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Water quality — Detection and enumeration of bacteriophages —

Part 2: Enumeration of somatic coliphages

Qualité de l'eau — Détection et dénombrement des bactériophages — Partie 2: Dénombrement des coliphages somatiques

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this part of ISO 10705 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 10705-2 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

ISO 10705 consists of the following parts, under the general title *Water quality — Detection and enumeration of bacteriophages*:

— *Part 1: Enumeration of F-specific RNA bacteriophages*

— *Part 2: Enumeration of somatic coliphages*

— *Part 3: Concentration methods*

— *Part 4: Enumeration of bacteriophages infecting* Bacteroides fragilis

Annex A forms a normative part of this part of ISO 10705. Annexes B and C are for information only.

Water quality — Detection and enumeration of bacteriophages —

Part 2:
Enumeration of somatic coliphages

1 Scope

This part of ISO 10705 specifies a method for the detection and enumeration of somatic coliphages by incubating the sample with an appropriate host strain. The method is applicable to all kinds of water, sediments and sludge extracts, where necessary after dilution. The method is also applicable to shellfish extracts.

In the case of low phage numbers, a preconcentration step may be necessary for which a separate International Standard will be developed.

NOTE It is desirable for International Standards to be adopted as widely as possible. This part of ISO 10705 includes reference to alternative procedures which obviate the need for expensive materials or equipment which may not be readily available in developing countries. Use of these alternatives will not affect the performance of this method.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this part of ISO 10705. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 10705 are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 31-0:1992, *Quantities and units — Part 0: General principles*.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*.

ISO 5667-1:1980, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes*.

ISO 5667-2:1991, *Water quality — Sampling — Part 2: Guidance on sampling techniques*.

ISO 5667-3:1994, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of samples*.

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination*.

ISO 8199:1988, *Water quality — General guide to the enumeration of micro-organisms by culture*.

ISO/IEC Guide 2, *Standardization and related activities — General vocabulary*.

3 Terms and definitions

For the purposes of this part of ISO 10705, the terms and definitions given in ISO/IEC Guide 2 and the following apply.

3.1
somatic coliphage
bacterial virus which is capable of infecting selected *Escherichia coli* host strains (and related strains) by attachment to the bacterial cell wall as the first step of the infection process

NOTE Somatic coliphages produce visible plaques (clearance zones) in a confluent lawn of host bacteria grown under appropriate culture conditions.

4 Safety precautions

The host strain used in this standard is non-pathogenic to man and animals, and should be handled in accordance with the normal (national or international) safety procedures for bacteriological laboratories. Somatic coliphages are also non-pathogenic to man and animals, but some types are very resistant to drying. Appropriate precautions should therefore be taken to prevent cross-contamination of test materials, particularly when examining or handling cultures of high titre or when inoculating cultures of the host strain. Such procedures shall be carried out in a biohazard cabinet or a separate area of the laboratory.

Chloroform is a carcinogenic substance. Observe relevant safety precautions or use an alternative method of equal efficacy.

5 Principle

The sample is mixed with a small volume of semi-solid nutrient medium. A culture of host strain is added and plated on a solid nutrient medium. After this, incubation and reading of plates for visible plaques takes place. The results are expressed as the number of plaque-forming particles, pfp (also termed plaque-forming units, pfu), per unit of sample volume.

6 Diluent, culture media and reagents

6.1 Basic materials

Use ingredients of uniform quality and chemicals of analytical grade for the preparation of culture media and reagents and follow the instructions given in annex A. For information on storage see ISO 8199, except where indicated in this part of ISO 10705. Alternatively, use dehydrated complete media and follow strictly the manufacturer's instructions.

For the preparation of media, use glass-distilled water or deionized water free from substances which might inhibit bacterial growth under the conditions of the test, and complying with ISO 3696.

NOTE Use of other grades of chemicals is permissible providing they are shown to be of equal performance in the test.

6.2 Diluent

For making sample dilutions, use peptone-saline solution (A.7) or another diluent complying with ISO 6887.

7 Apparatus and glassware

Usual microbiological laboratory equipment, including

7.1 Hot-air oven for dry-heat sterilization and an autoclave. Apart from apparatus supplied sterile, glassware and other equipment shall be sterilized according to the instructions given in ISO 8199.

7.2 Incubator or water bath, thermostatically controlled at (36 ± 2) °C.

7.3 Incubator or water bath, thermostatically controlled at (36 ± 2) °C and equipped with a shaking device, for example a rotating platform at (100 ± 10) r/min.

