99-8.

7.1.30 Chlorophenolred-β-D-galactopyranoside (CPRG), $C_{25}H_{22}Cl_2O_{10}S$, molecular weight 585,41 g/mol, CAS: 99792-79-7.

7.1.31 Lyticase from *Arthrobacter luteus* lyophilized powder, ≥2 000 units/mg protein, CAS: 37340-57-1.

7.1.32 DL-Dithiothreitol, $HSCH_2CH(OH)CH(OH)CH_2SH$, molecular weight 154,25 g/mol, CAS: 3483-12-3.

7.1.33 Sodium dodecyl sulfate, $CH_3(CH_2)_{11}OSO_3Na$, molecular weight 288,38 g/mol, CAS: 151-21-3.

7.1.34 Aceton (puriss p.a.), CH_3COCH_3, molecular weight 58,08 g/mol, CAS: 67-64-1.

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

If sterile water is needed, autoclave or sterilize by filtration (cellulose acetate, 0,2 µm). Water as specified here is also used for the stepwise dilution of the test sample.

7.3 Test strain.

The generation of the test strain is described in References [10] and [11]. It is derived from the strain *Saccharomyces cerevisiae* BJ3505 (protease deficient, MAT*α*, PEP4::HIS3, prb-1-delta1.6R, HIS3-delta200, lys2-801, trp1-delta101, ura3-52gal2can1). This strain harbours two plasmids. The construction of these plasmids is described in Reference [10]. The plasmid YEPE10 contains the *CUP1::hER* fusion which encodes the human estrogen receptor *α* cloned from the MCF-7 human cell lineage under the control of the metallothionein promoter *CUP1*. This plasmid is selected via the tryptophane auxothropy of the parent strain. The second plasmid is the reporter plasmid YRPEG3 which contains the fusion gene *2ERE-CyC1::lacZ*. This fusion gene expresses the *β*-galactosidase (encoded by *lacZ*) under the control of the iso1cytochrom c promoter from *S. cerevisiae* which is fused to two copies of the vitellogenin A2-gene from *Xenopus laevis*. This plasmid is selected via the uracil auxothropy of the parent strain.

7.4 Media.

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

7.4.1 10x SD-Medium.

Dissolve 67,0 g yeast nitrogen base without amino acids (7.1.1) and 200 g glucose (7.1.2) in water (7.2) and adjust the final volume with water (7.2) to 1 l. Sterilize the solution by filtration (cellulose acetate, 0,2 µm). Do not autoclave the solution because it contains vitamins such as Biotin. Store the solution in 50 ml aliquots in the dark at ≤ −18 °C no longer than 12 months.

7.4.2 10x McD-DO-Medium (McDonnell).

Dissolve

— 175 mg L-lysine-HCl (7.1.10);

— 120 mg L-histidine-HCl (7.1.7);

in water (7.2) and adjust the final volume with water (7.2) to 500 ml.

Sterilize by filtration (cellulose acetate, 0,2 µm) and store aliquots of 40 ml in the dark at ≤−18 °C no longer than six months.

7.4.3 Glucose solution.

Dissolve

— 144 g α-D-Glucose (7.1.2)

in water (7.2) and adjust the final volume with water (7.2) to 500 ml.

Sterilize by filtration (cellulose acetate, 0,2 µm) or autoclave and store the solution in the dark at 2 °C to 8 °C no longer than 12 months.

7.4.4 $CuSO_4$ solution, 10 mmol/l.

Dissolve 250 mg $CuSO_4 \cdot 5H_2O$ (7.1.17) in water (7.2) and adjust the final volume with water (7.2) to 100 ml.

Autoclave the solution. The solution may be used up to 12 months at room temperature.

7.4.5 Ampicillin stock solution.

Dissolve 1 g ampicillin sodium salt (7.1.18) in water (7.2) and adjust the final volume with water (7.2) to 10 ml.

Sterilize the solution by filtration (cellulose acetate, 0,2 µm) and store 1 ml aliquots in the dark at ≤−18 °C no longer than 12 months.

7.4.6 Streptomycin stock solution.

Dissolve 1 g streptomycin sulfate salt (7.1.19) in water (7.2) and adjust the final volume with water (7.2) to 10 ml.

Sterilize the solution by filtration (cellulose acetate, 0,2 µm) and store 1 ml aliquots in the dark at ≤−18 °C no longer than 12 months.

7.4.7 Growth medium (McDonnell).

Add

— 10 ml 10x SD-Medium (7.4.1);

— 10 ml 10x McD-DO-Medium (McDonnell, 7.4.2);

to 80 ml sterile water (7.2) under sterile conditions. Split the medium in 20 ml aliquots. Store the medium in the dark at 2 °C to 8 °C no longer than one week or in the dark at ≤–18 °C no longer than six months.

7.4.8 Exposure medium (McDonnell).

Mix

— 4,2 ml 10x SD-Medium (7.4.1);

— 4,2 ml 10x McD-DO-Medium DO-Medium (7.4.2);

— 1,6 ml Glucose solution (7.4.3);

— 99 µl $CuSO_4$-solution (7.4.4);

— 67 µl Ampicillin stock solution (7.4.5);

— 67 µl Streptomycin stock solution (7.4.6).

Prepare the exposure medium immediately before use. A volume of 5 ml per 96-well plate is required.

7.4.9 Aqueous solution of ethanol, volume fraction 0,3 %.

Add sterile water (7.2) to 300 µl ethanol (7.1.23) to a final volume of 100 ml.

7.4.10 Aqueous solution of glycerol, volume fraction 30 %.

Add water (7.2) to 3 ml glycerol (7.1.24) to a final volume of 10 ml. Sterilize the solution by filtration (Cellulose acetate, 0,2 µm) or autoclave.

7.4.11 17*β*-estradiol (E2) stock solution.

Dissolve 50 mg 17*β*-estradiol (E2) (7.1.25) in ethanol (7.1.23) and adjust the final volume with ethanol (7.1.23) to 10 ml. Store the stock solution in aliquots of 1 ml in the dark at ≤ –18 °C no longer than 12 months.

7.4.12 lacZ-buffer.

Dissolve in 950 ml of water (7.2):

— 10,67 g $Na_2HPO_4{\cdot}2H_2O$ (7.1.26);

— 0,75 g KCl (7.1.28);

— 0,25 g $MgSO_4{\cdot}7H_2O$ (7.1.29).

Before the adjustment of the pH the solution might be turbid. Adjust the pH to pH = 7,0 ± 0,2 by the addition of $NaH_2PO_4{\cdot}H_2O$ (7.1.27) (approximately 5,5 g).

Add and dissolve

— 1 g sodium dodecyl sulfate (7.1.33).

Adjust the final volume to 1 000 ml with water (7.2). Store the solution at room temperature no longer than six months.

7.4.13 lacZ reaction mixture

Add and dissolve per ml lacZ buffer:

— 0,4 mg chlorophenolred-β-D-galactopyranoside (CPRG) (7.1.30);

— 250 U Lyticase (7.1.31);

— 0,154 mg DL-Dithiothreitol (7.1.32).

For one 96-well plate 6 ml lacZ-reaction mixture are required. Prepare the lacZ-reaction mixture immediately before use.

Different batches of the lyticase may have a different specific lyticase-activity. Calculate the amount of lyticase needed for the reaction mixture according to:

$$m_{\text{lyticase}} = \frac{250\ \text{U}}{A_s} \tag{1}$$

where

m_{lyticase} is the mass (mg) of lyticase with a specific activity of A_s which is required for 1 ml lacZ-buffer;

A_s is the specific activity of the lyticase in U/mg.

8 Sampling and samples

8.1 General

This clause 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 (7.1.34). 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 (7.1.34). 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 [12] and [13].

Adjust the sample to a pH of 7,2 ± 0,2 using either HCl (7.1.21) or NaOH solution (7.1.22). Select the acid or alkali concentrations such that the added volumes are as small as possible. Avoid over-titration. The adjustment of the sample's pH might affect the sample. Report all visible changes caused by the adjustment of the pH value (ISO 5667-16).

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 overnight. 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 [14] and [15].

9 Procedure

9.1 Preparation of cryo-cultures for long-term storage

Store the culture aliquots in the dark at −75 °C ± 5 °C no longer than 12 months. Do not store the cells in liquid nitrogen because of a limited viability of the cells under this condition.

Measure the optical density of the culture after an overnight culture (9.2) at 600 nm ± 20 nm (OD600) in a 1→10 dilution with growth medium (7.4.7) and calculate the formazin attenuation units (FAU) for the undiluted culture according to ISO 7027. Use 600 nm for the FAU calibration.

NOTE FAU-value of about 1 200 is a usual cell density of an overnight culture and serves as a guidance value for the user. If the validity criteria according to Clause 10 are fulfilled, FAU-values that diverge from the guidance value are acceptable.

Mix 3,5 ml of this culture with 3,5 ml of a sterile aqueous solution of glycerol (7.4.10) with a volume fraction of 30 % under sterile conditions. Freeze 350 µl-aliquots of the cell suspension in cryogenic vials at −75 °C ± 5 °C.

Determine the EC_{50} of the reference compound 17β-estradiol with a frozen aliquot of the new cryo-culture according to the standard procedures described in 9.3 and 9.4. If the EC_{50} value, which is determined with the new cryo-culture, is not valid according to Clause 10 prepare new cryo-cultures.

9.2 Overnight culture

Culture yeast cells taken from a cryo-stock in growth medium (7.4.7) described as follows:

Under sterile conditions, pipette 5 ml of growth medium (7.4.7) into a 50 ml Erlenmeyer flask and inoculate the medium with 350 µl of a cryo-stock (9.1) immediately after thawing. Cover the flask with e.g. aluminium foil. Incubate the overnight culture for 22 h ± 1 h at 30 °C ± 1 °C under constant agitation. Prepare the overnight culture one day prior to the performance of the test. Other sterile flasks or containers might be used instead of an Erlenmeyer flask if it is proven that this change does not impact the performance of the test.

NOTE 1 5 ml overnight culture are sufficient for at least 50 96-well plates.

Measure the optical density of the culture after incubation in a 1→10 dilution with growth medium (7.4.7) at 600 nm ± 20 nm and calculate the FAU for the undiluted culture according to ISO 7027. Use 600 nm for the FAU calibration.

