SLOVENSKI STANDARD
SIST EN 14486:2005

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Kakovost vode - Detekcija ļ loveġkih enterovirusov z metodo enoplastnega plaka

Water quality - Detection of human enteroviruses by monolayer plaque assay

Wasserbeschaffenheit - Nachweis humaner Enteroviren mit dem Monolayer-Plaque-Verfahren

Qualité de l'eau - Détection des entérovirus humains par culture cellulaire par la méthode des plages

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English version

Water quality - Detection of human enteroviruses by monolayer plaque assay

Qualité de l'eau - Détection des entérovirus humains par culture cellulaire par la méthode des plages

Wasserbeschaffenheit - Nachweis humaner Enteroviren mit dem Monolayer-Plaque-Verfahren

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Management Centre: rue de Stassart, 36 B-1050 Brussels

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Contents

Page

Foreword

This document (EN 14486:2005) has been prepared by Technical Committee CEN/TC 230 “Water analysis”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by November 2005, and conflicting national standards shall be withdrawn at the latest by November 2005.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

Introduction

Enterovirus infection of man occurs all year round with a seasonal peak in the summer and autumn. These viruses are species specific and replicate primarily in the gastro-intestinal tract. Most infections are asymptomatic but some may result in a flu-like illness that in a minority of cases leads to meningitis or paralysis. Gastro-enteritis may occur as part of wider systemic disease. The viruses infect all ages but are particularly common in children. Many serotypes exist and the most common serotypes change from year to year.

Like all other viruses, enteroviruses are obligate intracellular parasites and will not replicate in the environment. Enteroviruses have been used as a marker for the presence of human faecal pollution for many years because they were the first isolated and still remain the most readily detectable human viruses in environmental samples.

BGM (Buffalo Green Monkey) cells have been shown to be sensitive to infection by enteroviruses including poliovirus and Coxsackievirus B (Dahling and Wright 1986, Morris 1985) and are in widespread use throughout the world. The BGM monolayer plaque assay is approximately half as sensitive as the suspended cell plaque assay (Dahling and Wright 1988) but utilises significantly fewer cells and is more practical for most laboratories. The Council Directive of 8 December 1975 concerning the quality of bathing water (76:160/EEC) specifies that the enumeration of enterovirus should be reported as confirmed 'Plaque forming units' per 10 l of original sample. The method described below for infectious enteroviruses, therefore meets the requirements of the EU Bathing Water Directive, is the most practical for most virus laboratories, is well established throughout the world and will detect a group of viruses known to be common in environmental samples.

WARNING — Enteroviruses are pathogens capable of causing illness. All samples and controls shall be handled by trained staff in a laboratory with appropriate equipment. Staff shall be fully vaccinated against poliovirus. Persons using this standard shall be familiar with normal virology laboratory practice. This standard does not purport to address fully all of the safety issues associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This European Standard describes a method for the detection of those serotypes of enterovirus that replicate and form plaques in BGM cells under agar. These will largely be poliovirus and Coxsackievirus B serotypes. Most serotypes of echovirus, Coxsackievirus A and animal enteroviruses are unlikely to produce plaques under these conditions. Reoviruses are unlikely to be detectable within the specified seven day incubation period. It is applicable to any type of water and processed water sample although toxic elements may interfere with cell culture.

The methods to confirm that the plaques seen contain enterovirus are given. Methods to identify which serotype of enterovirus is found in the plaque are not included as this is not required by the EU Bathing Water Directive.

Basic cell culture procedures including the preparation, maintenance and enumeration of BGM cell culture are not described but suggestions are made for media that may be used (see Annex A).

2 Normative references

The following referenced documents are indispensable for the application of this European Standard. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 8199, *Water quality — General guide to the enumeration of micro-organisms by culture*

3 Terms and definitions

For the purposes of this European Standard, the following term and definition applies.

3.1
enterovirus
virus that replicates and forms plaques in BGM cells under agar when incubated at (36 ± 2) °C for up to seven days and is confirmed by the production of a characteristic enterovirus cytopathic effect (CPE) when sub-cultured into fresh cells

4 Principle

4.1 Detection

Confluent monolayers of BGM cells in flasks or cell culture grade dishes are inoculated with sample and incubated for 1 h at (36 ± 2) °C or for 2 h at room temperature. Excess sample is removed from each flask or dish if necessary, and an overlay mixture containing agar is poured over the cell sheet and allowed to set. The cultures are incubated at (36 ± 2) °C for up to 7 d.

4.2 Enumeration

The pale areas of cell death (plaques) are counted daily. It is assumed that a plaque is the progeny of a single infectious unit of virus and the plaques counted and reported as 'plaque forming units (pfu)'.

The whole of the sample concentrate is assayed and the number of pfu in the original sample calculated.

4.3 Confirmation

The cells from a plaque are sub-cultured into fresh cells. An enterovirus in a plaque will be confirmed if it produces a characteristic cytopathic effect (CPE) when sub-cultured into fresh cells under liquid maintenance medium.