7.4 Water bath or heating block, thermostatically controlled at (45 ± 1) °C.

7.5 Water bath or equivalent device for melting of agar media.

7.6 pH meter.

7.7 Counting apparatus with indirect, oblique light.

7.8 Deep freezer, thermostatically controlled at (–20 ± 5) °C.

7.9 Deep freezer, thermostatically controlled at (–70 ± 10) °C or liquid nitrogen storage vessel.

7.10 Spectrophotometer, capable of holding cuvettes of 1 cm optical path length or side-arm of nephelometric flasks (7.17) and equipped with a filter for the range 500 nm to 650 nm with a maximum bandwidth of ± 10 nm.

Usual sterile, microbiological laboratory glassware or disposable plasticsware according to ISO 8199 and including

7.11 Petri dishes of 9 cm or 14 cm to 15 cm diameter, vented.

7.12 Graduated pipettes of 0,1 ml, 1 ml, 5 ml and 10 ml capacity and **Pasteur pipettes**.

7.13 Glass bottles of suitable volume.

7.14 Culture tubes with caps or suitable alternative.

7.15 Measuring cylinders of suitable capacity.

7.16 Conical flasks of 250 ml to 300 ml capacity, with cotton wool plugs or suitable alternative.

7.17 Cuvettes of optical path length 10 mm or **nephelometric conical flasks** with cylindrical side-arms which fit in the spectrophotometer (7.10) (see Figure 1); capacity 250 ml to 300 ml with cotton wool plugs or suitable alternative.