NOTE 2 FAU-value of about 1 200 is a usual cell density of an overnight culture and serves as a guidance value for the user. If the validity criteria according to Clause 10 are fulfilled, FAU-values that diverge from the guidance value are acceptable.

9.3 Test set up for aqueous samples

9.3.1 Preparation

Prepare the required volume of the exposure medium (7.4.8). A volume of 5 ml per 96-well plate is needed.

9.3.2 Preparation of the reference dilution series

Thaw an aliquot of the 17β-estradiol (E2) stock solution (7.4.11; 5 mg/ml). Dilute the thawed stock solution successively two times 1→100 and one time 1→3 with ethanol (e.g. 334 µl E2-solution + 666 µl ethanol). The resulting concentration is 166,6 µg E2/l. Close all vials used immediately after usage to avoid an evaporation of the ethanol.

Dilution series of the E2-reference:

Dilute the 166,6 µg/l ethanolic E2 solution 1→33,3 with sterile water to a concentration of 5 000 ng/l E2 (e.g. 30 µl E2 solution to 970 µl water). Store the prepared dilution of 17*β*-estradiol (166,6 µg E2/l) in the dark at ≤ −18 °C no longer than 12 month.

Prepare a final 1→10 dilution with water (7.2) by e.g. adding 400 µl of the 5 000 ng/l E2 to 3 600 µl sterile water (7.2) resulting in a concentration of 500 ng/l E2. Prepare this dilution in an amber glass vial with a cap which is lined with PTFE.

From this solution prepare six successive 1→3 dilutions with an aqueous solution of ethanol [volume fraction 0,3 % (7.4.9)], e.g. by adding 1 ml of the E2-solution to 2 ml of the aqueous solution of ethanol. Prepare these dilutions in glass vials with PTFE-caps also. Use the dilutions starting from 500 ng/l down to 0,66 ng/l for the determination of the concentration response curve of the E2 reference. 160 µl of every E2-dilution are sufficient for one test plate with two replicates per dilution level. The following Table 1 summarizes the preparation of the E2-dilution series.

Table 1 — Preparation of the E2-dilution series

E2 solution	c(E2)	Volume fraction ethanol	Dilution	Example	Used for reference concentration-response curve
1 [stock (7.4.11)]	5 mg/ml	100 %	1→100 with ethanol	10 µl E2 solution 1 + 990 µl ethanol	
2	50 µg/ml	100 %	1→100 with ethanol	10 µl E2 solution 2 + 990 µl ethanol	
3	500 µg/l	100 %	1→3 with ethanol	334 µl E2 solution 3 + 666 µl ethanol	
4	166,6 µg/l	100 %	1→33,3 with water	30 µl E2 solution 4 + 970 µl water	
5	5 µg/l	3 %	1→10 with water	400 µl E2 solution 5 + 3 600 µl water	
6	500 ng/l	0,3 %	1→3 with 0,3 % ethanol	1 ml E2 solution 6 + 2 ml 0,3 % ethanol	x
7	166,6 ng/l	0,3 %	1→3 with 0,3 % ethanol	1 ml E2 solution 7 + 2 ml 0,3 % ethanol	x
8	55,6 ng/l	0,3 %	1→3 with 0,3 % ethanol	1 ml E2 solution 8 + 2 ml 0,3 % ethanol	x
9	18,5 ng/l	0,3 %	1→3 with 0,3 % ethanol	1 ml E2 solution 9 + 2 ml 0,3 % ethanol	x
10	6,2 ng/l	0,3 %	1→3 with 0,3 % ethanol	1 ml E2 solution 10 + 2 ml 0,3 % ethanol	x
11	2 ng/l	0,3 %	1→3 with 0,3 % ethanol	1 ml E2 solution 11 + 2 ml 0,3 % ethanol	x
12	0,66 ng/l	0,3 %	1→3 with 0,3 % ethanol		x

NOTE After the addition of the yeast cells to the test well (9.3.7) the maximal final concentration of E2 is 333 ng/l. The final volume fraction of ethanol in the test wells with the reference compound is 0,2 %. This ethanol concentration can be tolerated by the yeast cells and has no impact on the test result.

9.3.3 Negative control

Use sterile water (7.2) as negative control (3.11). Each 96-well plate shall contain at least four replicates of the negative control. Use a volume of 80 µl per replicate.

9.3.4 Blank replicate

For each sample add at least four respective blank replicates (3.1), 80 µl each, on the same 96-well plate. The highest sample concentration is used for the blank replicates. In case of the negative control and the E2-reference one blank replicate is sufficient.

9.3.5 Sample dilution

Homogenize the sample before use by shaking. Prepare six successive dilutions of the test sample with sterile water (7.2) resulting in seven dilution levels (see Annex E for an example). Test at least four replicates for each dilution level.

9.3.6 Field blank

Prepare the field blank (3.6) similar to the samples, but without dilutions. For each sample a representative field blank has to be analysed at the same day using the same media, cell culture and instrumentation. 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 9.3.5. In this case proceed as described in 9.3.7 for the blank replicates.

If a field blank is representative for a series of samples, it may be tested only once.

9.3.7 Plate setup

Use a volume of 80 µl for each well (sample, dilutions, negative control, field blank, blank replica). Test each sample in seven dilution levels. Test at least four replicates of each dilution level on the same 96-well plate. Additionally, the sample plate shall contain at least four replicates of the negative control (9.3.3). As already described in (9.3.4) test for each sample at least four blank replicates on the same 96-well plate. In case of the negative control one blank replicate is sufficient. Test on each plate a concentration-response relationship of the reference 17β-estradiol (9.3.2) in duplicates. Test in total at least four replicates of the E2 dilutions series (9.3.2) in parallel. If only one 96-well plate is used for the assay, test at least four additional 17β-estradiol dilutions series (9.3.2) on a separate 96-well plate.

A recommended plate setup is shown in Annex B.

Inoculate the samples with the prepared yeast cells (see 9.3.8) within 2 h after the plate setup.

9.3.8 Inoculation of the test plate

Finish the plate setup (9.3.7) prior to the preparation of the inoculum.

Adjust the FAU of the cell suspension to 250 ± 10 % for example as described as follows:

Dilute an aliquot (e.g. 200 µl) of the overnight culture (9.2) 1→10 with growth medium (7.4.7) under sterile conditions. Transfer 300 µl in a well of a 96-well plate and measure the optical density of the 1→10 dilution at 600 nm ± 20 nm. Use 300 µl growth medium (7.4.7) for the background correction. Calculate the FAU of the diluted overnight culture according to ISO 7027. Use 600 nm for the FAU calibration.

Calculate the required volume of the 10-fold inoculum (resuspended yeast cells with adjusted FAU for the exposure) as follows:

$$V_{\text{10-fold inoculum}} = 0{,}5 + n \times 0{,}5 \qquad (2)$$

where

$V_{\text{10-fold inoculum}}$ is the volume in millilitres (ml) of the exposure medium (7.4.8) needed for the preparation of the 10-fold inoculum;

n is the number of 96-well plates used in parallel.

Calculate the needed volume of the overnight culture according to:

$$V_{\text{overnight-culture}} = \frac{250}{x_{\text{FAU}} \times 10} \times V_{\text{10-fold inoculum}} \quad (3)$$

where

$V_{\text{overnight-culture}}$ is the volume in millilitres (ml) of the overnight culture which is needed to prepare the 10-fold inoculum for the exposure;

x_{FAU} is the calculated FAU-value of the 1→10 diluted overnight culture;

$V_{\text{10-fold inoculum}}$ is the volume in millilitres (ml) of the exposure medium (7.4.8) needed for the preparation of the 10-fold inoculum.

Carry out all following steps under sterile conditions. Transfer the calculated volume of the overnight culture [Formula (3)] to a new test tube and pellet the yeast cells by centrifugation at 2 500 *g* for 10 min. Discard the supernatant and resuspend the yeast cells in the required volume of exposure medium (7.4.8). For quality control it is recommended to verify the FAU of the resulting cell suspension by a measurement of the optical density at 600 nm ± 20 nm. Use 300 µl of the suspension in a 96-well plate. Use exposure medium (7.4.8) for the background correction.

Dilute the prepared 10-fold inoculum 1→10 with exposure medium (7.4.8) to achieve a FAU-value of 25, e.g. by adding 4,5 ml of exposure medium (7.4.8) to 500 µl of the 10-fold inoculum.

It is not recommended to prepare directly a cell suspension with a FAU of 25 for exposure, because in this case the measurement of the OD600 for quality control results in low signals which are not reliable.

Add 40 µl exposure medium (7.4.8) to all wells which contain blank replicates. Add 40 µl of the diluted cell suspension (1-fold inoculum with a FAU-value of 25) to all other wells [negative control, samples and their dilutions, field blanks, and the dilution series of the reference compound (E2)].

Measure the optical density at 600 nm ± 20 nm and save the data for the subsequent data evaluation.

NOTE This measurement is needed for the assessment of possible bacterial growth in the assay. An increase of the optical density at 600 nm in the blank replicates (3.1) during the exposure indicates bacterial growth.

Cover the 96-well plate with a gas permeable foil (6.19) to prevent the test from drying out. Mix properly the content of each 96-well plate by vigorous shaking for about 30 s. Avoid spilling of culture medium during the agitation. Each laboratory has to define a suitable rotary speed which depends on the actual type of shaker in the laboratory.

Incubate the test at 30 °C ± 1 °C for 18 h ± 1 h without agitation. Do not cover the 96-well plates with a plastic lid and do not stack the prepared 96-well plates during this incubation.

9.4 Measurement

9.4.1 Measurement of the cell density

Resuspend the yeast cells after the incubation of 18 h thoroughly either by vigorous shaking or pipetting up and down. Measure the cell density for the determination of growth inhibition and/or cytotoxic effects at 600 nm ± 20 nm.

The proper resuspension of the yeast cells is crucial for the test procedure. Without homogeneously resuspended cells the measurement at 600 nm and the measurement of the reporter gene activity are highly error prone and will most likely lead to invalid test results.

