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

Part 3: In vitro human cell-based reporter gene assay

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

Partie 3: Essai in vitro sur cellules humaines avec gène rapporteur

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Foreword

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

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

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

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

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

Part 3: In vitro human cell-based reporter gene assay

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 utilizing stably transfected human cells. 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 0,3 ng/l and 1 ng/l 17β-estradiol equivalents (EEQ) based on the results of the international interlaboratory trial (see Annex F). The upper working range was evaluated [based on the results of the international interlaboratory trial (see Table F.3)] up to a level of 75 ng EEQ/l. Samples showing estrogenic potencies above this threshold have to be diluted for a valid quantification. Extraction and preconcentration 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*

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
culture medium
nutrients presented in a form and phase (liquid or solidified) which support cellular growth

[SOURCE: ISO 6107-6:2004, 24, modified — "cellular" replaces "microbiological"]

3.2
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]

3.3
dilution water
sterile water added to the test sample to prepare a series of defined dilutions

[SOURCE: ISO 20079:2005, 3.7]

3.4
EC_{50}
effective concentration of a compound which causes 50 % of an effect

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

3.5
extract
test sample after extraction and possible removal of extraction vehicle

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

[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"; "cells" replaces "strain".]

3.8
limit of quantification
LOQ
lowest value that can be determined with an acceptable level of accuracy and precision

[SOURCE: ISO 15839:2003, 3.18]

3.9
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"]

3.10
negative control
dilution water without test sample

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

3.11
passage number
the number of subcultures from cells in a new culture vessel (cell culture flask or micro titer plate)

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
relative light units
RLU
amount of reporter gene activity as measured by light produces using a luminometer, expressed as relative light units

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

3.15
stock culture
frozen culture of cells for the preservation of the characteristics of the cell line

[SOURCE: ISO 21427-2:2006, 13, modified — "the cell line" replaces "V79 cells"]

3.16
subculturing
transfer of part of a cell culture into a new cell culture vessel during cell culture

3.17
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 Interferences

Toxic effects present in the test samples may lead to a reduction of cell viability and hence to a reduction of the measured cellular response. Consequently, estrogenic effects of a sample may be masked by acute toxic effects leading to false negative test results (see Clause 9 for further information). In this case, the sample should be diluted further until no cytotoxicity is observed (see manual of cytotoxicity test used).

The use of inappropriate sampling devices and/or sampling flask may influence the test result because of the possible adsorption of active compounds on surfaces leading to false negative results. On the other hand, active compounds could be released into the sample from sampling flasks, especially if plastic ware is used, and false positive results might be generated. See Clause 7 for more information.

High salinity can cause toxic effects due to the resulting osmotic pressure. The ER(α) CALUX cells (References [10] to [16]) tolerates a conductivity of the sample up to 34,000 µS/cm (1,0 % *w*/*w* salinity). Bacterial and fungal contaminations can negatively influence the response of the cells. Therefore, antibiotics are added to the cell culture medium. Contamination of the cells is assessed by visual observation (microscope) when testing the sample. See Clause 9 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.

Anti-estrogenic compounds and other non-toxic inhibitory compounds might mask estrogenic effects. The presence of interfering compounds can be assessed by samples which are spiked with a defined amount of an estrogenic compound with defined properties (e.g. 17*β*-estradiol) leading to a known induction of the test system.

Compounds with estrogenic properties might be present as inactive conjugates. A chemical de-conjugation can be necessary in order to quantify the overall estrogenic potential of a sample.

5 Principle

Estrogen receptor (ER) - mediated signaling is essential in estrogen action and the mechanism of estrogen receptor signaling is well established. Upon estrogen binding the estrogen receptor becomes activated, and binds to recognition sequences in promoter regions of target genes, the so-called estrogen responsive elements (EREs). These EREs have been linked to a promoter element and a gene transcribing for the easily measurable protein luciferase. In these cells, the ligand-activated receptor will activate luciferase transcription, and the transcribed luciferase protein will emit light when a substrate is added. The signal dose-dependently increases as a result of increasing concentrations of ligand. The luciferase activity in cellular lysates is measured with a luminometer, allowing reliable, sensitive and quantitative measurements.

6 Apparatus and materials

Beside the equipment which is usually present in a laboratory for cell culture the following apparatus and materials are needed. For suitable sampling devices see Clause 8.

6.1 Laminar air flow cabinet, standard: “biological hazard”.

6.2 Water bath, 37 °C.

6.3 CO_2 incubator, 5 % CO_2, 37 °C, humidity 100 %.

6.4 Inverted phase-contrast microscope.

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

6.6 Shaking apparatus for micro-test plates.

6.7 Centrifuge.

6.8 Laboratory balance.

6.9 Sterile pipettes, 1 ml, 2 ml, 5 ml, 10 ml and 25 ml, glassware or plastics.

6.10 Pipette controller.

6.11 Cell culture flasks, 75 cm^2 with filter lids.

6.12 Sterile plastic containers, 12 ml and 50 ml with sterile cap.

6.13 Sterile plates with 12 wells.

6.14 Multi-channel multi-stepper pipette (repeater pipette), including 5 ml and 10 ml tips.

6.15 Pipettes, 1 µl, 50 µl, 200 µl and 1 000 µl, with sterile tips.

6.16 Multi-channel pipettes, up to 50 µl and up to 300 µl.

6.17 Sterile polystyrene 96-well plates, with flat transparent bottom and lid, appropriate for cell culture, volume 300 µl per well.

6.18 Microplate luminometer with two injectors, for addition of substrate and stop reagent.

6.19 Cell counter or hemacytometer.

6.20 pH meter.

6.21 Cryovials, sterile, 2 ml.

6.22 Liquid nitrogen container for long term cell storage.

6.23 Filter, cellulose acetate, 0,45 µm pore size.

7 Reagents, cells and media

7.1 Reagents

As far as possible, use "reagent grade" chemicals. If (different) hydrates are used that differ from the compounds specified, ensure that the appropriate mass of the main compound is employed.

7.1.1 Dimethyl sulfoxide (DMSO).

7.1.2 Glycerol for molecular biology, ≥99 %, molecular weight: 92,09 g/mol, CAS: 56-81-5.

7.1.3 17β-estradiol, ≥98 %, ($C_{18}H_{24}O_2$), molecular weight: 272,38 g/mol, CAS: 50-28-2.

7.1.4 Ethylene-diamine-tetra-acetate (EDTA), CAS: 6381-92-6.

7.1.5 Trypsine, CAS: 9002-07-7.

7.1.6 Fetal calf serum (FCS).

7.1.7 DMEM/F12 medium with phenol red as pH indicator.

7.1.8 Non-essential amino acids (100x).

7.1.9 Penicillin-streptomycin (100x), concentration used 5 000 units penicillin per ml / 5 000 µg streptomycin per ml).

7.1.10 Phosphate buffered saline pH 7,2 (PBS), without calcium and magnesium.

7.1.11 Adenosine-5'-triphosphate (ATP), CAS: 34369-07-8.

7.1.12 Trans-1, 2-diaminocyclohexane-N, N, N', N'-tetraacetic acid monohydrate (CDTA), CAS: 125572-95-4.

7.1.13 Dithiothreitol (DTT), CAS: 3483-12-3.

7.1.14 2-amino-2-(hydroxyl-methyl)-1,3-propanediol (TRIS), CAS: 77-86-1.

7.1.15 Dextran T500.

7.1.16 Activated charcoal.

7.1.17 Coenzyme A, free acid grade, Add CAS: 85-61-0.

7.1.18 D-Luciferin sodium salt, CAS: 103404-75-7.

7.1.19 Magnesium hydroxide carbonate pentahydrate, $C_4H_2Mg_5O_{14}$, CAS: 56378-72-4.

7.1.20 Magnesium sulfate, CAS: 7487-88-9.

7.1.21 Sodium bicarbonate, CAS: 144-55-8.

7.1.22 Cell culture medium powder, Sigma, D2902, without phenol red and sodium bicarbonate.

7.1.23 Tricine, CAS: 5704-04-1.

7.1.24 Acetone, (purity p.a.), CAS: 67-64-1.

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

7.1.26 Triton X-100, CAS: 9002-93-1.

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

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 Cell line.

A genetically modified human cell line, expressing the human estrogen receptor (hERα and/or hERβ) and containing a reporter plasmid for luciferase. More detailed descriptions of the individual cell lines are given in Annex C.

7.4 Media.

Always use sterile solutions, glassware, etc. Carry out all procedures under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). If autoclaving is necessary, always autoclave for 20 min at (121 ± 2) °C. Cover vessels loosely, never seal air tight.

