



DPC: 24/30478501 DC

BSI Group Headquarters

389 Chiswick High Road London W4 4AL

Tel: +44 (0)20 8996 9000

Fax: +44 (0)20 8996 7400

www.bsigroup.com

Date: 08 January 2024

Origin: International

Latest date for receipt of comments: 02 March 2024

Project No. 2023/01866

Responsible committee: EH/3/5 Biological Methods

Interested committees:

Title: Draft BS ISO 24295 Water quality - Determination of the dioxin-like activity of water and wastewater - Method using in vitro mammalian cell-based reporter gene assay.

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Introduction

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Submission of Comments

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Template for comments and secretariat observations

Date: xx/xx/20xx	Document: ISO/DIS xxxx
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1	2	(3)	4	5	(6)	7
MB	Clause No./ Subclause No./Annex (e.g. 3.1)	Paragraph/ Figure/Table/Note	Type of comment	Comment (justification for change) by the MB	Proposed change by the MB	Secretariat observations on each comment submitted
	3.1	Definition 1	ed	Definition is ambiguous and needs clarifying.	Amend to read '...so that the mains connector to which no connection...'	
	6.4	Paragraph 2	te	The use of the UV photometer as an alternative cannot be supported as serious problems have been encountered in its use in the UK.	Delete reference to UV photometer.	

DRAFT INTERNATIONAL STANDARD

ISO/DIS 24295

ISO/TC 147/SC 5

Secretariat: DIN

Voting begins on:
2024-01-08

Voting terminates on:
2024-04-01

Water quality — Determination of the dioxin-like activity of water and wastewater — Method using in vitro mammalian cell-based reporter gene assay

ICS: 13.060.50

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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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For an explanation of 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 www.iso.org/iso/foreword.html.

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

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs, dioxins), dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (DL-PCBs) are environmental contaminants that are largely produced in the process of combustion of domestic and industrial waste. Exposure to environmental media contaminated with these compounds is harmful to humans as well as wildlife. Effective concentration of the individual congeners of these compounds varies by orders of magnitude and one of the most harmful congeners is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD). The dioxin-like effects of these compounds on mammals are mediated via the aryl hydrocarbon receptor (AhR).

Contamination by dioxins and dioxin-like compounds in the environment are widely distributed, particularly in aquatic ecosystems. These hydrophobic environmentally persistent compounds accumulate in mammals including humans, via the food chain.^[27,28] AhR-mediated reporter gene assays have been developed for the determination of 2,3,7,8-TCDD equivalents (TCDD-EQ) of water and wastewater contaminated with dioxins and dioxin-like compounds. The TCDD-EQ value represents the dioxin-like activity, relative to 2,3,7,8 TCDD, of the water samples. The procedure described herein is applied to samples after extraction and a subsequent clean-up, to derive TCDD-EQ values, reflecting the integrated and combined effect of the structurally diverse chemical group of dioxins and dioxin-like compounds. Therefore, practical use of AhR-mediated reporter gene assays for monitoring of water and wastewater plays an important role on evaluation of dioxin-like activity of the water samples.

Water quality — Determination of the dioxin-like activity of water and wastewater — Method using in vitro mammalian cell-based reporter gene assay

WARNING — Dioxins and dioxin-like compounds are harmful to humans. Therefore, all work with these compounds require the utmost care; the national safety measures which correspond to those for harmful substances shall be strictly adhered to. The aryl hydrocarbon receptor (AhR)-mediated reporter gene assays utilize stably transfected mammalian cancer cell lines.

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 qualified staff.

1 Scope

This document specifies a method for the determination of the dioxin-like activity of water and wastewater by means of an AhR-mediated reporter gene assay utilizing stably transfected mammalian cell line. This reporter gene assay is based on the activation of the mammalian AhR signalling pathway.

This method is applicable to:

- drinking water;
- fresh water;
- groundwater;
- river water;
- waste water.

The limit of quantification (LOQ) at CV 20 % or less of this method is 0,5 pg and 1,0 pg 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) equivalents (TCDD-EQ)/l based on the results of laboratory trials using appropriately extracted and cleaned up water samples for dioxins and dioxin-like compounds in rat and mouse cell lines, respectively. The LOQ depends on the sample volume. For determination of TCDD-EQ derived from dioxins and dioxin-like compounds in water and wastewater, suitable extraction and clean-up are necessary for water samples, since AhR ligands/agonists are composed of a wide variety of structurally diverse compounds including natural, and anthropology-natural substances.