9.4.2 Measurement of the reporter gene activity

Transfer 30 µl from each well of the test plate to a new 96-well plate. Add 50 µl of the lacZ reaction mixture (7.4.13) to each well. Measure the optical density at 580 nm ± 20 nm. Incubate the plate for 1 h at 30 °C ± 1 °C under constant agitation. Due to the activity of the added enzyme lyticase, the yeast cells are lyzed during the incubation. Verify the cell lysis by an optical inspection. If the samples are turbid, pellet the remaining yeast cells and cell debris by a centrifugation at 4 000 *g* for 10 min. Transfer 60 µl of the cleared supernatant to a new 96-well plate for the measurement of the reporter gene activity.

If air bubbles are present in the wells before the measurement of the optical density, remove the air bubbles, e.g. by using the airflow of a hair-dryer.

Measure the optical density at 580 nm ± 20 nm.

If the measurement at 580 nm ± 20 nm exceeds the linear range of the photometer repeat the measurement at 540 nm ± 20 nm.

The first measurement at 580 nm is needed for the assessment of possible artificial cleavage of the substrate CPRG by compounds of the tested sample. An increase of the optical density at 580 nm in the blank replicates of the sample (3.1) during the incubation indicates artificial cleavage of the substrate.

Frequently photometers with filter are equipped with a filter for a measurement at 540 nm instead of 580 nm. If the measurement of the reporter gene activity has to be done at 540 nm it is recommended to double the volumes, i.e. 60 µl of exposed cells and 100 µl the lacZ reaction mixture (7.4.13).

9.5 Calculation of the corrected absorbance and the reporter gene induction

Calculate the corrected absorbance (A_C) for all dilutions of the samples (SD), all dilutions of the reference compound (RD) the negative control (NC) and the field blank according to Formula (4).

$$A_c(i) = \frac{\left[A_{580}(i) - \overline{B_{580}}(i)\right]}{\left[A_{600}(i) - \overline{B_{600}}(i)\right]} \tag{4}$$

where

$A_c(i)$ is the corrected absorbance for test *i* (sample dilutions SD, reference dilutions RD, negative control NC);

$A_{580}(i)$ is the absorbance at 580 nm for test *i*;

$\overline{B_{580}}(i)$ is the mean absorbance at 580 nm for blank replicates of test *i*;

$A_{600}(i)$ is the absorbance at 600 nm for test *i*;

$\overline{B_{600}}(i)$ is the mean absorbance at 600 nm for blank replicates of test *i*.

Calculate the mean A_C for the negative control (NC), the field blank and all tested dilutions of the sample and the reference compound (E2 dilution series) and the respective standard deviation [$\sigma(i)$]. For the assessment of the test results use these mean values. Use the mean corrected absorbance for a subsequent statistical evaluation and the construction of a concentration-response curve.

Calculate the induction rate (I) of the test with the maximal E2 concentration of the E2 dilution series (9.3.2) according to Formula (5). The induction rate quantifies the fold induction of the estrogen

receptor in the test compared to the basal receptor activity which is determined in the negative control. A value for a valid minimal induction rate is given in Clause 10.

$$q_I\left(\mathrm{E2_{max}}\right)=\frac{\overline{A_c}\left(\mathrm{E2_{max}}\right)}{\overline{A_c}\left(\mathrm{NC}\right)} \tag{5}$$

where

$q_I(\mathrm{E2_{max}})$ is the induction rate of the test with the highest E2 concentration of the E2 dilution series (9.3.2);

$\overline{A_c}\left(\mathrm{E2_{max}}\right)$ is the mean of the corrected absorbance of the two exposures with the highest E2 concentration of the E2 dilution series according to Formula (4);

$\overline{A_c}\left(\mathrm{NC}\right)$ is the mean of the corrected absorbances of the negative control replicates.

9.6 Calculation of the relative growth

The relative growth $G(\mathrm{i})$ is calculated in order to assess possible toxic effects of the sample. If the acute toxicity of the sample is too strong, estrogenic effects may be masked and cannot be quantified (see Clause 10 for further details). Calculate the relative growth $G(\mathrm{i})$ according to:

$$G\left(i\right)=\frac{\left[A_{600}\left(i\right)-\overline{B_{600}}\left(i\right)\right]}{\left[A_{600}\left(\mathrm{NC}\right)-\overline{B_{600}}\left(\mathrm{NC}\right)\right]} \tag{6}$$

where

$G(i)$ is the relative growth of test i (sample dilutions SD, reference dilutions RD, positive control PC);

$A_{600}(i)$ is the absorbance at 600 nm for test i;

$\overline{B_{600}}\left(i\right)$ is the mean absorbance at 600 nm for blank replicates of test i;

$A_{600}(\mathrm{NC})$ is the absorbance at 600 nm for negative control;

$\overline{B_{600}}\left(\mathrm{NC}\right)$ is the mean absorbance at 600 nm for blank replicates of the negative control.

9.7 Estimation of the EC_{50} of the reference compound by linear interpolation

Determine the EC50 of the 17β estradiol concentration response curve by an appropriate statistical method such as probit analysis, moving average or binomial methods (see References [16] and [17]).

For a graphical estimation on a Gaussian logarithmic diagram, see Reference [18].

10 Validity criteria

The overall test is valid if:

a) the EC_{50} of 17β-estradiol, derived from the 17β-estradiol concentration-response relationship is between 39 ng/l and 107 ng/l;

b) the mean corrected absorbance for the negative controls is in the range from 0,05 to 1,5;

c) the relative standard deviation of the corrected absorbance for the negative controls is <30 %;

d) the induction rate (9.5) induced by the highest E2 concentration is >10;

e) the mean OD 600 of the blank replicates (3.1) of a sample after exposure is not higher than the respective value before exposure (t-test with α = 0,05);

f) the mean OD 580 of the blank replicates (3.1) of a sample after measurement of the reporter gene activity (9.4.2) is not higher than the respective value before (t-test with α = 0,05), otherwise an artificial cleavage of the substrate might take place and the sample cannot be measured with this test procedure.

A dilution level of a sample is valid if:

a) the relative growth of yeast cells in this dilution level of the sample is >0,7 and <1,3;

b) the relative standard deviation of the corrected absorbance between the four replicates is <15 % (inter well deviation).

11 Assessment criteria

The test sample is defined to mediate an estrogenic activity if a significant concentration-related increase of reporter gene activation is measured which exceeds significantly the reporter gene activity in the field blank.

An example of statistical assessment is given in Annex H.

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/ 19040-1:2018;

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, source, date of arrival in the laboratory, storage conditions, date of stock culture preparation, and date of genotype checking (if this date deviates from stock culture preparation), obtained FAU of the overnight culture, adjusted FAU of the inoculum;

f) testing environment (address of testing laboratory, date of test, type of photometer used);

g) test results (mean corrected absorbance for field blank and each tested sample- and reference dilution with standard deviation, qualitative assessment of the estrogenicity of the sample (yes/no) with information about statistical evaluation, 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, further reporting is required as described in Annex I. If LID-values are calculated, further reporting is required as specified in Annex J.

Annex A
(normative)

Strain selection

A.1 General

Annex A describes the method for the selection of the *S. cerevisiae* strain.

A.2 Agar plates for strain selection

Suspend 2 g Agar (7.1.20) in 78 ml water.

Add a magnetic stirrer and autoclave the suspension for 20 min at 121 °C ± 2 °C.

Cool down the solution to 50 °C to 60 °C and add under sterile conditions:

— 10 ml 10x SD-Medium (7.4.1);

— 10 ml 10x McD-DO-Medium (7.4.2).

Mix the solution gently, avoid the formation of air bubbles in the mixture.

Add about 20 ml of the solution to a petri dish (Ø 9 cm) under sterile conditions. Cool down the prepared agar plates overnight.

A.3 Plating of the test strain

For strain selection streak a thawed stock culture in a 1→100 dilution with growth medium (7.4.7) on an agar plate for strain selection (A.2) and incubate the plate at 30 °C ± 1 °C overnight. Pick a single colony and inoculate 5 ml of the growth medium (7.4.7). Incubate the culture for 18 h overnight at 30 °C ± 1 °C under vigorous agitation. Use this culture for the preparation of stock cultures as described (9.1).

Annex B
(informative)

Plate set up

Annex B gives an example of a suitable plate set up for the YES.

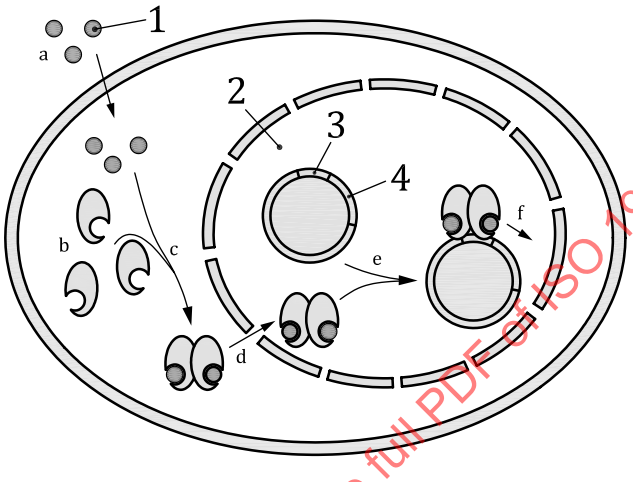
Table B.1 — B.Plate set up with two samples with seven successive dilution levels in four replicates

	1	2	3	4	5	6	7	8	9	10	11	12
A	E2, 0,66 ng/l	Negative control	Sample 1 (dilution level 12)				Field blank sample 1	Sample 2 (dilution level 12)				E2, 0,66 ng/l
B	E2, 2,0 ng/l		Sample 1 (dilution level 8)					Sample 2 (dilution level 8)				E2, 2,0 ng/l
C	E2, 6,2 ng/l		Sample 1 (dilution level 6)					Sample 2 (dilution level 6)				E2, 6,2 ng/l
D	E2, 18,6 ng/l		Sample 1 (dilution level 4)					Sample 2 (dilution level 4)				E2, 18,6 ng/l
E	E2, 55,6 ng/l		Sample 1 (dilution level 3)				Field blank sample 2	Sample 2 (dilution level 3)				E2, 55,6 ng/l
F	E2, 166,6 ng/l		Sample 1 (dilution level 2)					Sample 2 (dilution level 2)				E2, 166,6 ng/l
G	E2, 500 ng/l		Sample 1 (dilution level 1)					Sample 2 (dilution level 1)				E2, 500 ng/l
H	Blank replicate (E2, 500 ng/l)	Blank replicate NC	Blank replicates sample 1 (undiluted)					Blank replicates sample 2 (undiluted)				Blank replicate (E2, 500 ng/l)

Annex C
(informative)

Scheme of test principle

Annex C illustrates the underlying test principle of the YES. See Figure C.1.