7.4.1 Cell culture medium.

Add 5,4 ml non-essential amino acids (7.1.8), 42 ml FCS (7.1.6) and 1 ml of penicillin-streptomycin (7.1.9) to a bottle containing 500 ml DMEM/F12 medium with phenol red. Complete cell culture medium should be kept at 4 °C and stored for no longer than eight weeks.

7.4.2 Freezing medium.

Add 1 ml non-essential amino acids, 10 ml DMSO, 20 ml FCS and 1 ml penicillin-streptomycin (7.1.9) to 68 ml of DMEM/F12 medium with phenol red. Distribute 20 ml batches in a sterile manner over sterile tubes. Complete freezing medium should be kept at (−20 ± 1) °C until use and kept on ice on use.

7.4.3 Concentrated assay medium.

7.4.3.1 3x concentrated assay medium.

Dissolve the contents of one vial of cell culture medium powder (7.1.22) in 250 ml of water (at room temperature) and then dissolve 3,7 g sodium bicarbonate (7.1.21) in the same solution. Adjust the pH to 7,4 using a 1 mol/l HCl or 1 mol/l NaOH solution. Add water to a final volume of 300 ml and filter sterilize. Add 12 ml of non-essential amino acids (7.1.8), 55 ml stripped serum (7.4.8) and 37 ml penicillin-streptomycin (7.1.9). Complete media should be kept at (4 ± 1) °C and stored for no longer than eight weeks.

7.4.3.2 1x concentrated assay medium.

Dilute 100 ml of 3x concentrated assay medium with 200 ml of water and filter sterilize. Complete media should be kept at (4 ± 1) °C and stored for no longer than eight weeks.

7.4.4 Trypsin solution.

Add 20 ml of trypsin (7.1.5) to 980 ml PBS (7.1.10) and add 0,2 g EDTA (7.1.4). Sterile filter the trypsin solution and aliquot into 50 ml portions. The expiration date is 3 months from the day of preparation. Store the tubes of trypsin at −20 °C.

7.4.5 Stop reagent.

Dissolve 8,0 g of sodium hydroxide in one litre of demineralized water, to achieve a final concentration of 0,2 M.

7.4.6 Substrate mixture.

Completely dissolve tricine (7.1.23) and magnesium sulfate (7.1.20) in 500 ml of demineralized water, until the solution is clear and colourless. Add the remaining chemicals from Table 1 and add 400 ml of water. Adjust the pH to 7,8 using 1 mol/l solution of HCl and/or NaOH. Adjust to a final volume of 1 l. Store at (−20 ± 1) °C for a maximum of 3 months or (−80 ± 1) °C for a maximum of 12 months.

Table 1 — Preparation of substrate mixture (1 000 ml)

Compound	Weight g	Molecular weight g/mol	Molarity Mmol/l
Tricine	3,58	179,2	20
$C_4H_2Mg_5O_{14}$	0,52	485,69	1,1
MgSO4	0,31	120,37	2,6
EDTA	0,037	372,23	0,10
DTT	5,14	154,2	33,3
Coenzyme A	0,21	767,6	0,27
Luciferin	0,15	320,32	0,47
ATP	0,29	551,1	0,53

7.4.7 Lysis mixture.

Completely dissolve the compounds listed in Table 2 into 500 ml of demineralized water. Adjust pH to 7,8 using 1 mol/l HCl and/or 1 mol/l NaOH solutions. Adjust final volume to 1 l and aliquot into 40 ml portions. Store at ≤–18 °C (change for all T issues) for a maximum of 1 year, store at 4 °C for a maximum of one month.

Table 2 — Composition of lysis mixture (1 000 ml)

Compound	Weight g	Volume ml	Molecular weight	Concentration
Tris	3,0		121,1	25 mmol/l
DTT	0,31		154,2	2,0 mmol/l
CDTA	0,73		364,35	2,0 mmol/l
Glycerol		100		10 %
Triton X-100		10		1 %

7.4.8 Stripped fetal calf serum.

Dissolve 0,6 g Tris (7.1.14) in 50 ml of demineralized water in a 500 ml glass beaker and adjust the pH to 8,0. Add 450 ml of demineralized water, 0,25 g Dextran T500 (7.1.15) and 2,5 g of activated charcoal (7.1.16). Close the bottle and stir the mixture overnight at 4 °C. Also thaw a bottle of FCS (7.1.6) overnight at 4 °C.

The next day, transfer 200 ml of thawed FCS to a sterile glass bottle and heat for 30 min at 56 °C in a water bath. Transfer the charcoal suspension to 12 clean 50 ml centrifuge tubes and centrifuge the tubes for 20 min at 1 000 *g*. Decant the supernatant. Divide FCS over six tubes with charcoal pellets and resuspend. Incubate this suspension for 45 min at 45 °C while shaking. Centrifuge the tubes for 20 min at 1 000 *g* and decant the serum into 6 other tubes with fresh charcoal pellets. Incubate this suspension for another 45 min at 45 °C while shaking. Centrifuge the tubes for 20 min at 1 000 *g* and decant the serum into six clean tubes. Centrifuge again for 20 min at 1 000 *g* to remove residual charcoal. Pool serum, filter sterilize and aliquot into 55 ml portions. Store at –20 °C for a maximum of six months.

7.4.9 17*β*-estradiol (E2) stock solution.

Dissolve 13,6 mg 17*β*-estradiol (E2) (7.1.3) in 10 ml DMSO (7.1.1). Dilute the stock 1 000x, e.g. by pipetting 1 ml to 9 ml DMSO and sequentially 1 ml to 99 ml DMSO (final concentration stock solution: 5,0E-6 M). Store 1 ml aliquots at ≤–18 °C.

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) preferably with polypropylene caps or polytetrafluoroethylene (PTFE) lined caps. To avoid photo-degradation of compounds of interest, preferably 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 (7.1.24). 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 (7.1.24). 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 sample

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 [20] and [21].

Adjust the sample to a pH of 7,2 ± 0,2 using either HCl (7.1.27) or NaOH solution (7.1.25). 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 [22] and [23].

9 Procedure

9.1 Cell culture maintenance

All steps are performed under sterile conditions using a laminar flow cabinet.

9.1.1 Freezing cells

Trypsinate cells that are subconfluent as indicated in 9.1.3 and resuspend into 10 ml cell culture medium (7.4.1). Before proceeding to cell freezing, prepare in advance cryovial: Label each cryovial: type of cell, batch number, passage number, date of freezing. Keep track of passage number. Transfer the cells to a 12 ml cell culture tube. Centrifuge the cells (250 x *g*, 5 min), remove the supernatant medium and resuspend the cells into 4 ml of ice cold freezing medium (7.4.2). Mix cells and freezing medium not more than 3 times. Transfer 1 ml of this cell suspension into cryovials (6.21) on ice, close the vials quickly. Place the cryovials in a polysterene box, cover the cryovials with tissue to facilitate a graduate freezing of the cells and store at −80 °C overnight. The next day, transfer the cryovials to a liquid nitrogen container for long term storage.

9.1.2 Starting a new cell culture

Thaw cells that are stored in liquid nitrogen quickly at 37 °C. Transfer the cell suspension to a 15 ml tube and add 10 ml of cold cell culture medium in a drop wise manner. Centrifuge the cell suspension (250 x *g*, 5 min) and remove the supernatant. Resuspend the cells in 10 ml of culture medium (room temperature), transfer to a new cell culture flask (6.11) and incubate until subconfluent in a CO_2 incubator (5 % CO_2, 37 °C, 100 % humidity).

9.1.3 Culturing cells

Remove cells in a cell culture flask from the incubator. Remove the cell culture medium and wash the cells twice with approximately 5 ml of PBS (7.1.10). Add 2 ml of trypsin to the cells, swirl around and remove the trypsin. Leave the cells in the incubator to trypsinate until the cells have detached from the surface (about 2 min). Resuspend the cells in 10 ml of cell culture medium, transfer a small part to a new cell culture flask (see subculture ratio defined in Annex C, count the cells if necessary using a cell counter (6.19). Add cell culture medium up to a final volume of 10 ml and place the cell culture flask in the CO_2 incubator.

9.2 Human cell reporter gene assay test procedure

IMPORTANT — After thawing cells from a stock culture, passage two to three times before using the cells in the test.

The procedure takes two days: seeding the cells on day 1 and exposure on day 2. After exposure, the cells are incubated for 24 h. One plate can accommodate 10 dilutions of one sample. A reference compound dose-response curve is included on every plate. All steps are performed under sterile conditions using a laminar flow cabinet.

