
**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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Published in Switzerland

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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).

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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.

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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

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 17 β -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 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*

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

EC₅₀

effective concentration of a compound which causes 50 % of an effect

Note 1 to entry: In the sense of the present document the EC₅₀ 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 $\mu\text{S}/\text{cm}$ (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 CO₂ incubator, 5 % CO₂, 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 cm² 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 %, (C₁₈H₂₄O₂), 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.