NOTE In this document, TCDD-EQ is used instead of TEQ which represents toxicity equivalent quantity.

2 Normative references

There are no normative references in this document.

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](https://www.iso.org/obp)
- IEC Electropedia : available at <https://www.electropedia.org/>

3.1

confluence

indicate stage of cell growth

Note 1 to entry: The culture flask should be covered by cells when the cell line is cultivated in the culture flask. 100 % confluence indicates full growth. Visual confirmation of the stage of cell growth can be observed under a microscope.

3.2

dilution water

D

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

Note 1 to entry: The composition of the water is specified in the respective standard.

[SOURCE: ISO 20079:2005,^[18] 3.7, modified — “Note 1 to entry” has been added; ISO 5667-16:2017,^[5] 3.7]

3.3

culture medium

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

[SOURCE: ISO 6107:2021,^[1] 3.147, modified — “microbiological” replaced by “cellular”]

3.4

dilution factor

dilution factor is the ratio of the solute to dimethyl sulfoxide (DMSO)

EXAMPLE If 10 µl of a solution is diluted with DMSO to 100 µl of a solution, the resulting dilution factor is 10 (x10)

Note 1 to entry: For undiluted water or wastewater, 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.

3.5

EC50

effect 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 enzyme activity which can be achieved by this compound.

3.6

limit of detection

LOD

minimum concentration of a compound that can be detected by an AhR-mediated reporter gene assay with a given statistical probability

Note 1 to entry: Usually the detection limit is given as three times the standard deviation of noise in the system.

3.7

limit of quantification

LOQ

lowest value that can be determined by an AhR-mediated reporter gene assay with an acceptable level of accuracy and precision

[SOURCE: ISO 15839:2003,^[2] 3.18]

3.8**passage number**

the number of times the same batch of cells have been sub-cultured

3.9**procedure blank (PB)**

a complete analysis, including extraction and clean-up, conducted using the solvents and reagents only, in the absence of any sample

3.10**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] 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"]

3.11**relative light units****RLU**

amount of reporter luciferase activity as measured by emitted light produced using a luminometer

3.12**reporter gene expression**

quantitative inducible expression of a (reporter) gene attached to the ligand-activated receptor recognition sequence followed by the promoter sequence of another gene

3.13**stock culture**

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

[SOURCE: ISO 21427-2:2006,^[4] 13, modified — "the cell line" replaces "V79 cells"]

3.14**subculturing**

a subculture is a new cell culture made by transferring a part of cells from a previous culture to fresh growth medium

Note 1 to entry: This action is called subculturing the cells.

3.15**vehicle control****VC**

DMSO control solution on a 96-well plate at exposure

4 Principle

The AhR-mediated signalling pathway is activated when a ligand such as 2,3,7,8-TCDD binds to the AhR. This activity is directly related to inducible expression of metabolizing enzyme genes including cytochrome P450 1a1 in mammalian cells. Upon entering cells by diffusion, a ligand binds to a cytosolic AhR. The ligand-activated AhR complex translocate into the nucleus of the cell, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). The ligand, AhR and ARNT complex binds to its specific recognition DNA sequence, the so-called dioxin responsive element (DRE), and thus induces the expression of DRE-associated genes (e.g. cytochrome P450 1a1 gene and many others). These biochemical effects are probably associated with subsequent toxic responses of 2,3,7,8-TCDD and related compounds. Characteristics and details of AhR-mediated reporter gene assay using transfected mammalian cell lines are described in [Annex C](#).

The DREs have been linked to a promoter followed by a gene coding for the reporter luciferase. The reporter gene constructed was transfected into mammalian hepatoma cells. In the transfected cells, the ligand-activated AhR complex induces the expression of the reporter luciferase gene, and then the produced luciferase emits light when a luciferin as substrate is added. The response increases as a result of increasing luciferase activity in a ligand concentration-dependent manner.

There are species differences in AhR ligands/agonists specificity in the assay with cell lines of different mammalian species since affinity of AhR species toward these compounds is different. Also, mammalian cell lines show species differences in growth rate and sensitivity to chemical, physical and biological conditions. For photographs showing typical mammalian cells observed by a culture microscope see [Figure 1](#).