Key

1 ER-agonist
2 nucleus
3 ERE
4 reporter gene
a The ER-agonist (xenoestrogen) diffuses into the yeast cell.
b The human estrogen receptor alpha (hERα) is heterologously expressed [e.g. via a plasmid which contains the respective gene under the control of a constitutive promoter (not shown)].
c The ER-agonist binds to the estrogen receptor and induces a dimerization of hER*α*.
d Due to receptor binding and dimerization a nuclear localization signal is exposed and the dimer is imported in the cell nucleus.
e The receptor dimer binds to and activates a promoter that contains an estrogen responding element (ERE). The reporter construct might be integrated in the yeast genome or can be part of a plasmid as shown here.
f The expression of the reporter gene is proportional to the activation of the hERα by the (xeno)estrogens.

Figure C.1 — Basic test principle of the YES

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. Dissolve final extracts preferably in dimethylsulfoxide (DMSO), alternatively ethanol can be used. Prepare reference compound concentrations for the concentration-response curve using the same solvent as the samples.

D.3 Test with diluted organic solutions or extracts

The procedure is identical to the description in Clause 9 with the following exceptions:

Use ethanol, methanol or DMSO as organic solvent for extraction procedures or the preparation of organic solutions of chemicals. Prepare an aqueous dilution of the organic extract or solution that the maximal volume fraction of the organic solvent is 1 %.

Use an aqueous solution with the same volume fraction of the organic solvent:

— for the preparation of the dilution series of the sample;

— for the negative control;

— for the preparation of the E2 reference and the dilution series of the E2 reference.

Use 80 µl of the aqueous organic solution for each well and add 40 µl of the prepared cell suspension (9.3.8) to the negative control, the sample dilutions the dilution series of the reference compound and the field blank. To prepare 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.

D.4 Direct testing of organic solutions or extracts

The procedure is identical to the description in Clause 9 with the following exceptions:

Use ethanol or methanol as organic solvent for extraction procedures or the preparation of organic solutions of chemicals. Organic solutions or extracts can be directly added to and diluted with the organic solvent on the 96-well plate using volumes between 20 µl and 80 µl per well. An example with 40 µl organic solution or extract per well is presented below using a 96-well plate layout that is analogous to the layout shown in Table B.1.

Column 2 receives 40 µl organic solvent and serves as the negative control. Column 7 receives 40 µl of the field blanks.

In columns 3 to 6 and 8 to 11, each well in rows B to H receives 40 µl organic solvent. 40 µl organic solution or extract are pipetted to rows A and B. The 80 µl volume in the row B is mixed by pipetting 40 µl up and down and repeating this three times. 40 µl are then transferred from row B to row C, mixed and transferred to row D and so on to create a 1 in 2 dilution series. Finally, the 40 µl coming out of row H are discarded.

To prepare the E2-reference use the 83,3 µg/l ethanolic E2-solution (9.3.2) and dilute it 40-fold with the organic solvent (e.g. add 20 µl E2-solution to 780 µl organic solvent) to 2,08 µg/l. Pipette 100 µl of organic solvent in each of 8 wells of a separate 96-well plate. Add 100 µl of the 2,08 µg/l E2-solution to the first well and make a 1 in 2 dilution series over 7 wells. 20 µl of the dilution series is pipetted to the plate, i.e. in columns 1 and 12 over rows A to G. The volume used for the E2-reference (i.e. 20 µl per well) needs to be matched to the volume of the organic solutions or extracts (i.e. in the range of 20 µl to 80 µl). In the example above, with 40 µl organic solution or extract, an additional 20 µl of organic solvent needs to be added to each well of the E2-reference dilution series.

Evaporate the organic solvent under sterile conditions. Add 80 µl sterile water (7.2) and 40 µl of the cell suspension (9.3.8) to the negative control, the sample dilutions, the dilution series of the reference compound and the field blank. Cover the 96-well plate with a gas permeable foil which prevents the test from drying out. Redissolve the samples by vigorous shaking for 2 min (avoid spilling of culture medium during the agitation).

NOTE The way how a sample is transferred to the plate (as an aqueous sample, a diluted extract or a redissolved extract) can affect results (see References [19] and [20]).

D.5 Data from literature

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:

$$P_r = \frac{\mathrm{EC}_{50}(R)}{\mathrm{EC}_{50}(C)} \times 100 \qquad \text{(D.1)}$$

where

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

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

$\mathrm{EC}_{50}(C)$ is the EC_{50} of the compound.

Table D.1 shows the relative potencies to 17β-estradiol for selected compounds together with the respective reference.

Table D.1 — Summary of relative potencies to 17β-estradiol for selected compounds

Compound	Relative potency (P_r) to 17β-estradiol (%)	Reference
17α-estradiol	5,25	[21]
17β-ethinylestradiol	88,8	[21]
17β-estradiol	100	[21]
17β-estradiol-3(beta-D-glucuronide)	0,32	[21]
17β-estradiol-3-glucuronide-17-sulfate	0	[21]
17β-estradiol-3-sulfate	0,01	[21]
2',3',4',5'-Tetrachloro-4-biphenylol	0,82	[21]

Table D.1 *(continued)*

Compound	Relative potency (P_r) to 17β-estradiol (%)	Reference
2,3,7,8-Tetrachlorodibenzo-p-dioxin	0,26	[21]
2',4',6'-Trichloro-4-biphenylol	1	[21]
2',5'-dichloro-4-biphenylol	0,62	[21]
2'-Chloro-4-biphenylol	0,003 7	[21]
2-tert-Butylphenol	0,000 218 06	[22]
3,3',5,5'-Tetrachloro-4–4'-biphenyidiol	0,016	[21]
4'-Chloro-4-biphenylol	0,06	[21]
4-Hydroxytamoxifen	0,007 3	[21]
4-Nonylphenol (straight chain)	0,002 2	[21]
4-Nonylphenol (technical grade)	0,005	[21]
4-Octylphenol	0,003	[21]
4-tert-Butylphenol	0,004 617 65	[22]
4-tert-Octylphenol	0,000 36	[21]
α-Zearalanol (zearanol)	1,3	[21]
α-Zearalenol	8,7	[21]
Androstenediol	0,023	[21]
Androstenedione	0	[21]
β-Sitosterol	0,000 45	[23]
β-Zearalanol	0,46	[21]
β-Zearalenol	0,066	[21]
Bis(2-ethylhexyl)phthalate	0	[21]
Bisphenol A	0,005	[21]
Bochanin A	0,009 1	[21]
Butylbenzylphthalate	0,000 4	[21]
Butylhydroxytoluene	0	[21]
Chlorophene	0,009 9	[21]
Cholesterol	0	[21]
Clomiphene	0,002 3	[23]
Cortisol	0	[21]
Coumestrol	0,67	[21]
Daidzein	0,001 3	[21]
DDT	0,000 03	[21]
Dehydroepiandrosterone	0,001 8	[21]
Dienestrol	25,4	[21]
Diethylstilbestrol	74,3	[21]
Dihydrotestosterone	0,05	[23]
Di-*n*-butylphthalate	0	[21]
D-Norgestrel	0,000 4	[21]
Equol	0,085	[21]
Estriol	0,63	[21]
Estrone	9,6	[21]
Formononetin	0,005 6	[21]
Genistein	0,049	[21]
Hexestrol	30,6	[21]

Table D.1 *(continued)*

Compound	Relative potency (P_r) to 17β-estradiol (%)	Reference
ICI 164,384	0,001 6	[23]
ICI 164,384	0,001 6	[23]
Mestranol	7,3	[21]
Methoxychlor	0,003 3	[21]
Nafoxidine	0	[21]
o,p'-DDD	0,000 006 7	[23]
o',p'-DDE	0,000 04	[21]
o',p'-DDT	0,000 11	[21]
Progesterone	0	[21]
Stigmasterol	0,000 22	[22]
Tamoxifen	0,004 7	[21]
Testosterone	0,001	[21]
Zearalenone	0,26	[21]

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	Final percentage of the sample in the test
1	1→1 No pre-dilution. The sample is used as it is.	1→1,5 80 µl sample + 40 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.	66 %
2	1→1,33 e.g. 1,5 ml sample + 0,5 ml dilution water	1→1,5	1→2	50 %
3	1→2 e.g. 1,0 ml sample + 1,0 ml dilution water	1→1,5	1→3	33,3 %
4	1→2,66 e.g. 0,75 ml sample + 1,25 ml dilution water	1→1,5	1→4	25 %
6	1→4 e.g. 0,5 ml sample + 1,5 ml dilution water	1→1,5	1→6	16,7 %
8	1→5,33 e.g. 0,375 ml sample + 1,625 ml dilution water	1→1,5	1→8	12,5 %
12	1→8 e.g. 0,25 ml sample + 1,75 ml dilution water	1→1,5	1→12	8,3 %

Annex F
(informative)

Performance data

F.1 Design of the interlaboratory trial

F.1.1 General

An interlaboratory test based on the procedure described in this document was carried out in November/December 2016 for the generation of these validation data (see Reference [24]). The aim of this study was to demonstrate the suitability of the proposed method for the determination of the estrogenic potential of water and waste water by means of a reporter gene assay utilizing modified yeast cells (*Saccharomyces cerevisiae*). The statistical evaluation of the data was performed according to ISO 5725-2.

F.1.2 Description of samples

In total eight samples were analysed.