9.2.1 Seeding the cells (day 1)

Remove cells that are subconfluent (85 % to 95 % confluency) from cell culture flasks by enzymatic digestion (trypsin/EDTA) (9.1.3) and resuspend into 1x concentrated assay medium (7.4.3). Transfer the cell suspension to a sterile 50 ml tube and determine the cell concentration using a cell counter (6.19). Dilute the cell suspension with 1x concentrated assay medium to a concentration of 5 000 to 20 000 cells per well, depending on the type of cell line used. Use this cell suspension to fill the inner 60 wells of 96-well plates (see Figure 1; 100 µl cell suspension per well). Fill the outer 36 wells with 200 µl PBS. Transfer the microtiter plates to the CO_2 incubator and incubate for 16 h to 24 h (overnight). The next day, examine each plate under a phase contrast microscope to ensure that cells are subconfluent, cell growth is relatively even and that there are no contaminations.

	1	2	3	4	5	6	7	8	9	10	11	12
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	C	C	C	C	C	C	C	C	C	C	P
C	P	C	C	C	C	C	C	C	C	C	C	P
D	P	C	C	C	C	C	C	C	C	C	C	P
E	P	C	C	C	C	C	C	C	C	C	C	P
F	P	C	C	C	C	C	C	C	C	C	C	P
G	P	C	C	C	C	C	C	C	C	C	C	P
H	P	P	P	P	P	P	P	P	P	P	P	P

Figure 1 — Schematic illustration of the microtitre plate after subculturing

NOTE P is filled with 200 µl PBS and C with 100 µl cell suspension.

9.2.2 Preparation of the E2-reference (day 2)

Thaw an aliquot of the 17β-estradiol (E2) stock solution (7.4.9); (5,0E-06 mol/l). Dilute the thawed stock 1→100 in water (e.g. 100 µl E2-solution + 9 900 µl water, resulting stock 5,0E-8 mol/l E2). Dilute the 1→100 diluted stock another 1→100 in water (final E2 concentration of 5,0E-10 mol/l; DMSO concentration is 0,01 %, which does not influence the assay results). Prepare serial dilutions from this 5,0E-10 M stock, according to the schedule in Table 3. All dilutions should be prepared in glass tubes with polytetrafluoroethylene (PTFE) caps. Discard 150 µl of concentrations tube (7) and tube (8), so that the final volume is 1 350 µl in all tubes. Add 675 µl of 3x concentrated assays medium (7.4.3) to each of the tubes and mix. The volume of E2-reference prepared, is sufficient for three 96-well plates.

Table 3 — Dilution steps for the reference compound 17β-estradiol (E2)

Tube	Water	Add	[E2] mol/l	[E2] mol/l	[E2] ng/l
			in water	**in medium**	**in medium**
(1)	0 µl	1 500 µl of 5,0E-10 mol/l E2 stock	5,0E-10	3,3E-10	90,8
(2)	1 050 µl	450 µl of 5,0E –10 mol/l E2 stock	1,5E-10	1,0E-10	27,2
(3)	1 350 µl	150 µl of (1)	5,0E-11	3,3E-11	9,08
(4)	1 350 µl	150 µl of (2)	1,5E-11	1,0E-11	2,72
(5)	1 350 µl	150 µl of (3)	5,0E-12	3,3E-12	0,908
(6)	1 350 µl	150 µl of (4)	1,5E-12	1,0E-12	0,272
(7)	1 350 µl	150 µl of (5) (+ discard 150 µl)	5,0E-13	3,3E-13	0,090 8
(8)	1 350 µl	150 µl of (6) (+ discard 150 µl)	1,5E-13	1,0E-13	0,027 2
(9)	1 350 µl	—	0,0E+00	0,0E+00	0,0

NOTE The volume of E2-reference concentration prepared is sufficient for three 96-well plates.

9.2.3 Preparation of the sample dilutions (day 2)

Homogenize the sample before use by shaking. Prepare nine successive dilutions of the test sample with sterile water resulting in ten dilution levels (see Table 4). Less dilution levels can be used, provided that the signal obtained can be used for the calculation of the 17β-estradiol equivalents for the test samples. Prepare all dilutions in glass tubes with polytetrafluoroethylene (PTFE) caps. Discard 500 µl of the last two dilutions (tubes (9) and (10)) so that the final volume is 500 µl in all tubes. Add 250 µl of the 3x concentrated medium (7.4.3) to each tube. A recommended set up for chemicals and extracts is shown in Annex D and Annex E.

Table 4 — Dilution steps for the water samples

Tube	Water	Add	Dilution factor (D)	% sample
(1)	0 µl	500 µl of sample	1x	100,0
(2)	500 µl	500 µl of sample	2x	50,0
(3)	667 µl	333 µl of sample	3x	33,3
(4)	500 µl	500 µl of (2)	4x	25,0
(5)	500 µl	500 µl of (3)	6x	16,7
(6)	500 µl	500 µl of (4)	8x	12,5
(7)	500 µl	500 µl of (5)	12x	8,3
(8)	500 µl	500 µl of (6)	16x	6,3
(9)	500 µl	500 µl of (7) (+ discard 500 µl)	24x	4,2
(10)	500 µl	500 µl of (8) (+ discard 500 µl)	32x	3,1

9.2.4 Field blank

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

9.2.5 Exposing the cells (day 2)

Use a volume of 200 µl for each well (sample, dilutions, controls, field blank). Test each sample at ten dilutions, with each dilution level in triplicate on the same plate. Additionally, the sample plate shall contain at least a triplicate of the negative control. Include a dose-response relationship of the reference compound 17β-estradiol on each plate.

To expose the cells, proceed in two steps. First, remove the medium from the wells B2-D11 (wells for reference compound concentrations and field blank) by aspiration. Add 200 µl of each of the concentration of the reference compounds (9.2.2) (in triplicate) according to the layout in Annex B. Add 200 µl of field blank (in triplicate), according to the layout in Annex B. Secondly, remove the medium from the wells E2-G11 (wells for sample dilutions) by aspiration. Add 200 µl of each of the sample dilutions (9.2.3) (in triplicate) according to the layout in Annex B. Ensure that the cells are not without a medium for more than 2 min. After exposure, transfer the micro well plate to the CO_2 incubator (100 % humidity, 5 % CO_2, 37 °C) and incubate for 24 h. A recommended plate set-up is shown in Annex B.

9.2.6 Harvesting the cells (day 3)

After 24 h treatment, examine each plate under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Record changes in the morphology of the cells due to cytotoxic effects of the test sample extract, but do not use these records for any quantitative measure of estrogenic activity. Undesirable growth characteristics of control cells can indicate experimental error and can be cause for rejection of the assay.

To harvest the cells, remove the medium from the cells and add 30 µl lysis reagent to each of the inner 60 wells (see Figure 1). Shake the plate for 15 min at room temperature for measurement of luminescence (9.2.7) or store at <–20 °C for up to two weeks.

9.2.7 Measurement of luminescence (day 3)

Thaw the substrate mixture (7.4.6) and allow it to reach room temperature. If frozen plates are to be measured, allow these to reach room temperature before continuing with the measurement. Measure the luminescence of the inner 60 wells only of the 96-well plate (well by well) using a luminometer and the following settings:

— add 100 µl of substrate mixture to a well (7.4.6);

— measure the amount of luminescence for 4 s;

— add 100 µl of stop reagent (7.4.5);

— continue with the next well.

Continue until all plates have been measured. Export all measured data to files that can be imported later on into calculation programmes. A recommended settings of the luminometer is shown in Annex A.

9.3 Data analysis

9.3.1 Calculation of the reporter gene induction

The induction rate (I(i)) quantifies the fold induction of the estrogen receptor mediated response of sample dilutions and E2 reference concentrations in the test compared to the basal receptor activity, which is determined in the reference solvent control. Calculate the induction rate of sample dilutions and E2 reference concentrations in test (i) according to the following Formula (1):

$$\mathrm{I}(i) = \frac{\mathrm{RLU}(i)}{\mathrm{RLU}(\mathrm{NC})} \tag{1}$$

where

I(i) is the induction rate of a sample dilution or E2 reference concentration in test i;

RLU(i) is the estrogenic response (relative light units) of a sample dilution or E2 reference concentration in test i;

RLU(NC) is the mean estrogenic response (relative light units) of the reference solvent control (0 mol/l E2 concentration) in test i.

Calculate the mean I(i) for all tests and the respective standard deviation (sigma (i)). For the assessment of the test results use these mean values. Use the mean induction rate for a subsequent statistical evaluation and the construction of a dose response curve. Use the mean induction rate as well for the calculation of 17β-estradiol equivalents as described in Annex H.