When two different species of mammalian cell lines are used simultaneously for assays of water samples, data shows species differences, which are useful for toxicological evaluation of dioxin-like activity.

5 Interferences

The stage of cell growth can affect the sensitivity of the assay. Therefore, the confluency of cell line is observed under a culture microscope, and the cell number in the culture medium is always adjusted to ensure sensitivity consistency, prior to the use of the assay.

The use of inappropriate sampling devices and/or sampling flasks may influence the test results because of the possible adsorption of active compounds and contamination on surfaces leading to false negative results. On the other hand, active compounds could be released into the sample from sampling flasks, especially if wares used are contaminated with active compounds, and false positive results might be generated.

Dioxins and dioxin-like compounds are hydrophobic and adsorbed on suspended solid particles in water samples and on surfaces of sample flasks. For extraction, dissolving and rinsing of these compounds, organic solvents such as dichloromethane, toluene and DMSO are used. These solvents in test samples can cause toxic effects on mammalian hepatoma cells for the AhR-mediated reporter gene assay and need to be removed as much as possible in test samples. For example, the cells tolerate up to 1,5 % w/w of DMSO, as used for dissolving test compounds in test sample solutions.

Bacterial and fungal contaminations can negatively influence the cells. Therefore, it is recommended to add antibiotics to the cell culture medium. Contamination of the cells is assessed by visual observation (microscope) when testing the samples. If the sample is filtered to remove bacteria, suspended solid particles are also removed from the sample. Thus, substances with dioxin-like effects which are adsorbed on particles might not be detected.

AhR antagonistic compounds might mask dioxin-like effects. The presence of interfering compounds can be assessed by additional testing using samples which are spiked with a defined amount of a dioxin-like compound with defined properties (e.g. 2,3,7,8-TCDD) leading to a known induction of the test system.

Cytotoxic 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, dioxin-like 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 (using an appropriate cytotoxicity test)^[30].

6 Apparatus and materials

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

6.2 Water bath, (37 ± 3) °C.

- 6.3 **CO₂ incubator**, ($5 \pm 0,5$) % CO₂, (37 ± 3) °C and humidity 100 %.
- 6.4 **Inverted phase-contrast microscope.**
- 6.5 **Culture microscope.**
- 6.6 **Freezer**, at least $\leq (-20 \pm 2)$ °C and at least $\leq (-80 \pm 2)$ °C.
- 6.7 **Shaking apparatus for micro-test plates.**
- 6.8 **Centrifuge**, suitable for centrifugation of 15 ml and 50 ml tubes at 250G to 500G.
- 6.9 **Laboratory balance with a minimum accuracy of 3 decimals.**
- 6.10 **Sterile pipettes**, 10 ml glassware or plastics.
- 6.11 **Pipette controller.**
- 6.12 **Cell culture flasks**, 75 cm² and 175 cm² with filter lids.
- 6.13 **Conical tube**, 15 ml and 50 ml, suitable for centrifugation.
- 6.14 **Multi-channel multi-stepper pipette (repeater pipette)**, including 5 ml and 10 ml tips.
- 6.15 **Pipettes**, 20 µl, 200 µl and 1 000 µl, with sterile tips.
- 6.16 **Multi-channel pipette**, up to 50 µl and up to 200 µl.
- 6.17 **Sterile polystyrene 96-well plates**, with flat transparent bottom and lid, appropriate for cell culture and volume 300 µl per well.
- 6.18 **Microplate luminometer**, detector of 340 nm to 650 nm, with two injectors for addition of substrate and stop reagent.
- 6.19 **Counting chamber.**
- 6.20 **Cryovials**, sterile, 2 ml.
- 6.21 **Liquid nitrogen container for long-term cell storage.**
- 6.22 **Autoclave.**
- 6.23 **Vacuum pump.**
- 6.24 **Mixer.**
- 6.25 **Glass tube**, 5 ml to 10 ml.
- 6.26 **pH meter.**
- 6.27 **Filter**, cellulose acetate, 0,45 µm pore size.

6.28 Nitrile gloves.

6.29 Sterile transparent polystyrene 12- and 48-well plates.

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 Water, grade 3, as defined in ISO 3696; water with a conductivity up to 5 $\mu\text{S}/\text{cm}$ 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.