Table F.1 — Description of samples

Sample number	Sample type	Description	Expected estrogenic activity	Nominal EEQ-value [ng/l]
S1	Aqueous	Effluent of a municipal sewage treatment plant (STP)	±	
S2	Aqueous	Same as sample 1 but spiked with 15 ng/l 17β-ethinylestradiol (EE2)	+	S1 + 15 ng EEQ/l
S3	Aqueous	Influent of a municipal STP (same STP as for samples 1 and 2)	++	
S4	Aqueous	Surface water rhine	–	
S5	Aqueous	Same as sample S4, but spiked with 20 ng/l EE2	+	S4 + 20 ng EEQ/l
S6	Aqueous	Blanc water (de-ionized water)	–	
S7	Ethanolic	Mix 1: 800 ng/l E2 + 5 000 ng/l E1, which have been diluted 1- > 100 which results to final concentrations of 8 ng/l E2 + 50 ng/l E1 in 1 % ethanolic solution	+	14 ng EEQ/l
S8	Ethanolic	Mix 2: 5 mg/l Bisphenol A + 80 mg/l tert-Butylphenol + 100 mg/l Benzylbutylphthalat;, which have been diluted 1- > 100 which results to final concentrations of 50 µg/l Bisphenol A + 800 µg/l tert-Butylphenol + 1 mg/l Benzylbutylphthalat in 1 % ethanolic solution	++	

NOTE Based on historical screening data, the expected estrogenic activity of the samples was scored "+" expected low activity (close to the assay LOQ), "++" expected significant activity (quantified between the assay LOQ and EC_{50} of the reference curve), "-" no activity expected.

F.1.3 Participating laboratories

16 laboratories provided data for the distributed samples. The samples S1 to S6 have been tested by all participating laboratories. For the samples S7 and S8 which could be tested optionally, ten laboratories provided data as indicated in the Table F.2. The samples were coded and tested at least two times independently. A third testing was optional.

Table F.2 — Overview of tested samples

Laboratory	YES Mc Donnell	
	Samples S1 to S6	Samples S7 and S8
L01	X	X
L02	X	
L03	X	
L04	X	X
L05	X	
L06	X	X
L07	X	X
L08	X	X
L09	X	X
L10	X	
L11	X	X
L12	X	
L13	X	X
L14	X	
L15	X	X
L16	X	X
Number of data sets	**16**	**10**

F.2 Results of the interlaboratory trial

F.2.1 General

All laboratories carried out either two or three repeated measurements per sample. Two samples were tested on the same 96-well plate together with the respective controls, thus depending on the number of test repetitions per lab a total of six or nine repeated measurements were available for the control measurements, i.e. negative control, concentration response curve of the reference. According to Annex E of this document the samples were tested at dilution levels D1, D2, D3, D4, D6, D8 and D12 by all laboratories.

For the samples S1 to S6 all 16 laboratories provided data resulting in 41 to 44 individual measurements per sample. For the samples S7 and S8 ten laboratories provided data resulting in 23 individual measurements per sample. Based on the validity criteria given in Clause 10 invalid measurements were identified an excluded from the further data evaluation. For the samples S1 to S8 the percentage of invalid test results varied between 14 % and 26 %. In total 17,2 % percent of all measurements were excluded because of an invalid EC_{50} value of the E2 concentration response curve. Furthermore, 4,5 % of the measurements were excluded because the variability of the respective corrected absorbance of the negative control (*CV* % Ac(NC)) was >30 %. In sum the overall percentage of invalid test results was 19,1 %.

All measurements of L09 were excluded because of invalid EC_{50}-values of the reference. In case of L03 and L05 50 % of the measurements were invalid either because of invalid EC_{50}-values of the reference or *CV* % Ac(NC) > 30 %. In case of L07 the percentage of invalid test results because of EC_{50}-values

of the reference was 44 %. The percentage of invalid test results of the laboratories L10, L15 and L16 varied between 11 % and 33 %. All measurements from the remaining nine laboratories were valid.

F.2.2 Summary of 17β-estradiol equivalent concentrations (EEQ)

The median of all reported LOQ-values for the reference compound E2 was 10,8 ng/l. As expected with few exceptions the quantifications of the 17β-estradiol equivalent concentrations for the low- and unpolluted samples S1 (effluent of a municipal sewage treatment plant), S4 (surface water Rhine) and S6 (de-ionized water) were below the LOQ and thus no further statistical analysis was performed for these samples.

In some cases results below LOQ were reported for the contaminated samples S2, S3 and S5. These values were omitted from further calculations e.g. the calculation of the laboratory mean value because of the inherent uncertainty.

From all valid single measurements for the EEQ-values (samples S2, S3 and S5) four results were identified as outliers and omitted from further analysis. No further data based on the laboratory mean values were identified as outliers.

The results are summarized in Table F.3 and shown in Figure F.1. The samples S2, S3 and S5 showed geometric mean values for the equivalence concentration of 17,9 ng/l, 35,5 ng/l and 22,4 ng/l EEQ, respectively. The different contaminated levels were reflected very well by the laboratory results. This holds true as well for the ethanolic samples S7 and S8 with mean EEQ-values of 14,6 ng/l and 29,4 ng/l.

The relative reproducibility standard deviation (s_R) for the aqueous samples S2, S3 and S5 were 26,7 %, 40,9 % and 28,0 %. In case of the ethanolic samples S7 and S8 that were tested optionally by the laboratories the respective s_R-values were 46,1 % and 54,7 %. This results in a mean standard deviation of reproducibility of 31,9 % for the aqueous samples.

Compared to water samples without ethanol, the testing of a mixture of reference compounds in an aqueous ethanolic solution of 1 % resulted in a higher variability of results that has to be taken into account for data interpretation.

Table F.3 — Summary of EEQ results [ng/l] of the Yeast Estrogen Screen (YES, McDonnell[10])

	Sample							
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
Number of laboratories	16	16	16	16	16	16	8	8
Number of measurements	42	42	41	44	43	43	23	23
Invalid measurements	8	7	10	8	7	6	6	6
% Invalid measurements	19,0	16,7	24,4	18	16,6	14,0	26,0	26,0
Number of valid measurements < LOQ	34	5	2	34	2	33	3	-
Outliers excluded (single measurements)	n.d.	1 (L15)	—	n.d.	1 (L10)	n.d.	1 (L07)	1 (L07)
Outliers excluded (means)	n.d.	—	—	—	—	—	—	—
Number of laboratories for statistics	14	14	13	15	13	15	7	8

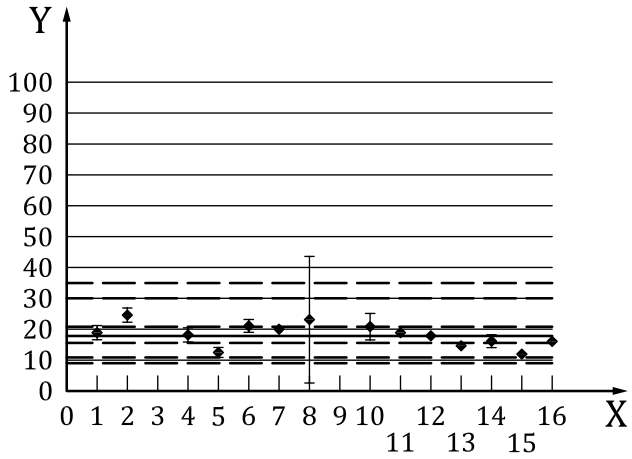
Table F.3 *(continued)*

Number of valid measurements > LOQ, without outliers	0	29	29	2	33	4	13	16
Laboratory geometric mean (expected value) - *n*								
L01	< LOQ *n* = 3	18,7 (18,9) *n* = 3	36,0 (36,0) *n* = 2	< LOQ *n* = 3	23,9 (24,1) *n* = 3	< LOQ *n* = 3	16,8 (16,8) *n* = 3	41,2 (42,1) *n* = 3
L02	< LOQ *n* = 3	24,5 (24,6) *n* = 3	52,1 (52,7) *n* = 3	9,2 / < LOQ *n* = 3	27,6 (27,6) *n* = 3	< LOQ *n* = 3	—	—
L03	—	—	—	5,7 *n* = 1	< LOQ *n* = 1	24,2 / < LOQ *n* = 2	—	—
L04	< LOQ *n* = 3	18,3 (18,5) *n* = 3	37,8 (39,0) *n* = 3	< LOQ *n* = 2	26,4 (26,6) *n* = 3	< LOQ *n* = 3	< LOQ *n* = 1	32,9 *n* = 1
L05	< LOQ *n* = 1	12,0 (12,1) *n* = 2	—	< LOQ *n* = 1	—	< LOQ *n* = 2	—	—
L06	< LOQ *n* = 3	21,1 (21,2) *n* = 3	49,9 (50,2) *n* = 3	< LOQ *n* = 3	23,5 (23,5) *n* = 3	14,8 / < LOQ *n* = 3	11,0 (13,4) *n* = 2	23,1 (27,3) *n* = 3
L07	< LOQ *n* = 1	19,8 *n* = 1	28,9 *n* = 1	< LOQ *n* = 3	28,4 (28,9) *n* = 3	< LOQ *n* = 1	29,6 (29,9) *n* = 2	56,1 (56,2) *n* = 2
	Sample							
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
L08	< LOQ *n* = 3	16,8 (22,8) *n* = 2	30,7 (38,3) *n* = 3	< LOQ *n* = 3	19,6 (20,6) *n* = 3	< LOQ *n* = 3	10,3 (10,4) *n* = 3	14,7 (17,7) *n* = 3
L09	—	—	—	—	—	—	—	—
L10	< LOQ *n* = 3	20,2 (20,7) *n* = 3	31,8 (31,8) *n* = 2	< LOQ *n* = 2	15,7 (16,0) *n* = 2	< LOQ *n* = 3	—	—
L11	< LOQ *n* = 3	18,8 *n* = 1	56,5 *n* = 1	< LOQ *n* = 3	18,7 (18,9) *n* = 3	< LOQ *n* = 3	18,0 *n* = 1	31,3 (31,4) *n* = 2
L12	< LOQ *n* = 3	17,9 *n* = 1	28,5 (31,8) *n* = 3	< LOQ *n* = 3	25,5 (26,6) *n* = 3	< LOQ *n* = 3	—	—
L13	< LOQ *n* = 2	14,4 *n* = 1	30,4 (30,7) *n* = 2	< LOQ *n* = 2	15,5 *n* = 1	< LOQ *n* = 1	11,8 *n* = 1	41,4 *n* = 1
L14	< LOQ *n* = 2	16,1 (16,2) *n* = 2	32,8 (33,1) *n* = 2	< LOQ *n* = 2	18,8 (18,8) *n* = 2	11,2 / < LOQ *n* = 2	—	—
L15	< LOQ *n* = 2	11,8 *n* = 1	40,4 (60,1) *n* = 2	< LOQ *n* = 2	22,8 (31,7) *n* = 2	< LOQ *n* = 2	—	—
L16	< LOQ *n* = 2	16,0 (16,1) *n* = 3	20,5 (20,6) *n* = 2	< LOQ *n* = 3	19,8 (20,1) *n* = 2	6,4 / < LOQ *n* = 3	11,8 *n* = 1	27,3 *n* = 1
min – max		11,8 – 24,5	20,5 – 52,1		15,5 – 28,4		10,3 – 29,6	14,7 – 56,1
factor max/min		2,1	2,5		1,8		2,9	3,8