7.18 Membrane filter units for decontamination, pore size 0,2 µm.

7.19 Plastics vials, lidded, of 1,5 ml to 3 ml capacity.

7.20 Refrigerator, temperature set at (5 ± 3) °C.

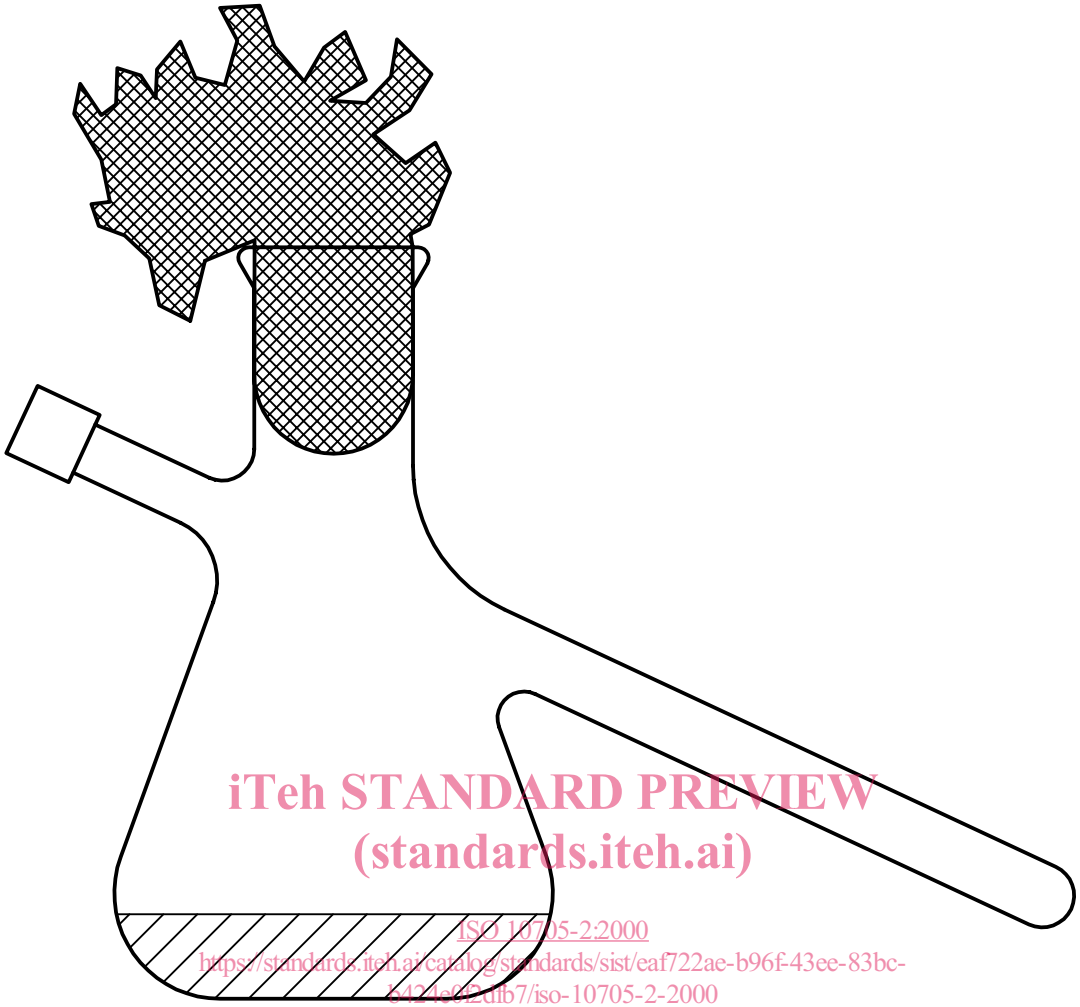

Figure 1 — Nephelometric conical flask for culturing the host strain

8 Microbiological reference cultures

For samples with low bacterial content (drinking water, unpolluted natural waters), use *Escherichia coli* strain C, ATCC 13706. Samples containing large numbers of bacteria (polluted natural waters, wastewater) should be examined using the nalidixic acid resistant mutant *E. coli* strain CN (ATCC 700078 [1]), also known as WG5 [2].

Use bacteriophage ϕX174 (ATCC 13706-B1) for the preparation of reference material (11.6.1).

NOTE The ATCC strains are available from the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110.

9 Sampling

Take samples and deliver them to the laboratory in accordance with ISO 8199, ISO 5667-1, ISO 5667-2 and ISO 5667-3.

10 Preparation of test material

10.1 Culturing and maintenance of host strains

10.1.1 General

The culturing and maintenance of host strains involves several stages which are summarized in Figure 2.

For culturing of the host strains in the several stages, it is best to gently shake the cultures. In addition to increasing the growth rate of bacteria, shaking ensures that all the cells are actively growing and no stationary-phase cells develop, which could decrease the efficiency of plating. Therefore, inoculum cultures should be repeatedly shaked by hand if a shaker is not available.

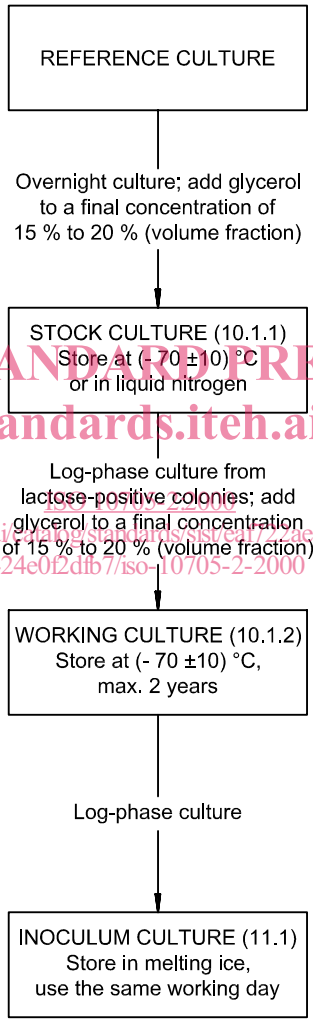

Figure 2 — Scheme for culture and maintenance of host strains

10.1.2 Preparation of stock cultures

Rehydrate the contents of a lyophilized ampoule of the reference culture of the host strains in a small amount (*ca.* 3 ml) of Modified Scholtens' Broth (M.S.B.) (A.1) using a Pasteur pipette (7.12). Transfer the suspension to a 300 ml conical flask (7.16) containing (50 ± 5) ml of MSB. Incubate for (20 ± 4) h at (36 ± 2) °C while gently shaking using an incubator or water bath (7.3). Add 10 ml [i.e. a final concentration of 15 % to 20 % (volume fraction)] of sterile glycerol (A.5) and mix well. Distribute into plastics vials (7.19) in *ca.* 0,5 ml aliquots and store at (–70 ± 10) °C or in liquid nitrogen.

NOTE This first passage of the host strains should be stored as a reference in the laboratory.

10.1.3 Preparation of working cultures

Remove a vial of stock culture (10.1.2) from frozen storage, allow to equilibrate to room temperature (15 °C to 30 °C) and inoculate on a plate of McConkey agar (A.6) or another lactose-containing medium in such a way that single colonies are obtained. Incubate at (36 ± 2) °C for (20 ± 4) h. The remaining content of the vial of stock culture can be used to inoculate more plates on the same working day (if necessary), otherwise it should be treated as contaminated waste.