9.3.2 Calculation of the percentage of maximum response

The percentage of maximum response (% Max) quantifies the response of the estrogen mediated response in the samples compared to the maximum response of the reference compound 17β-estradiol. It has the advantage that results between labs and assays can be compared, regardless of date and location specific variations in relative light units or induction factors of the individual assays. The maximum response is set to 100 %, while the background is set to 0 %. Calculate the % Max of a test (i) according to the following Formula (2):

$$\%\mathrm{Max}(i)=\frac{I_{\mathrm{Sample}}-1}{I_{\mathrm{PC}}-1}\times 100 \qquad (2)$$

where

% Max(i) is the relative response of a sample compared to the maximum response of the PC in test i;

I_{Sample} is the fold induction rate of a sample in test i;

I_{PC} is the mean fold induction value of the 1E-10 mol/l E2 concentration of test i.

9.3.3 Calculation of the dose-response curve

Every 96-well plate includes a full dose-response curve of the reference compound 17β-estradiol (E2).

Calibration dose-response curve: Calculate a calibration curve using the percentage of maximum induction from the various 17β-E2 concentrations, based on the formula given in Annex H.

Sample dilution-response curve: Calculate the dilution-response curve using the percentage of maximum induction from the various sample dilutions, based on the Formula (H.1) given in Annex H.

10 Validity criteria

10.1 Validity criteria for the assay

The test is valid if:

a) the EC_{50} concentration range of 17β-estradiol is in the range from 1,6 to 34 pmol/l;

b) the minimum fold induction of the highest 17β-estradiol concentration, with respect to the reference solvent control, is equal to or higher than 5;

c) the correlation R2 of the 17β-estradiol concentration response curve is equal to or higher than 0,98.

If any of these criteria are not met, the entire test plate is invalid.

10.2 Validity criteria for samples

If the criteria for the test plate are valid, results of the different dilutions of a sample are valid if:

a) the relative standard deviation of the selected triplicate is <15 % (inter well deviation). In case the tested sample concentration shows no response above the LOD in all triplicate wells, the relative standard deviation is allowed to be >15 % but <30 %;

b) no other types of interfering conditions have been observed, e.g. contamination, cytotoxicity etc.

If these criteria are not met, the sample shall be retested. If the highest dilution of the samples still shows a response above the LOQ, higher dilutions need to be retested to determine the dilution factor D. In that case, the samples with the two highest dilutions factors are again included in the repeated analysis. Higher dilutions factors can be prepared according to the schedule given in Annex E. Validation data from an interlaboratory test based on the procedure described in this document are described in Annex F.

11 Assessment criteria

The test sample is assessed 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 19040-3:2018;

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 and time (if not tested directly) and other manipulations);

e) cell line used, including passage number/cell batch;

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

g) test results (mean induction rates for field blank, mean induction rates for each tested sample concentration and reference dilution with percentage standard deviation, qualitative assessment of the estrogenicity of the sample (yes/no) with information about statistical evaluation), indication of toxic effects (if any) and other observations (e.g. precipitation, contamination). If EEQ values for the sample or sample dilutions are calculated, further reporting is required as described in Annex H. If LID values are calculated further, reporting is required as specified in Annex I.

Annex A
(informative)

Settings of the luminometer

To measure the luminescence response in the transparent 96-well plates, the luminometer needs to have two injectors available: one for adding the substrate and a second one to add the sodium hydroxide solution to quench the luminescence signal. The following settings are used:

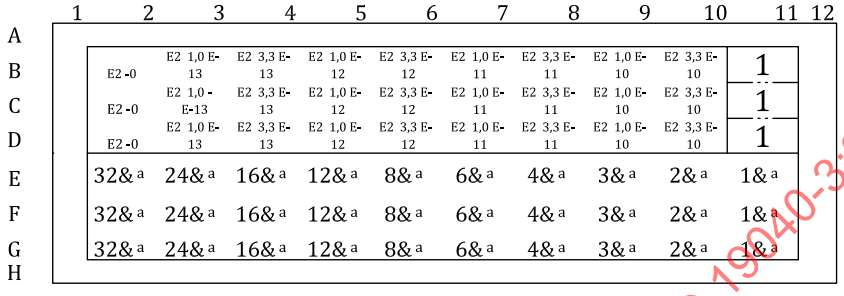
— volume dispenser I: 100 µl luciferase substrate solution (to produce the light);

— volume dispenser II: 100 µl 0,2 mol/l NaOH solution (to quench the signal);

— measuring time after the addition of 100 µl luciferase preparation: 4 s.

Annex B
(informative)

Plate setup



Key

1 field blank

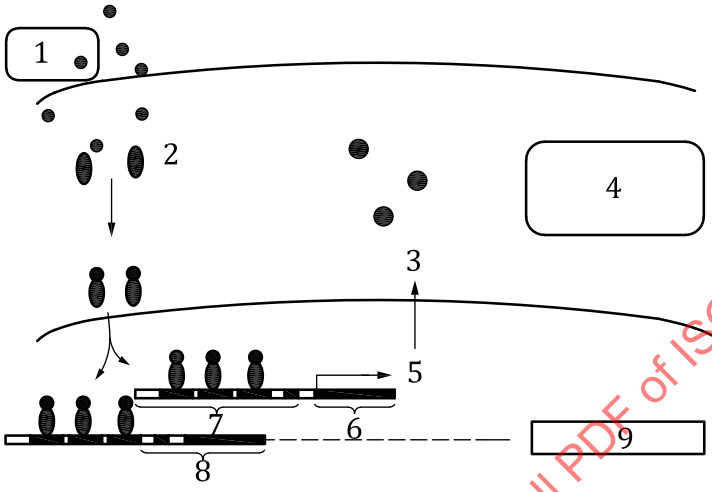
[a] Sample.

Figure B.1 — Plate set-up for one sample with 10 successive dilution levels (in triplicate)

Include a full dose-response curve of the reference compound 17β-estradiol (E2) on every plate.

Annex C
(informative)

Bioassay characteristics and details



Key

1 chemical (mix)
2 receptor
3 luciferase protein
4 light signal proportional to amount of biological active chemical in sample
5 luciferase mRNA
6 luciferase
7 receptor binding elements
8 endogenous gene
9 biological effect

Figure C.1 — Test principle

Estrogenic compounds, present in a complex mixture, diffuse into the cell where they can bind to the human estrogen receptor (hER). Due to receptor binding, the activated estrogen receptors dimerize and are actively transported into the nucleus. The receptor dimer binds to and activates a promoter that contains estrogen responding elements. As a result, the cell produces the enzyme luciferase, which can be quantified by adding the substrate luciferin and measuring the light production. The expression of the reporter gene, assessed by amount of light produced, is proportional to the activation of the human estrogen receptor by the (xeno)estrogen (see Figure C.1).

ERalpha CALUX

The ERalpha CALUX expresses the human estrogen receptor alpha (hERα) in human osteoblastic osteosarcoma U2-OS cells. The cell line contains a plasmid with repeats of estrogen responsive elements (EREs) controlling the expression of the reporter gene luciferase (see References [10] to [18]).The assay is highly selective for estrogens because multimerized hormone-responsive elements in the reporter constructs are cloned upstream of a TATA box only to prevent interference by other promoter elements (References [17] and [18]). Regarding the instructions for the subculture ratio, see Table C.1.

Table C.1 — Instructions for the subculture ratio

Time to next subculture	75 cm^2 bottle
3 d	1:4
4 d	1:8

MELN

The MELN cell line is derived from the MCF-7 human breast cancer cell line that have been stably transfected with a plasmid containing the luciferase gene driven by an ERE in front of a β-globin promoter (Reference [19]). The cells express both ERα and ERβ endogenously. A slight adaptation is required for this cell line: on day 2 the medium is refreshed and on day 3 the cells are exposed (day 2 in the protocol). Regarding the instructions for the subculture ratio, see Table C.2.

Table C.2 — Instructions for the subculture ratio

Time to next subculture	75 cm^2 bottle
3 d	1:3
4 d	1:5

ER CALUX

The ER CALUX cell line is derived from the T47D human breast cancer cell line that have been stably transfected with a plasmid containing the luciferase gene (see References [12],[14],[15]) Regarding the instructions for the subculture ratio, see Table C.3.

Table C.3 — Instructions for the subculture ratio

Time to next subculture	75 cm^2 bottle
3 d	1:3
4 d	1:5

This document is based on the evaluation and interlaboratory study using the above mentioned in vitro human cell-base reporter gene assays. Other reporter cell lines, e.g.: HELN-ERalpha, HELN-ERbeta, HeLa-9903, T47D-kbLuc, can be used if they fulfil the described general requirements as mentioned in Clause 10. In addition, laboratory proficiency shall be proven by the continuous successful participation in interlaboratory studies for the determination of estrogenic potential for water and waste water.