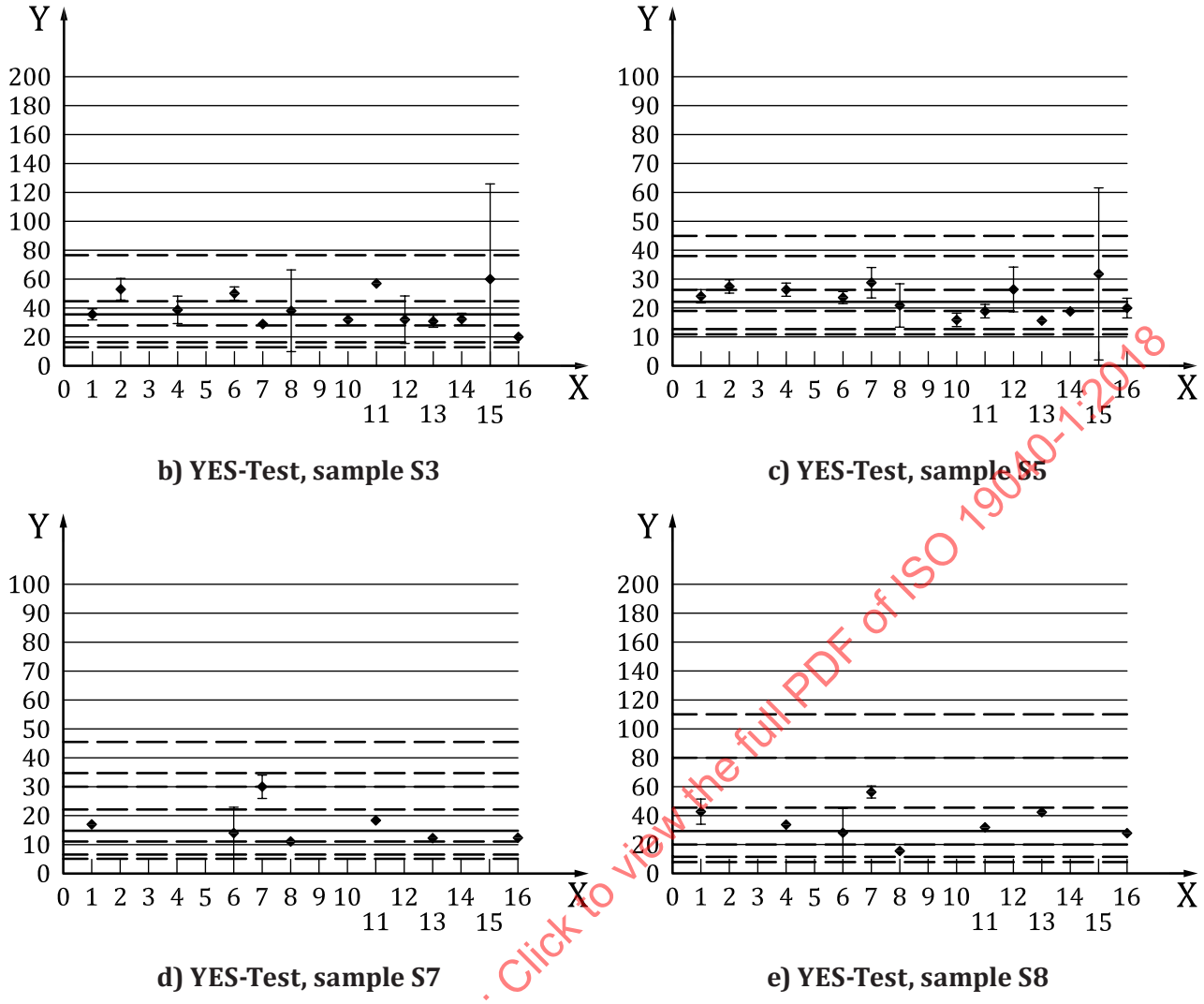
Table F.3 *(continued)*

geometric mean	n.d.	17,9	35,5	n.d.	22,4	n.d.	14,6	29,4
expected value		18,6	38,4		23,3		16,1	33,5
95 % confidence interval		15,4 – 20,9	28,0 – 45,1		19,0 – 26,4		10,6 – 21,9	19,2 – 45,1
95 % prediction interval		10,7 – 30,0	16,4 – 76,8		13,1 – 38,4		6,2 – 34,5	10,1 – 80,2
99 % prediction interval		9,1 – 35,2	12,9 – 97,6		11,1 – 45,4		4,7 – 45,1	7,9 – 109,6
s_r (repeatability)		4,41	13,1		5,75		3,90	6,33
s_L (interlaboratory variability)		2,26	8,65		3,05		6,31	17,22
s_R (reproducibility)		4,96	15,7		6,51		7,42	18,35
s_r %		23,8	34,1		24,7		24,2	18,9
s_L %		12,2	22,5		13,1		39,2	51,4
s_R %		26,7	40,9		28,0		46,1	54,7
s_R / s_r		1,1	1,2		1,1		1,9	2,9

s_r	repeatability standard deviation
s_R	reproducibility standard deviation
s_L	laboratory standard deviation
n.d.	not determined
n	number of measurements



a) YES-Test, sample S2



b) YES-Test, sample S3

c) YES-Test, sample S5

d) YES-Test, sample S7

e) YES-Test, sample S8

Key

X	laboratory
Y	EEQ (ng/l)
black marked symbols	laboratory specific expected values with standard deviation (Whisker)
black solid line	interlaboratory geometric mean
black dotted lines (small)	95 % confidence interval of the interlaboratory geometric mean
black dotted lines (big)	95 % tolerance range of the interlaboratory geometric mean
black dashed lines	99 % tolerance range of the interlaboratory geometric mean

Figure F.1 — Summary of 17β-estradiol equivalent concentrations (EEQ) [ng/l] of samples S2, S3, S5, S7 and S8

F.2.3 Summary of lowest ineffective dilutions LID

The lowest ineffective dilution is determined as described in Annex J. for each independent experiment. Dilutions are prepared according to Table E.1. All valid single test results were used for the statistical analysis. The test result of laboratory 11 for sample 3 was identified as an outlier based on the laboratory mean values and was omitted from the further statistical evaluation.

The low- and unpolluted samples S1 (effluent of a municipal sewage treatment plant), S4 (surface water Rhine) and S6 (deionized water) showed LID-values of 1, i.e. no statistical significant stimulation of the assay by the undiluted samples.

The samples S2, S3 and S5 showed mean LID-values of 2,5, 6,4 and 3,4, respectively. The different contaminated levels were reflected by these laboratory results. This holds true as well for the ethanolic samples S7 and S8 with mean LID-values of 2,0 and 4,9.

The relative reproducibility standard deviation (s_R) for the aqueous samples S2, S3 and S5 were 49,7 %, 44,5 % and 46,6 %. In case of the ethanolic samples S7 and S8 that were tested optionally by the laboratories the respective s_R-values were 42,0 % and 35,0 %. This results in a mean standard deviation of reproducibility of 46,9 % for the aqueous samples.

The results are summarized in Table F.4 and shown in Figure F.2.

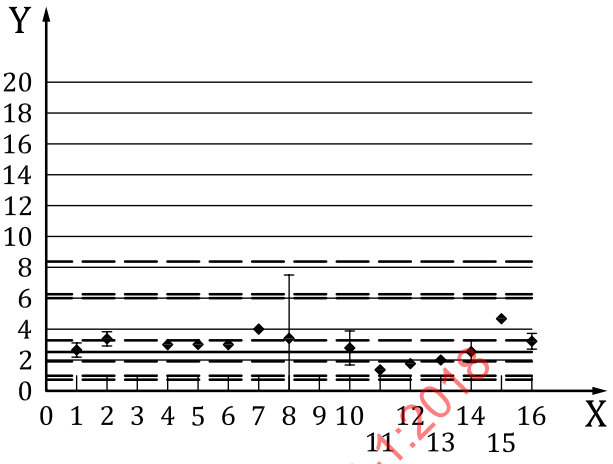
Table F.4 — Summary of LID results of the Yeast Estrogen Screen (YES, McDonnell[10])

	Sample							
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
Number of laboratories	16	16	16	16	16	16	16	16
Number of measurements	43	42	41	44	43	43	23	23
Invalid measurements	8	7	10	8	7	6	6	6
% Invalid measurements	18,6	16,6	24,4	18,2	16,3	14,0	26,1	26,1
Number of valid measurements "G > 12"	—	—	—	—	—	—	—	—
Number of valid measurements G = 1	35	5	2	34	2	33	3	0
Outliers excluded (single measurements)	n.d.	—	—	n.d.	—	n.d.	—	—
Outliers excluded (means)	n.d.	—	L11	n.d.	—	n.d.	—	—
Number of laboratories for statistics	14	14	12	15	14	15	8	8
Number of measurements for statistics	35	35	28	36	35	37	17	17
Laboratory geometric means, (expected values) – *n*								
L01	1 *n* = 3	2,6 (2,7) *n* = 3	6,9 (7,1) *n* = 2	1 *n* = 3	3,8 (4,1) *n* = 3	1 *n* = 3	2,3 (2,4) *n* = 3	5,2 (5,4) *n* = 3
L02	1 *n* = 3	3,3 (3,3) *n* = 3	10,5 (10,8) *n* = 3	1,3 (1,4) *n* = 2	4,6 (4,7) *n* = 3	1 *n* = 3	—	—
L03	—	—	—	2 *n* = 1	1 *n* = 1	2,5 (5,4) *n* = 2	—	—
L04	1 *n* = 3	3,0 (3,0) *n* = 3	8,0 (8,0) *n* = 3	1 *n* = 2	4,8 (5,2) *n* = 3	1 *n* = 3	1 *n* = 1	4 *n* = 1
L05	1 *n* = 1	3,0 (3,0) *n* = 2	—	1 *n* = 1	—	1 *n* = 2	—	—
L06	1 *n* = 3	3,0 (3,0) *n* = 3	7,3 (7,4) *n* = 3	1 *n* = 3	3,3 (3,3) *n* = 3	1,3 (1,4) *n* = 3	1,8 (2,1) *n* = 3	7,3 (7,4) *n* = 3
L07	1 *n* = 1	4 *n* = 1	6 *n* = 1	1 *n* = 3	4,2 (4,4) *n* = 3	1 *n* = 1	2,9 (3,1) *n* = 3	6,0 (6,0) *n* = 3