The identification of the serotype of the enterovirus may be done by a serum neutralisation test, by immunofluorescence using specific monoclonal antibodies, or by enterovirus specific polymerase chain reaction (PCR). The details of these methods are beyond the scope of this European Standard.

5 Apparatus

5.1 General

All plastics ware and glassware shall be sterile.

Except for disposable glassware which is delivered sterile, glassware shall be sterilised in accordance with instructions given in ISO 8199.

5.2 Incubator, capable of being maintained at (36 ± 2) °C, with 5 % (volume fraction) CO_2 supply if Petri dishes are used.

5.3 Water bath, capable of maintaining (45 ± 2) °C.

5.4 Water bath, boiling.

5.5 Refrigerator, capable of maintaining (5 ± 3) °C.

5.6 Deep freeze, capable of maintaining (-20 ± 5) °C.

5.7 Petri dishes, sterile, tissue culture grade, with a nominal diameter of 90 mm or 60 mm.

5.8 Tissue culture flasks, sterile, volume 25 cm^3, 75 cm^3, 80 cm^3

5.9 Pipettes, sterile, graduated, volumes 1 ml and 10 ml.

5.10 Micropipette, with sterile pipette tips, 1 ml to 5 ml.

5.11 Measuring cylinder, sterile, volume 50 ml.

5.12 Vertical laminar air-flow cabinet, optional for cell culture procedures.

5.13 Liquid nitrogen, optional for cell culture storage facility.

6 Media

Media shall be suitable for the culture and maintenance of BGM cells. Examples are given in Annex A.

7 Cell culture

7.1 Stock culture

Grow stock BGM cells on a seven-day cycle using local cell culture protocols.

Growth medium (A.1) may be replaced with maintenance medium (A.2) on day four or five.

Use the harvest of cells to produce confluent monolayers of BGM cells (7.2) for the sample inoculation procedure.

7.2 Preparation of cell culture monolayer

Prepare a fresh cell suspension from a stock culture (7.1) by conventional trypsination and re-suspend the cells to give a final concentration of $4 \times 10^6 \pm 2 \times 10^2$ per 60 mm/90 mm Petri dish or per 75 cm^2/80 cm^2 flask.

NOTE An example of counting cells using Fuchs-Rosenthal counting chamber is given in Annex B.

A total of (10 ± 2) ml of growth medium per 60 mm/90 mm Petri dish, or (30 ± 5) ml growth medium per 75 cm²/80 cm² flask is needed. Add enough growth medium to the cell suspension to make up to this total volume.

Place flasks in an incubator (5.2) until a confluent cell sheet is formed. Place Petri dishes in an incubator (5.2) that has the atmosphere moistened to prevent evaporation from the Petri dishes.

8 Assay procedure

8.1 Sample inoculation

It is good practice to divide the sample to be tested between assays on different days in case of technical problems. For the most accurate estimation of the number of enterovirus plaque forming units in the original sample, the whole concentrate should be assayed. The total number of plaques counted in the whole of the concentrate equals the total number of plaque forming units in the original sample.

Prepare sufficient Petri dishes (5.7) or flasks (5.8).

The sample concentrate under investigation shall be at room temperature.

Discard the media from a confluent monolayer of BGM cells (7.2). For the Petri dishes remove the medium using a sterile pipette or equivalent. Treat medium as potentially infected material and discard appropriately.

If necessary, wash the cells with (3 ± 1) ml of phosphate buffered saline (PBS, A.5) and discard the PBS. For the Petri dishes, remove the PBS as above.

Add the sample concentrate. Add (1 ± 0,5) ml to 75 cm²/80 cm² tissue culture flasks (5.8) and to 60 mm/90 mm Petri dishes (5.7) or (0,5 ± 0,2) ml to 25 cm² tissue culture flasks (5.8). Rock to ensure an even spread.

Place the inoculated cells in the incubator (5.2) for at least 1 h or at room temperature for 2 h. The cultures may be gently rocked every 15 min to ensure maximum adsorption of the virus.

Prepare enough (see Table 1) overlay medium (A.4) for each flask (5.8) or Petri dish (5.7) and place in a water bath (5.3). Allow time to equilibrate to the temperature of the water bath.

Melt enough (see Table 1) agar (A.3) for the assay in a boiling water bath (5.4) and then place in the water bath at (45 ± 2) °C (5.3). Allow time to equilibrate to the temperature of the water bath.

Table 1 — Volumes of sample, agar and overlay medium needed for different culture containers

Volume in millimeters

Culture container	Agar	Overlay Medium
75 cm²/80 cm² tissue culture flask	(10 ± 2)	(10 ± 2)
25 cm² tissue culture flask	(4 ± 1)	(4 ± 1)
60 mm/90 mm Petri dish	(6 ± 2)	(6 ± 2)

Add the appropriate volume (see Table 1) of the molten agar into the overlay medium, making sure that the agar is not cooled sufficiently for it to set. Swirl the resulting overlay mixture and leave in the water bath (5.3) until needed.

After the adsorption time, remove the flasks or Petri dishes from the incubator and carefully discard the inoculum, if necessary appropriately for infective material.