7.1.2 DMSO, molecular weight 78,13 g/mol, CAS: 67-68-5.

7.1.3 2,3,7,8-TCDD, molecular weight 321,97 g/mol, CAS: 1746-01-6 (50 $\mu\text{g}/\text{ml}$ of DMSO).

7.1.4 Trypsin 0,25 %, CAS: 9002-07-7 or Trypsin 2,5 % CAS: 15090-46.

7.1.5 Fetal bovine serum (FBS), stored at $(-20 \pm 1) ^\circ\text{C}$.

7.1.6 Penicillin streptomycin (100x), concentration used 5 000 units penicillin per ml / 5 000 μg streptomycin per ml, stored at $(-20 \pm 1) ^\circ\text{C}$.

7.1.7 Tricine, CAS: 5704-04-1.

7.1.8 Magnesium hydroxide carbonate pentahydrate, $\text{C}_4\text{H}_2\text{Mg}_5\text{O}_{14}$, CAS: 56378-72-4.

7.1.9 Magnesium sulfate, MgSO_4 , CAS: 7487-88-9.

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

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

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

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

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

7.1.15 2-Amino-2(hydroxyl-methyl)-1,3-propanediol (Tris), CAS: 77-86-1.

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

7.1.17 MEM α medium, with phenol red as pH indicator.

7.1.18 Glycerol, molecular weight; > 99 %, 92,09 g/mol, CAS: 56-81-5.

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

7.1.20 Propanone (acetone), molecular weight 58,08 g/mol, CAS: 67-64-1.

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

7.1.22 Hydrochloric acid solution, 1 M (HCl), CAS: 7647-01-0.

7.1.23 Sodium bicarbonate, NaHCO₃, CAS: 144-55-8.

7.1.24 Ethanol, CAS: 64-17-5.

7.2 Cell line(s)

Genetically modified mammalian cell lines such as mouse cell lines and rat cell line, producing native mammalian AhR and containing a ligand-activated receptor recognition sequence followed by a promoter-linked reporter gene for luciferase, is used. More detailed descriptions of the individual cell lines are given in [Annex C](#).

7.3 Media

Always use sterile solutions and glassware, etc. Carry out all procedures under aseptic conditions and in the sterile environment of a laminar flow cabinet ([6.1](#)). If autoclaving is necessary, always autoclave for 20 min at $(121 \pm 2) ^\circ\text{C}$. Cover vessels loosely and never autoclave sealed vessel.

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

7.3.2 MEM-alpha medium with L-glutamine, stored at $4 ^\circ\text{C}$ to $8 ^\circ\text{C}$.

7.3.3 Cell culture medium

Add 44 ml FBS ([7.1.5](#)) and 5 ml of penicillin-streptomycin ([7.1.6](#)) to a bottle containing 500 ml MEM-alpha medium with L-glutamine ([7.3.2](#)) for mouse cell line. For the rat cell line, add 55 ml FBS and 1 ml of penicillin-streptomycin to a bottle containing 500 ml MEM-alpha medium. Complete cell culture medium should be kept at $(5 \pm 3) ^\circ\text{C}$ and stored for no longer than eight weeks.

7.3.4 Freezing medium

For the mouse cell line, prepare freezing medium by adding 6 ml DMSO ([7.1.2](#)) to 34 ml of cell culture medium ([7.3.3](#)). For rat cell lines, remove 142,5 ml from a new MEM-alpha bottle and add 37,5 ml DMSO and 100 ml FBS ([7.1.5](#)). Distribute aliquot 20 ml batches in a sterile manner over sterile tubes. Complete freezing medium should be kept at $\leq (-18 \pm 2) ^\circ\text{C}$ and no longer than one year. When in use, always keep freezing medium cooled (for example on ice).

7.3.5 Lysis mixture

Lysis mixture can be made as follows; completely dissolve the compounds listed in [Table 1](#) in 500 ml of water ([7.1.1](#)). Adjust pH to 7,8 using 1 mol/l NaOH solutions. Adjust final volume to 1 l and aliquot into 40 ml portions. The prepared lysis mixture should be stored at $(5 \pm 3) ^\circ\text{C}$ for a maximum of one month or at $\leq (-18 \pm 2) ^\circ\text{C}$ for a maximum of 1 year.

NOTE Lysis mixture is commercially available.