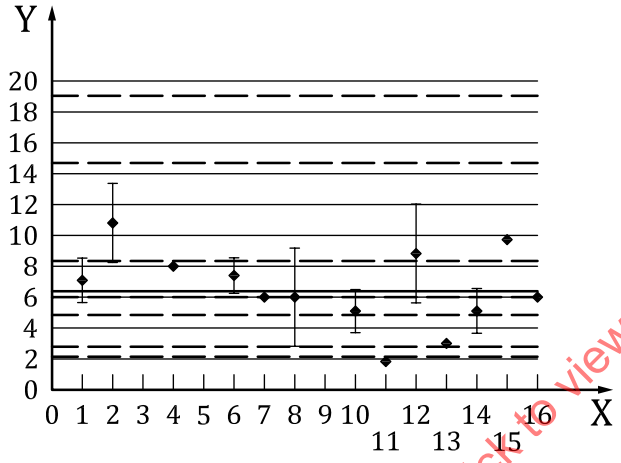
Table F.4 *(continued)*

	Sample							
	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**
L08	1 $n = 3$	2,3 (3,4) $n = 3$	5,2 (6,0) $n = 3$	1 $n = 3$	2,9 (3,1) $n = 3$	1 $n = 3$	2,3 (2,4) $n = 3$	3,3 (3,3) $n = 3$
L09	—	—	—	—	—	—	—	—
L10	1 $n = 3$	2,5 (2,7) $n = 3$	4,9 (5,1) $n = 2$	1 $n = 2$	3,3 (3,9) $n = 3$	1 $n = 3$	—	—
L11	1 $n = 3$	1,3 (1,4) $n = 3$	—	1 $n = 3$	2 $n = 3$	1 $n = 3$	1,4 (1,6) $n = 2$	3,5 (3,5) $n = 2$
L12	1 $n = 3$	1,5 (1,8) $n = 3$	8,3 (8,8) $n = 3$	1 $n = 3$	5,2 (5,4) $n = 3$	1 $n = 3$	—	—
L13	1 $n = 2$	2 $n = 1$	3 (3) $n = 2$	1 $n = 2$	1,4 (1,6) $n = 2$	1 $n = 1$	2 $n = 1$	6 $n = 1$
L14	1 $n = 2$	2,4 (2,6) $n = 2$	4,9 (5,1) $n = 2$	1 $n = 2$	3,0 (3,0) $n = 2$	1,4 (1,6) $n = 2$	—	—
L15	1 $n = 2$	3,5 (4,7) $n = 2$	6,0 (9,7) n $= 2$	1 $n = 2$	3,5 (4,7) $n = 2$	1 $n = 2$	—	—
L16	1 $n = 2$	3,3 (3,3) $n = 3$	6,0 (6,0) $n = 2$	1 $n = 3$	3,5 (3,5) $n = 2$	1,3 (1,4) $n = 3$	2 $n = 1$	4 $n = 1$
min - max		2,0 – 3,5	3,0 – 10,5	1 – 1,3	1,4 – 5,2	1 – 2,5	1,0 – 2,9	3,3 – 7,3
Factor max/min		1,75	3,50	1,3	3,7	2,5	2,9	2,2
Geometric mean	n.d.	2,5	6,4	n.d.	3,4	n.d.	2,0	4,9
Expected value		2,8	7,0		3,8		2,2	5,2
95 % CI		1,9 – 3,3	4,9 – 8,4		2,6 – 4,4		1,8 – 2,8	3,7 – 6,5
95 % PI		1,0 – 6,3	2,8 – 14,7		1,4 – 8,1		0,9 – 4,4	2,5 – 9,5
99 % PI		0,8 – 8,4	2,1 – 19,0		1,1 – 10,6		0,7 – 5,7	2,0 – 11,6
s_r (repeatability)		1,12	2,45		1,36		0,86	0,76
s_L (interlaboratory variability)		0,82	1,92		1,10		0,31	1,62
s_R (reproducibility)		1,40	3,12		1,75		0,92	1,79
s_r %		40,1	35,0		36,2		39,1	14,7
s_L %		29,3	27,5		29,4		14,4	31,5
s_R %		49,7	44,5		46,6		41,6	34,8
s_R/sr		1,3	1,3		1,3		1,3	2,4

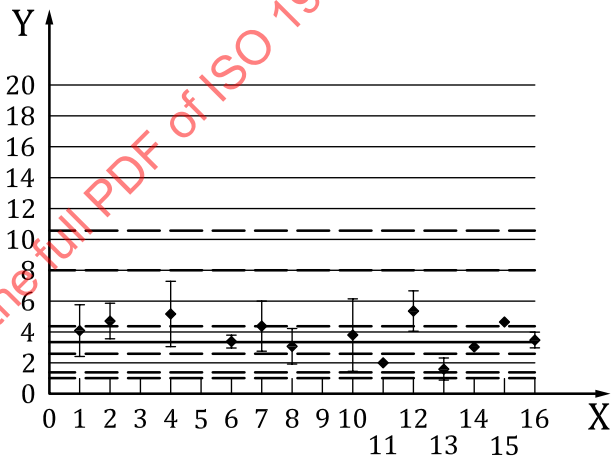
s_r	repeatability standard deviation
s_R	reproducibility standard deviation
s_L	laboratory standard deviation
n.d.	not determined
n	number of measurements



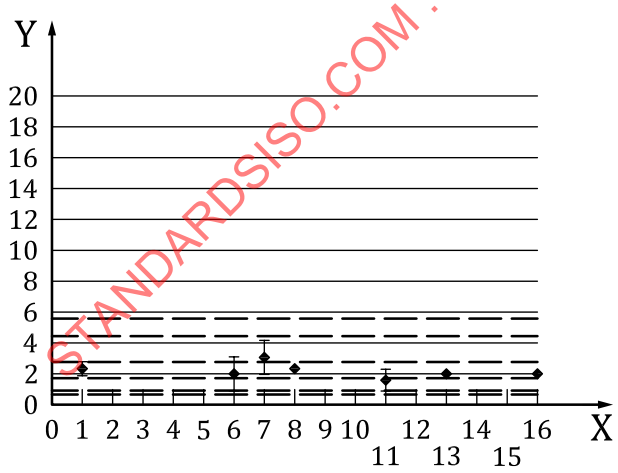
a) YES-Test, sample S2



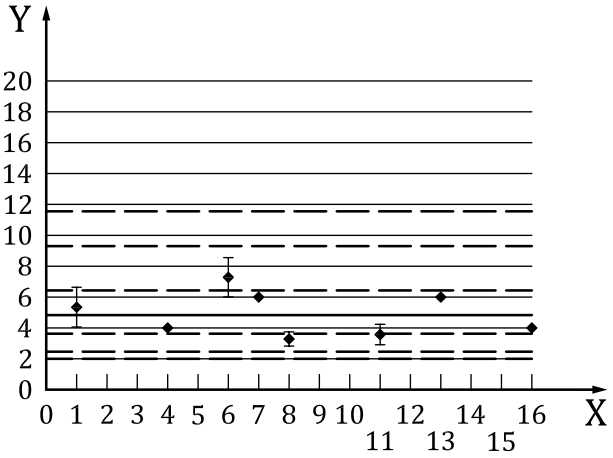
b) YES-Test, sample S3



c) YES-Test, sample S5



d) YES-Test, sample S7



e) YES-Test, sample S8

Key

X	laboratory
Y	lowest ineffective dilution LID
black marked symbols	laboratory specific expected values with standard deviation (Whisker)
black solid line	interlaboratory geometric mean
black dotted lines (small)	95 % confidence interval of the interlaboratory geometric mean

black dotted lines (big)	95 % tolerance range of the interlaboratory geometric mean
black dashed lines	99 % tolerance range of the interlaboratory geometric mean

Figure F.2 — Summary of lowest ineffective dilution (LID) of samples S2, S3, S5, S7 and S8

F.2.4 Trueness of results

For the samples S2, S3 and S8 the nominal value for the estrogenicity is not available. Therefore, only a qualitative statement concerning the trueness of the results is possible for these samples. In sum the reported results are in good agreement with the expectations (see Table F.1). The sample 2 was an effluent of a municipal waste water treatment plant spiked with 15 ng/l EE2. The mean value of the results for the estradiol equivalent concentration is 17,9 ng/l EEQ (95 % confidence interval 15,4 ng/l to 20,9 ng/l). Sample S3 was an influent of a municipal waste water treatment plant and showed a mean value of 35,5 ng/l EEQ (95 % confidence interval 28,0 ng/l to 45,1 ng/l). This finding is in accordance of the expectation that the influent of a waste water treatment plant shows a considerable estrogenic activity. Sample S8 was a mixture of three non-steroidal xenoestrogens. Due to the composition of this mixture it was to be expected that this sample should result in EEQ-values > sample S7 (14 ng/l). The mean value for sample S8 was 29,4 ng/l EEQ (95 % confidence interval 19,2 ng/l to 45,1 ng/l).