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 organic solutions or extracts

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

Use 1x concentrated assay medium as test medium, e.g. by diluting the 3x concentrated medium (7.4.3) with demineralized water. For exposure, pipette 1 µl of each sample dilution into 1 ml of test medium (final solvent concentration 0,1 %). This process is repeated until all samples have been transferred to the medium. Mix the sample with the medium, e.g. by mixing them 10 min at 300 rpm, at room temperature. Similarly, prepare enough test medium containing the 17β-estradiol standards for all plates (600 µl per plate is required, make at least 300 µl extra to allow for pipetting losses). For exposure, replace the medium on the cells by 200 µl of exposure medium containing the compounds/extracts of interest, using the layout of Figure B.1. Typical levels of detection (LOD) and quantification (LOQ) of estradiol equivalents (EEQs) in water samples are around 0,3 ng EEQ/l (LOD) and 1,0 ng EEQ/l (LOQ).

D.4 Data from literature

Chemicals can also be tested in the human cells based reporter gene assay.

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:

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

where

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

$EC_{50}(R)$ is the EC50 of the reference compound 17β-estradiol;

$EC_{50}(C)$ 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 (Pr) to 17β-estradiol for selected compounds

Compound	U2OS-ERα	Reference	T47Dαβ	Reference
17β-estradiol	1		1	
17α-ethinylestradiol	1,3 to 1,5	[10] [11] [25]	1,2	[12] [14] [15]
17α -estradiol	0,1	[10] [11] [16]	0,016	[12] [14] [15]
Estrone	0,02	[10] [11] [16]	0,056	[12] [14] [15]
4-Nonylphenol	5,9E-04	[10] [11]	2,3E-05	[12] [14] [15]
Dimethyl-phtalate			1,1E-05	[12] [14] [15]
Genistein	1,1E-04	[11]	6,0E-05	[12] [14]
o,p-DDT	1,9E-05	[11]	9,1E-06	[12] [14]
Methoxychlor	1,8E-06	[11]	1,0E-06	[12] [14]
Bisphenol-A			7,8E-06	[12] [14] [15]
Nonylphenol ethoxylate			3,8E-06	[12] [14] [15]
4-Octylphenol			1,4E-06	[12] [14] [15]
Di-ethylphtalate			3,2E-08	[12] [14] [15]
Di-*n*-butylphtalate			1,8E-08	[12] [14] [15]
Equol	7,6E-04	[10] [11]		
Norethynodrel	0,015	[10]		
Di(2-Ethylhexyl)phthalate			> 6,0E-07	[12] [14] [15]
Estriol	0,017	[11]		

Annex E
(informative)

Preparation of dilution series

Table E.1 — Dilution steps for the water samples

Dilution level (D)	% sample	
1x	100	500 µl sample
2x	50,0	500 µl sample + 500 µl water
3x	33,3	333 µl sample + 667 µl water
4x	25,0	500 µl 2x + 500 µl water
6x	16,7	500 µl 3x + 500 µl water
8x	12,5	500 µl 4x + 500 µl water
12x	8,3	500 µl 6x + 500 µl water
16x	6,3	500 µl 8x + 500 µl water
24x	4,2	500 µl 12x + 500 µl water
32x	3,1	500 µl 16x + 500 µl water
48x	2,1	500 µl 24x + 500 µl water
64x	1,6	500 µl 32x + 500 µl water
96x	1,0	500 µl 48x + 500 µl water
128x	0,78	500 µl 64x + 500 µl water
192x	0,52	500 µl 96x + 500 µl water
256x	0,39	500 µl 128x + 500 µl water
384x	0,26	500 µl 192x + 500 µl water
512x	0,20	500 µl 256x + 500 µl water
768x	0,13	500 µl 384x + 500 µl water
1 024x	0,10	500 µl 512x + 500 µl water

NOTE Samples are diluted using two-fold dilution steps in order to accurately assess the dilution level *D*.

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 December 2015 for the generation of this validation data (see Reference [26]). 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 stably transfected human cells, which is the focus of the scope. The statistical evaluation of the data was performed according to ISO 5725-2.

F.1.2 Description of samples and participating laboratories

In total eight samples were analysed (see Table F.1):

Table F.1 — Description of samples

Sample number	Sample type	Description	Expected estrogenic activity	Assigned EEQ value ng/l
S1	Aqueous	Effluent of a municipal sewage treatment plant (STP)	+	
S2	Aqueous	Same as sample S1, but spiked with 10 ng/l EE2	++	S1 + 15 ng EEQ/l
S3	Aqueous	Influent of a municipal sewage treatment plant (same STP as for the samples 1 and 2)	+++	
S4	Aqueous	Surface water Rhine		
S5	Aqueous	Same as sample S4, but spiked with 15 ng/l EE2	++	S4 + 22,5 ng EEQ/l
S6	Aqueous	Blanc water (de-ionized water)		
S7	Ethanolic	Mix 1: 400 ng/l E2 + 4000 ng/l E1, which have been diluted 1- > 100 which results to final concentrations of 4 ng/l E2 + 40 ng/l E1 in 1 % ethanolic solution	++	4,8
S8	Ethanolic	Mix 2: 4 mg/l bisphenol a + 100 mg/l *tert*-butylphenol + 100 mg/l benzylbutylphthalat;, which have been diluted 1- > 100 which results to final concentrations of 40 µg/l bisphenol a + 1 mg/l *tert*-butylphenol + 1 mg/l benzylbutylphthalat in 1 % ethanolic solution (REPs only known from bisphenol a)	++	ca. 3,5

NOTE 1 Based on historical screening data the expected estrogenic activity of the samples were scored "+" expected low activity (close to the assay LOQ), "++" expected significant activity (quantified between the assay LOQ and EC_{50} of the reference curve), "+++" expected high activity (quantified above the EC_{50} of the reference curve) or"-" no activities expected.

Twelve laboratories provided data for the distributed samples. The in vitro human reporter gene assay can be carried out with various cell lines. In the present round robin test, only laboratory number L07 used cell line T47D, all other laboratories used the cell line U2OS. The data interpretation of laboratory

L07 with T47D cell didn't show any systematically different EEQ values compared to all other laboratories using the U2OS cell line.

The samples S1 to S6 have been tested from all twelve participating laboratories. For samples S7 and S8, only 4 measured values of 3 laboratories were available. A statistical analysis was therefore not possible. Still, for sample S7 and S8 the geometric mean values for orientation are given in Table F.3.

Table F.2 — Overview of samples which each laboratory was testing

Laboratory	Sample S1 to S6	Sample S7 to S8
L01	x	x
L02	x	
L03	x	x
L04	x	
L05	x	
L06	x	
L07	x	
L08	x	
L09	x	x
L10	x	
L11	x	
L12	x	
Number of data sets	12	3

NOTE 2 Most laboratories used ERα CALUX based on stable transfected U2OS – cells. Only laboratory L07 used ER CALUX based on stable transfected T47D –cells.

In most cases each sample was analysed by all laboratories at least twice, in independent experiments. The number of laboratories which provided data for a specific sample is indicated as well as the number of independent tests in Table F.2.

F.1.3 Summary of 17β-estradiol equivalent concentrations (EEQ)

All laboratories carried out either two or three repeated measurements per sample. Here each sample measurement was assigned its own control, thus in case of samples S1 to S6 per lab a total of 12 or 18 repeated measurements were available for evaluation. The dilution steps used for the evaluation of the EEQ and LID value were for all laboratories: undiluted, 2-, 4-, 8-, 16-, 32-, 64-, 128-, 256- and 512-times diluted.

The level of estrogenic activity of samples with a response below LOQ are set to the LOQ level for calculation purposes. In case no activity is detected in any of the replicates, the average LOQ is expressed for the replicate measurements.

For the samples 1 to 6, respectively 26 to 27, individual measurements were available from a total of 12 laboratories; for the samples 7 and 8 are each 4 individual measurements of 3 laboratories. Based on the specified validity invalid individual measurements were identified and excluded from further analysis.

All assays were classified as valid. Of all the 161 sample measurements, total 5 results were classified as not valid for (slightly) too high variability of parallel measurements:

— Sample 1: L11, measurement 1 and 2;

— Sample 3: L08, measurement 2;

— Sample 5: L09 measurement 2 and L10 measurement 2.

This corresponds to an amount of 3 % based on all measurements; based on the respective samples, the proportion of invalid measurements was between zero and 7,7 %. The lab-specific LOQ values were indicated from participants between 0,32 ng/l and 0,71 ng/l. The lab-specific LOD values were reported between 0,17 ng/l and 0,35 ng/l.

For the aqueous sample S6 (deionized water, unloaded) no effects were measured. Also for the sample S4 (surface water Rhine, not or very lightly loaded) results were found below the level of quantification (LOQ; at around 1,0 ng EEQ/l) and below the level of detection (LOD; at around 0,3 ng EEQ/l).