A quantitative evaluation of the trueness of the assay is possible with the spiked sample S5 and the compound mixture in sample S7. The assigned EEQ values were calculated based on the relative potencies (see Table D.1) of the spiked compounds in the samples S5 and S7. The trueness was calculated according to ISO 5725-2. Additionally, the standard uncertainty associated with the method and laboratory bias was calculated as the square root of the mean deviation squares between the individual measurement results and the respective assigned (true) value.

The assigned EEQ value of the spiked sample S5 was determined for each laboratory as a sum of the measured EEQ value of the unspiked sample S4 and the theoretical value of the spiking. If a laboratory reported a result „< LOQ" for the unspiked sample, the corresponding EEQ value was assumed to be zero.

In case of sample S5 the results from L03 and in case of sample S7 the results from L07 show extreme deviations from the assigned EEQ value of about 100 %. In contrast, the results from the other participating laboratories show a high accordance with the assigned values. Given also the comparatively high percentage of invalid test results from the laboratories L03 and L07, these results might be regarded as highly questionable and it seems justified to omit them from the calculation of ‘measurement uncertainty’ and method bias” (trueness). Nevertheless, for transparency reasons Table F.5 shows also the respective results based on all values in brackets ‘[]’, i.e. extreme values are included in the calculations.

For the sample S5 the 95 % confidence interval of the relative bias from mean recovery of the method ranged from −3,5 % (underestimation) to +20,8 % (overestimation) [-19,2 % to +21,1 %]. For the samples S7 the 95 % confidence interval ranged from −29,6 % to +19,3 % [-33,7 % to +56,7 %]. The relative bias from mean recovery for the sample S5 was +8,7 % [+0,9 %] and for the sample S7 −5,1 % [+11,5 %]. The overall bias of the method is +4,3 % [+4,4 %]. In sum these results indicate that the evaluated method shows no systematic bias, because the value “zero”, i.e. no deviation from the true result, is included in the calculated confidence intervals for both samples.

The results for the estimation of the trueness are summarized in Table F.5 and shown in Figure F.3.

Table F.5 — Summary of trueness evaluation for samples S5 and S7 [Yeast Estrogen Screen (YES, McDonnell[10])]

	Sample					
	S5			S7		
Laboratory	EEQ assigned [ng/l] = EEQ P4 + 20	EEQ [ng/l] real	Diff real – assigned (%)	EEQ assigned [ng/l]	EEQ [ng/l] real	Diff real – assigned (%)
L01	20	23,9	3,9 (+19,5 %)	14	16,8	2,8 (+20,0 %)
L02	23,1	27,6	4,5 (+19,5 %)	—	—	—
L03	25,7	< LOQ	25,7 (–100 %)	—	—	—
L04	20	26,4	6,4 (+32 %)	—	—	—
L06	20	23,5	3,5 (+17,5 %)	14	11	–3 (–21,4 %)
L07	20	28,4	8,4 (+42 %)	14	29,6	15,6 (+111,45 %)
L08	20	19,6	–0,4 (–2,0 %)	14	10,3	–3,7 (–26,4 %)
L10	20	15,7	–4,3 (–21,5 %)	—	—	—
L11	20	18,7	–1,3 (–6,5 %)	14	18	4 (+28,6 %)
L12	20	25,5	5,5 (+27,5 %)	—	—	—
L13	20	15,5	–4,5 (–22,5 %)	14	11,8	–2,2 (–15,7 %)
L14	20	18,8	–1,2 (–6,0 %)	—	—	—
L15	20	22,8	2,8 (+14,0 %)	—	—	—
L16	20	19,8	–0,2 (–1,0 %)	14	11,8	–2,2 (–15,7 %)
Mean SU	4,3 ng/l = 21,2 % [8,0 ng/l = 38,9 %]			3,1 ng/l = 21,9 % [6,5 ng/l = 46,7 %]		
Mean SU across samples S5 and S7	3,9 ng/l [7,6 ng/l]					
RB (trueness)	+8,7 % [+0,9 %]			–5,1 % [+11,5 %]		

SU = term standard uncertainty associated with the method and laboratory bias.

RB = relative bias from mean recovery.

NOTE 1 The absolute laboratory-specific differences between assigned and measured EEQ-values are shown (in parenthesis '()': percentage differences from the assigned value).

NOTE 2 In case of sample S5 the result from L03 and in case of sample S7 the result from L07 are excluded as questionable values from the calculation of the measurement uncertainty and the method bias. The respective values shown in brackets '[]' are based on all reported values, i.e. extreme values included.

Table F.5 *(continued)*

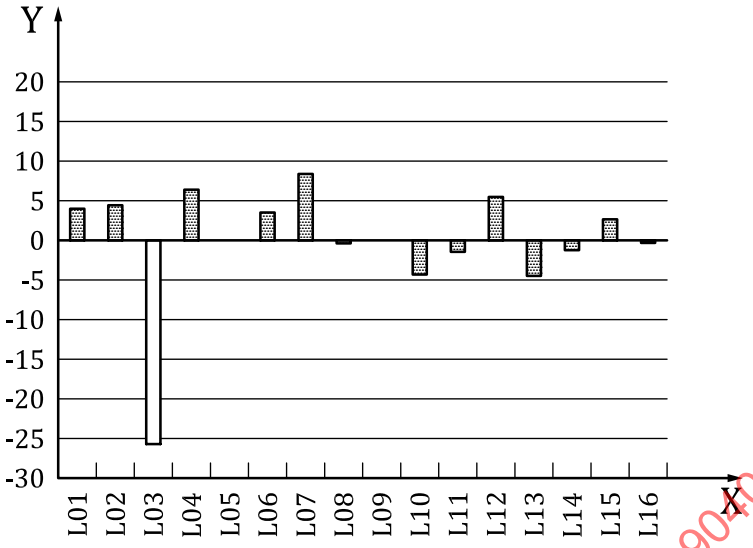
	Sample					
	S5			S7		
Laboratory	EEQ assigned [ng/l] = EEQ P4 + 20	EEQ [ng/l] real	Diff real – assigned (%)	EEQ assigned [ng/l]	EEQ [ng/l] real	Diff real – assigned (%)
95 % CI	−3,5 % to +20,8 % [-19,2 % to +21,1 %]			−29,6 % to +19,3 % [-33,7 % to +56,7 %]		
RBacross samples S5 and S7	+4,3 % [+ 4,4 %]					
95 % CI	−6,1 % to +14,7 % [-13,4 % to +22,3 %]					

SU = term standard uncertainty associated with the method and laboratory bias.

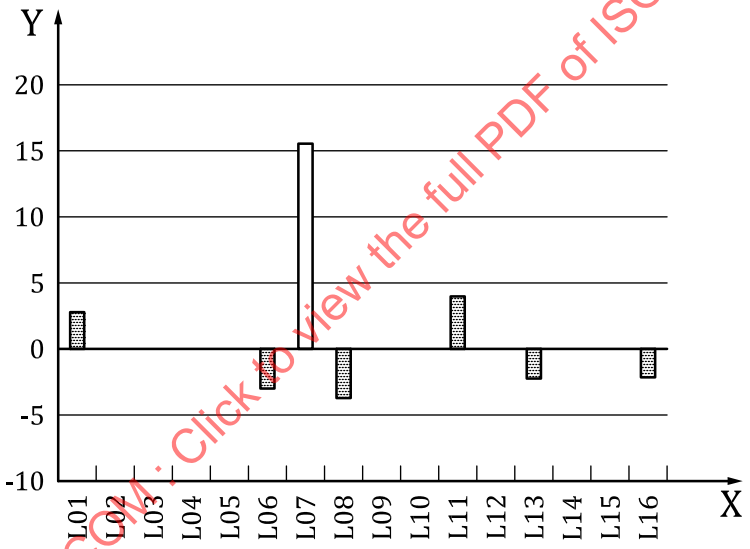
RB = relative bias from mean recovery.

NOTE 1 The absolute laboratory-specific differences between assigned and measured EEQ-values are shown (in parenthesis '()': percentage differences from the assigned value).

NOTE 2 In case of sample S5 the result from L03 and in case of sample S7 the result from L07 are excluded as questionable values from the calculation of the measurement uncertainty and the method bias. The respective values shown in brackets '[]' are based on all reported values, i.e. extreme values included.



a) Sample S5, YES, assigned = P4 + 20



b) Sample S7, YES, assigned = 14

Key

X	laboratory
Y	difference to assigned EEQ (ng/l)
white columns	extreme values

Figure F.3 — Summary of the trueness for samples S5 and S7 (expressed in absolute differences to the assigned value)

Annex G
(informative)

Use of other yeast strains based on *Saccharomyces cerevisiae*

G.1 General

Annex G describes the use of other test strains based on *S. cerevisiae*, especially the test strain according to Routledge and Sumpter 1996 [Reference (25)].

G.2 Test strain according to Routledge and Sumpter [Reference (25)]

G.2.1 General

This test strain has a higher sensitivity for steroidal compounds with estrogen activity like the natural hormones 17β-estradiol, estrone and 17β-ethinylestradiol but is less sensitive for e.g. 4-iso-nonylphenol.

G.2.2 Strain description

The generation of the test strain is described in References [25] and [26]. The *S. cerevisiae* strain BJ1991 was used for construction. The human estrogen receptor (hER) is expressed under the control of the yeast copper metallothionein promoter (CUP1). The CUP1 promoter is entirely under the control of exogenously added copper to the media. The respective construct is stably integrated into the yeast genome. Additionally, the strain contains a PGK-ERE-lacZ construct as reporter plasmid. The estrogen responsive element (ERE) from Xenopus vitellogenin A2 gene was fused to the gene lacZ which encodes the enzyme β-galactosidase.

G.2.3 Media

G.2.3.1 10x Su-DO-Medium (Sumpter).

Dissolve in the following order:

— 2 000 mg **L**-serine (7.1.13);

— 1 000 mg **L**-threonine (7.1.14);

— 750 mg **L**-valine (7.1.16);

— 500 mg **L**-leucine (7.1.9);

— 250 mg **L**-phenylalanine (7.1.12);

— 150 mg **L**-isoleucine (7.1.8);

— 150 mg **L**-tyrosine (7.1.15);

— 100 mg Adenine (7.1.3);

— 100 mg **L**-arginine (7.1.4);

— 500 mg **L**-aspartic acid (7.1.5);

— 500 mg **L**-glutamic acid (7.1.6);