The low polluted sample S1 (effluent WWTP) showed a detectable estrogenic potential of 1,7 ng/l (laboratory geometric mean). The samples S2, S3 and S5 yielded geometric EEQ means of 18,7 ng/l, 51,5 ng/l and 23,6 ng/l. The different contaminated levels were reflected very well by the laboratory results. The total variability of EEQ values expressed in coefficients of variation was lying between 18 % and 42 %. Table F.3 summarizes the results. Figure F.1 shows the results graphically. An examination of the accuracy is carried out in F.1.4.

Table F.3 — Summary of EEQ results of the *in vitro* human reporter gene assay

	HRGA **EEQ** [ng/l]							
Sample	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
Number of laboratories	12	12	12	12	12	12	3	3
Number of measurements	26	27	27	27	27	27	4	4
Invalid measurements	2	—	1	—	2	—	—	—
% Invalid measurements	7,7	—	3,7	—	7,4	—	—	—
Number of measurements < LOD	1	—	—	26	—	27	—	—
Outliers excluded (single measurements)	1 (L05)	—	1 (L08)	n.d.	1 (L03)	n.d.	n.d.	n.d.
Outliers excluded (means)	—	1 (L07)	—	n.d.	—	n.d.	n.d.	n.d.
Number of laboratories for statistics	11	11	12	1	12	0	3	4
Number of measurements for statistics	22	25	25	1	24	0	4	4
	Laboratory geometric mean (expected value), *n*							
L01	1,5 (1,6), 3	19,7 (19,7), 3	65,1 (66,0), 3	< LOQ	25,3 (26,4), 3	< LOD	6,6 - 1	5,3 - 1
L02	1,3 (1,3), 2	20,4 (20,7), 2	75,3 (75,9), 2	< LOQ	31,9 (32,7), 2	< LOD	—	—
L03	2,1 (2,1), 2	19,6 (22,4), 2	40,1 (42,9), 2	< LOQ	22,6 - 1	< LOD	3,2 - 1	4,5 - 1
L04	1,2 (1,2), 2	16,1 (16,9), 2	36,9 (38,1), 2	< LOQ	15,6 (17,5), 2	< LOD	—	—
L05	2,6 - 1	19,0 (19,0), 2	69,3 (69,3), 2	< LOQ	24,2 (24,7), 2	< LOD	—	—
L06	1,2 - 1	14,9 (15,1), 2	51,3 (51,3), 2	< LOD	19,8 (19,9), 2	< LOD	—	—
L07	1,9 (1,9), 2	10,5 (10,5), 2	42,9 (43,1), 2	< LOD	14,0 (14,0), 2	< LOD	—	—
L08	1,4 (1,4), 3	19,1 (19,5), 3	57,2 - 1	< LOQ	30,7 (30,7), 3	< LOD	—	—
L09	1,9 (2,1), 3	20,3 (20,4), 3	67,3 (68,4), 3	< LOQ	25,5 (25,5), 2	< LOD	5,4 - 2	5,7 - 2
L10	2,4 (2,4), 2	21,5 (21,5), 2	21,5 (21,5), 2	< LOQ - 0,65	27 - 1	< LOD	—	—
L11	—	18,5 (18,5), 2	38,2 (39,9), 2	< LOQ	27,9 (31,2) 2	< LOD	—	—
L12	1,8 - 1	16,5 (16,6), 2	72,0 (72,0), 2	< LOQ	24,0 (24,0) 2	< LOD	—	—
min - max	1,2 to 2,6	14,9 to 21,5	36,9 to 75,3		14,0 to 31,9			

Table F.3 *(continued)*

factor max/min	2,2	1,4	2,0		2,3			
	HRGA **EEQ** [ng/l]							
Sample	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
Geometric mean	**1,7**	**18,7**	**51,5**	**n.d.**	**23,6**	**n.d.**	**5,0**	**5,3**
Expected value	1,8	19,0	55,9		24,9		—	—
95 % CI	**1,4 to 2,1**	**16,5 to 21,1**	**39,9 to 66,6**		**19,3 to 28,9**		—	—
95 % PI	0,9 to 3,0	13,1 to 26,7	23,4 to 113,8		12,7 to 44,1		—	—
99 % PI	0,8 to 3,7	29,9 to 11,7	18,5 to 145,6		10,4 to 53,5		—	—
s_r (repeatability)	0,317	3,044	7,261		4,462		—	—
s_L (interlaboratory variability)	0,444	1,720	22,399		6,777		—	—
s_R (reproducibility)	0,546	3,501	23,547		8,114		—	—
s_r %	18,1	16,0	13,0		17,9		—	—
s_L %	25,4	9,1	40,1		27,3		—	—
s_R %	**31,2**	**18,4**	**42,1**		**32,6**		—	—
s_R/s_r	1,72	1,15	3,24		1,82		—	—

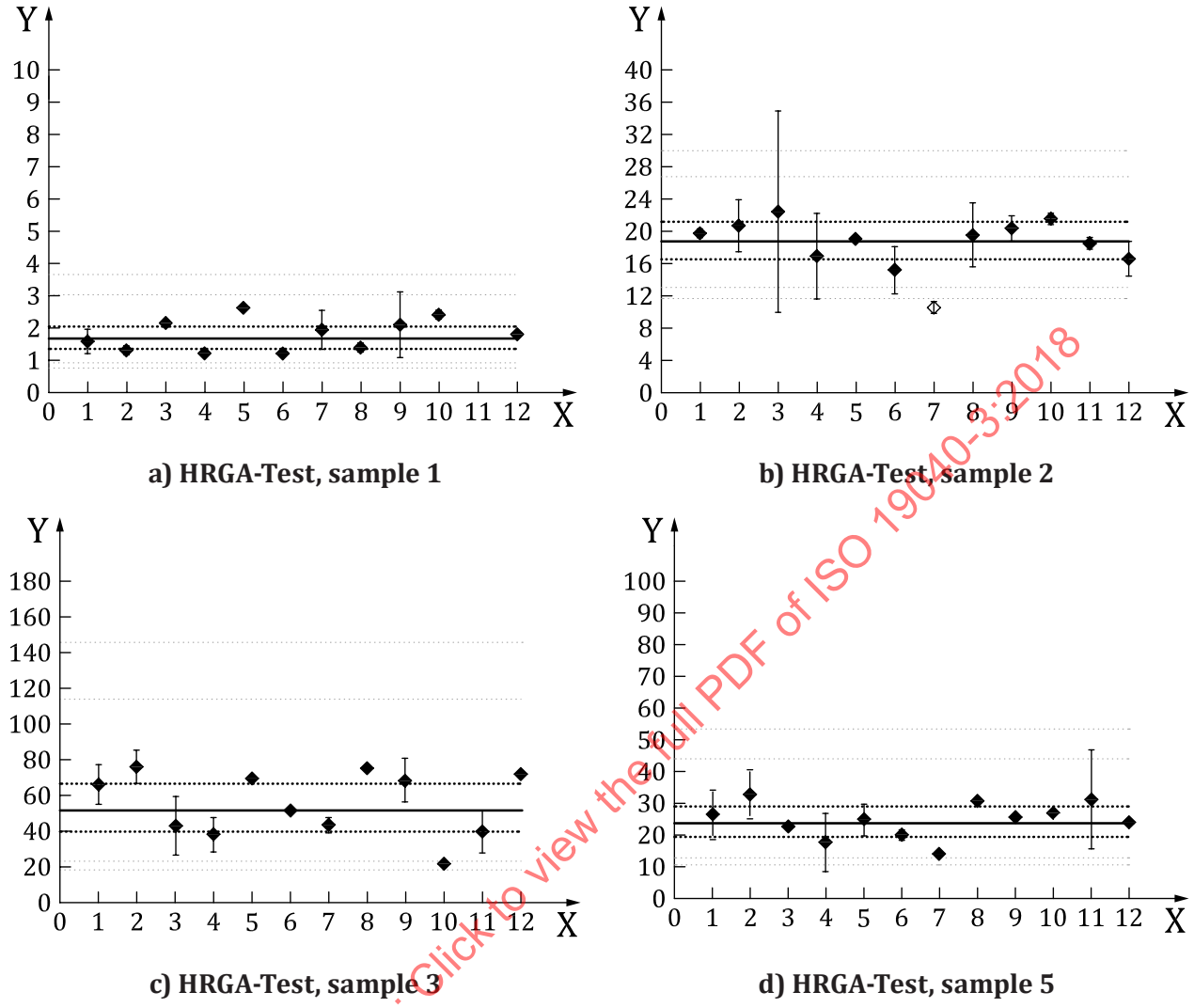
NOTE Confidence interval (95 % CI) and prediction interval (95 % PI und 99 % PI) are related to the geometric means.

s_r standard deviation of repeatability

s_R standard deviation of reproducibility

s_L laboratory standard deviation

n.d. not detected



Key

X	laboratory
Y	EEQ (ng/l)
black marked symbols	laboratory specific expected values with standard deviation (Whisker)
empty symbols	outliers
black solid line	interlaboratory geometric mean
black dotted lines	95 % confidence interval of the inter-laboratory geometric mean
grey dotted lines	95 % and 99 % tolerance range of the inter-laboratory geometric mean

Figure F.1 — Summary of 17β-estradiol equivalent concentrations (EEQ) [ng/l] in aqueous samples S1, S2, S3 and S5

F.1.4 Summary of lowest ineffective dilutions LID

The lowest ineffective dilution is determined as described in Annex I. I.6 for each independent experiment. Dilutions are prepared according to Table F.4. For the valid single results, both with the original raw data as well as with the individual laboratory mean results and outlier analysis was performed. No values were identified as outliers. Table F.4 summarizes the results. Figure F.2 shows the results graphically.

The overall geometric means of the LID analysis presents well the different levels of estrogenic activity in the samples S1 to S6. The laboratory means scattered depending on the sample and covered up to five

dilution levels. The total variability of the results expressed in coefficients of variation was between 69 % and 132 %.

Table F.4 — Summary of determined lowest ineffective dilutions (LID)

	Summary of determined lowest ineffective dilutions (LID) LID							
Sample	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
Number of laboratories	12	12	12	12	12	12	3	3
Number of measurements	26	27	27	27	27	27	4	4
Invalid measurements	2	—	1	—	2	—	—	—
% Invalid measurements	7,7	—	3,7	—	7,4	—	—	—
Number of measurements = 1	—	—	—	21	—	24	1x > 16	1x > 16
Outliers excluded (single measurements)	1 (L07)	—	—	—	1 (L02)	—	—	—
Outliers excluded (means)	—	—	—	—	—	—	—	—
Number of laboratories for statistics	11	12	12	12	12	12	3	3
Number of measurements for statistics	23	27	26	27	24	27	3	3
	Laboratory geometric mean (expected value), *n*							
L01	5,0 (5,5), 3	80,6 (87,4), 3	203 (220), 3	1,3	128 (163), 3	1	4 - 1	8 - 1
L02	11,3 (12,8), 2	64 (133,4), 2	256 (256), 2	1,4	128 - 1	1,4	—	—
L03	5,7 (6,4), 2	45,3 (51,0), 2	64 (64), 2	1,4	128 (128), 2	1,4	8 - 1	8 - 1
L04	11,3 (12,8), 2	90,5 (102,1), 2	512 (512), 2	2	128 (128), 2	1	—	—
L05	5,7 (6,4), 2	22,6 (25,5), 2	64 (64), 2	1	45,3 (51,0), 2	1	—	—
L06	4 (4), 2	45,3 (51,0), 2	362 (408), 2	1	45,3 (51,0), 2	1,4	—	—
L07	8 - 1	90,5 (102,1), 2	362 (408), 2	1	64 (64), 2	1	—	—
L08	3,2 (3,4), 3	50,8 (55,0), 3	181 (204), 2	1	128 (128), 3	1	—	—
L09	10,1 (13,9), 3	128 (128), 3	406 (440), 3	1	181 (204), 2	1	16 - 1	32 - 1
L10	4 (4), 2	32 (32), 2	32 (32), 2	2	32 - 1	1	—	—
L11	-	22,6 (25,5), 2	32 (32), 2	1	22,6 (25,5), 2	1	—	—
L12	16 - 1	90,5 (102,1), 2	256 (256), 2	2	64 (64), 2	1	—	—
min - max	4 to 11,3	22,6 to 128	32 to 512	1 to 2	22,6 to 181	1 to1,4	4 to 16	8 to 32
factor max/min	2,8	5,7	16,0	2	8	1,4	4,0	4,0

Table F.4 *(continued)*

	Summary of determined lowest ineffective dilutions (LID) **LID**							
Sample	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
Geometric mean	**6,3**	**57,8**	**167,1**	**1,3**	**80,6**	**1,1**	**8,0**	**12,7**
Expected value	7,6	72,1	276,7	n.d.	103,8	n.d.	n.d.	n.d.
95 % CI	4,1 to 9,5	37,8 to 88, 2	88,3 to 316,3		51,3 to 126,7			
95 % PI	1,9 to 21,2	15,6 to 213,2	23,3 to 1196,5		20,0 to 324,6			
99 % PI	1,3 to 30,9	10,4 to 320,1	12,7 to 2207,9		13,0 to 500,8			
s_r (repeatability)	4,089	35,844	67,254		33,408			
s_L (inter-laboratory variability)	3,240	40,282	358,992		77,199			
s_R (reproducibility)	5,218	53,920	365,238		84,118			
s_r %	53,7	49,7	24,3		32,2			
s_L %	42,5	55,9	129,7		74,4			
***s_R* %**	68,5	74,8	132,0		81,0			
s_R/s_r	1,28	1,50	5,43		2,52			

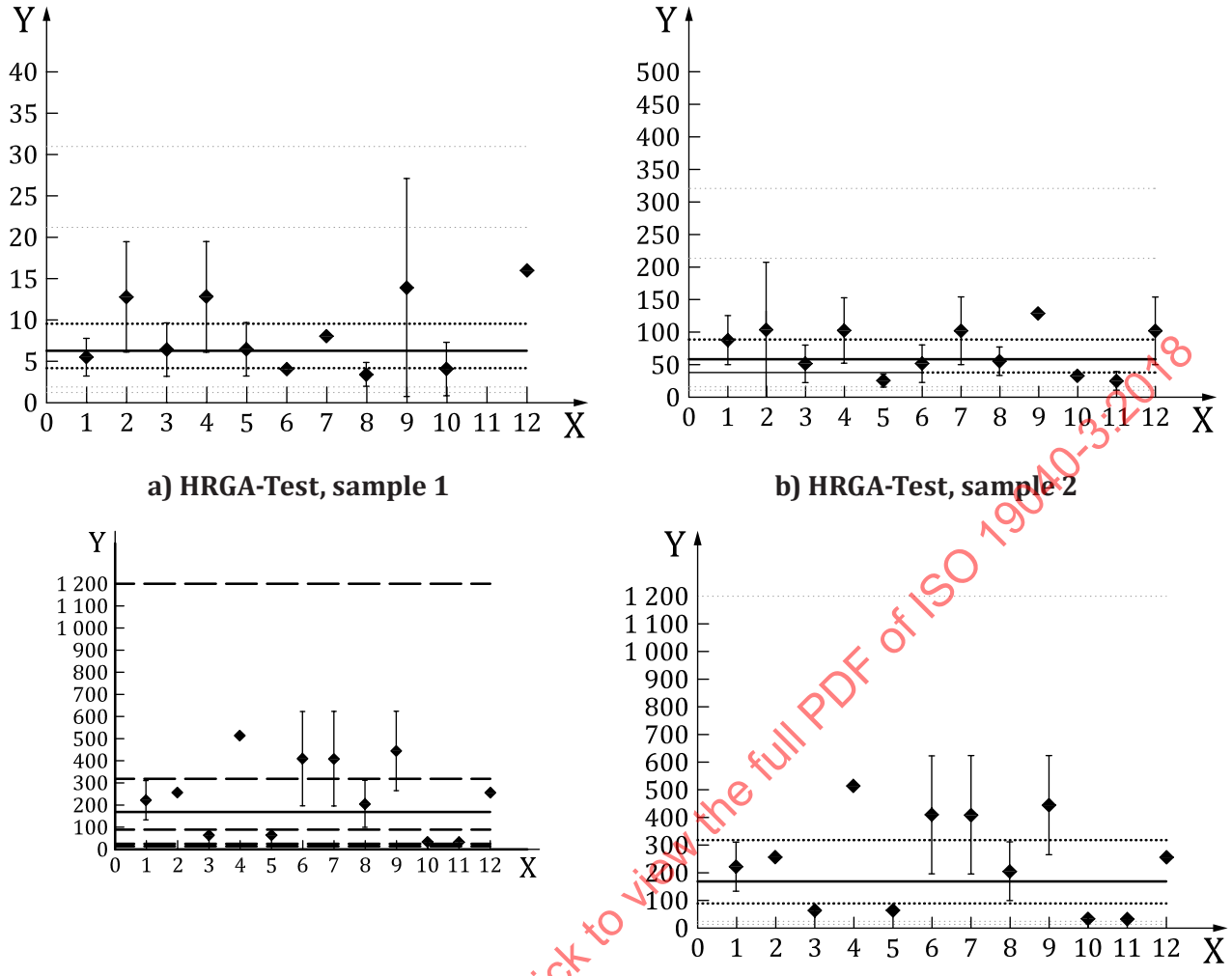
NOTE 1 Confidence interval (95 % CI) and prediction interval (95 % PI und 99 % PI) related to the geometric means.
NOTE 2 Laboratory 7 used the ER CALUX bioassay (T47D cells); all other laboratories used the ERα CALUX bioassay (U2OS cells).

s_r standard deviation of repeatability

s_R standard deviation of reproducibility

s_L laboratory standard deviation

n.d. not detected



a) HRGA-Test, sample 1

b) HRGA-Test, sample 2

c) HRGA-Test, sample 3

d) HRGA-Test, sample 5

Key

X	laboratory
Y	LID
black marked symbols	laboratory specific expected values with standard deviation (Whisker)
black solid line	interlaboratory geometric mean
black dotted lines	95 % confidence interval of the inter-laboratory geometric mean
grey dotted lines	95 % and 99 % tolerance range of the inter-laboratory geometric mean

Figure F.2 — Summary of determined lowest ineffective dilutions (LID) of aqueous samples S1, S2, S3 and S5

F.1.5 Trueness of results

A quantitative evaluation of the accuracy is possible with the spiked samples S2 and S5; for the remaining samples only a qualitative statement is possible. The assigned EEQ values were calculated from the relative potencies of the spiked samples S2 and S5 and the mixtures of reference chemicals in ethanol solution (S7 and S8). For the sample S7 the assigned EEQ value was 4,8 ng/l; for sample S8 3,5 ng/l.

The assigned EEQ value of the spiked samples was determined for each laboratory as a sum of the measured EEQ value of the unspiked sample 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.

For the following evaluation of the theoretical EEQ values, a relative equivalent potencies (REPs) of 1,5 was used for 17α-ethinyl-estradiol (see Table D.1). Therefore, in case of samples S2 and S5, the assigned EEQ values are resulting from the sum of the EEQ value of the aqueous sample S1 plus 15 ng/l (S2) or S4 plus 22,5 ng/l (S5).

For samples S2 and S5, 19 of 21 results with the U2OS cell line were in the range of ±30 % of the assigned values (90 %). Two results (10 %) differed between 31 % to 50 % from the assigned EEQ value. The mean percentile difference was +11,7 % for sample S2 (95 % confidence interval between −3,3 % and +20,1 %) and +10,6 % for sample S5 (95 % confidence interval between −3,2 % and +24,4 %). Across the samples, the mean accuracy was +11,1 % (95 % confidence interval between +3,6 % and +18,6 %).

As to be expected, for the high polluted sample S3 (influent WWTP), high EEQ values were found (37 ng/l to 75 ng/l). The results for the spiked mix samples S7 and S8 were in a range of −33 % to +63 % of the assigned values.

Table F.5 and Figure F.3 summarize the laboratory specific differences between mean and assigned EEQ values.

Table F.5 — Summary of trueness evaluation for samples S2 and S5

	S2				S5			
Lab	**EEQ assigned** [ng/l] **= EEQ P1 + 15**	**EEQ** [ng/l] **real**	**Diff real - assigned**	**Diff real – assigned** (%)	**EEQ assigned** [ng/l] **= EEQ P4 + 22,5**	**EEQ** [ng/l] **real**	**Diff real - assigned**	**Diff real – assigned** (%)
L01	16,5	19,7	**3,2 (19 %)**	**19,4**	22,5	25,3	**2,8 (12 %)**	**12,4**
L02	16,3	20,4	**4,1 (25 %)**	**25,2**	22,5	31,9	**9,4 (42 %)**	**41,8**
L03	17,1	19,6	**2,5 (15 %)**	**14,6**	22,5	22,6	**0,1 (0,4 %)**	**0,4**
L04	16,2	16,1	**−0,1 (−0,6 %)**	**−0,6**	22,5	15,6	**−6,9 (−31 %)**	**−30,7**
L05	17,6	19	**1,4 (8 %)**	**8,0**	22,5	24,2	**1,7 (8 %)**	**7,6**
L06	16,2	14,9	**−1,3 (−8 %)**	**−8,0**	22,5	19,8	**−2,7 (−12 %)**	**−12,0**
L07	16,9	10,5	**−6,4 (−38 %)**		22,5	14	**−8,5 (−38 %)**	
L08	16,4	19,1	**2,7 (16,5 %)**	**16,5**	22,5	30,7	**8,2 (36 %)**	**36,4**
L09	16,9	20,3	**3,4 (20 %)**	**20,1**	22,5	25,5	**3 (13 %)**	**13,3**
L10	17,4	21,5	**4,1 (24 %)**	**23,6**	23,15	27	**3,9 (17 %)**	**16,8**
L11	—	—	—		22,5	27,9	**5,4 (24 %)**	**24,0**
L12	16,8	16,5	**−0,3 (−1,8 %)**	**−1,8**	22,5	24	**1,5 (7 %)**	**6,7**

MU standard uncertainty associated with the method and laboratory bias.

NOTE 1 Assigned and real analysed EEQ-values as well as laboratory-specific differences between assigned and real analysed EEQ-values (In brackets: percentage differences from the assigned value).

NOTE 2 L07 used the cell line T47D, all other laboratories the cell line U2OS; results of laboratory L07were not considered for the calculation of mean, 95 % confidence interval CI and 95 % prediction-interval PI. The relative equivalent potency of EE2 here used is 1,5.

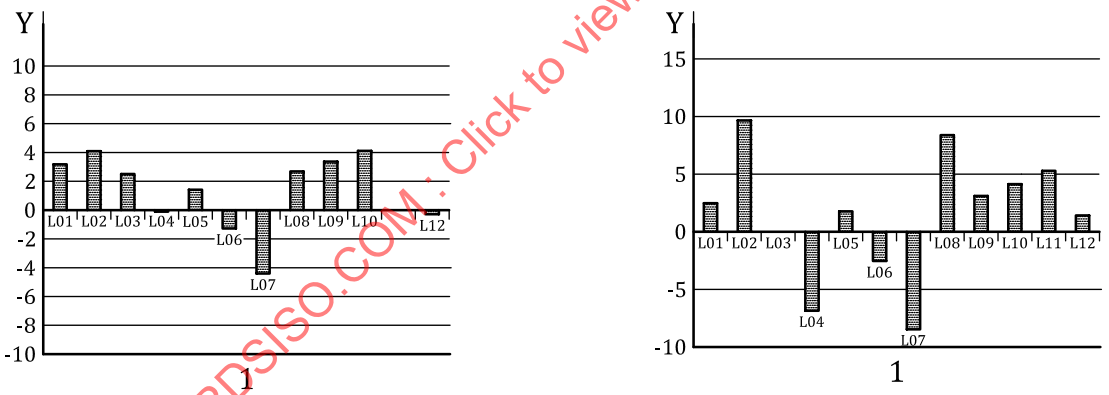
Table F.5 *(continued)*

Lab	S2				S5			
	EEQ assigned [ng/l] **= EEQ P1 + 15**	**EEQ** [ng/l] **real**	**Diff real - assigned**	**Diff real – assigned** (%)	**EEQ assigned** [ng/l] **= EEQ P4 + 22,5**	**EEQ** [ng/l] **real**	**Diff real - assigned**	**Diff real – assigned** (%)
Mean MU	16,1 %				22,2 %			
Mean MU across samples S2 and S5	19,2 %							
Relative bias from mean recovery	+11,7 %				+10,5 %			
95 % ci	+3,3 % - +20,0 %				−3,2 % - +24,4 %			
Relative bias from mean recovery across samples S2 and S5	**+ 11,1 %**							
95 % CI	**+3,6 % - +18,6 %**							

MU standard uncertainty associated with the method and laboratory bias.

NOTE 1 Assigned and real analysed EEQ-values as well as laboratory-specific differences between assigned and real analysed EEQ-values (In brackets: percentage differences from the assigned value).

NOTE 2 L07 used the cell line T47D, all other laboratories the cell line U2OS; results of laboratory L07were not considered for the calculation of mean, 95 % confidence interval CI and 95 % prediction-interval PI. The relative equivalent potency of EE2 here used is 1,5.



a) Sample 2, HRGA, assigned = sample 1 + 15 **b) Sample 5, HRGA, assigned = sample 4 + 22,5**

Key

1 laboratory

Y difference to assigned EEQ (ng/l)

Figure F.3 — Summary of the accuracy of samples S2 and S5 (expressed in absolute differences to the assigned value)