Add (50 ± 5) ml of MSB to a conical flask of 300 ml (7.16) and warm to at least room temperature (faster growth will occur if the broth is prewarmed to 37 °C). Select three to five lactose-positive colonies from the McConkey agar and inoculate material from each of these colonies in the flask with MSB. Incubate for (5 ± 1) h at (36 ± 2) °C while gently shaking using an incubator or water bath (7.3). Add 10 ml of sterile glycerol (A.5) and mix well. Distribute in plastics vials (7.19) in *ca.* 1,2 ml aliquots and store in a deep freezer at (–70 ± 10) °C (7.9) for a maximum of two years.

NOTE If a great number of tests is anticipated, several conical flasks can be inoculated in parallel.

10.2 Calibration of absorbance measurements for counts of viable microorganisms

Remove a vial of working culture from the deep freeze (7.9) and allow to equilibrate to room temperature (15 °C to 30 °C). Add (50 ± 5) ml of MSB to a nephelometric conical flask (7.17), warm to at least room temperature (faster growth will occur if the broth is prewarmed to 37 °C). Adjust the spectrophotometer reading to zero on the filled flask side-arm. Alternatively, add (50 ± 5) ml of MSB (A.1) to a plain conical flask (7.16) and aseptically transfer a portion to a cuvette (7.17). Using this cuvette, adjust the spectrophotometer reading to zero. Discard the broth transferred to the cuvettes used to measure absorbance.

Inoculate MSB with 0,5 ml of working culture. Incubate at (36 ± 2) °C with gentle shaking in an incubator or water bath (7.3) for up to 3,5 h. Every 30 min measure absorbance as indicated above and withdraw a 1 ml aliquot for viable counts, ensuring that the flask is removed from the incubator for as short a time as possible.

Dilute aliquots to 10^{-7} and count colony-forming units (cfu) in 1 ml volumes of the 10^{-5}, 10^{-6} and 10^{-7} dilutions by the standard pour-plate procedure in nutrient agar or Modified Scholtens' Agar (MSA) (A.2.1), in duplicate. Alternatively, perform membrane filtration with 1 ml volumes of the same dilutions and count cfu by the standard membrane filter procedure on nutrient agar or MSA (A.2.1), in duplicate. Incubate at (36 ± 2) °C for (20 ± 4) h (using 7.2). Count the total number of colonies in/on each plate yielding between 30 and 300 colonies and calculate the number of cfu/ml (consult ISO 8199 if necessary).

NOTE 1 This procedure should be carried out several times (approx. two to three times) to establish the relationship between absorbance measurements and colony counts. Once sufficient data have been obtained, further work can then be based only on absorbance measurements.

NOTE 2 If the cell density of approx. 10^8 cfu/ml is not reached within 3,5 h of incubation, 1 ml of working culture may be inoculated instead of 0,5 ml.

11 Procedure

11.1 Preparation of inoculum cultures

Remove a vial of working culture from the deep freeze (7.9) and allow to equilibrate to room temperature (15 °C to 30 °C). Add (50 ± 5) ml of MSB to a nephelometric conical flask (7.17) or plain conical flask (7.16), and prewarm to at least room temperature (faster growth will occur if the broth is prewarmed to 37 °C). Adjust the spectrophotometer reading to zero as indicated in 10.2.

Inoculate 0,5 ml of working culture into MSB. Incubate at (36 ± 2) °C with gentle shaking in an incubator or water bath (7.3). Measure absorbance every 30 min as indicated in 10.2. At an absorbance corresponding to a cell density of

approximately 10^8 cfu/ml (based on data obtained in 10.2), take the inoculum culture from the incubator and quickly cool the culture by placing it in melting ice. Use the inoculum culture within the same working day.

NOTE An alternative (but less controlled) way to prepare an inoculum culture is the following:

Inoculate 0,5 ml of working culture, thawed as indicated above, into (50 ± 5) ml of MSB prewarmed at room temperature. Incubate for (3 ± 1) h at (36 ± 2) °C with gentle shaking. Alternatively, inoculate typical colonies from an agar plate, or a loopful of growth from an agar slant [incubated for not longer than (20 ± 4) h at (36 ± 2) °C and stored at (5 ± 3) °C for not longer than a working day], into (50 ± 5) ml of MSB prewarmed at room temperature and incubate for (3 ± 1) h at (36 ± 2) °C with gentle shaking. Use immediately or take the inoculum culture from the incubator and quickly cool to 5 °C to 10 °C, preferably by placing onto melting ice. Use this inoculum culture within the same working day. Whatever the preparation procedure may be, the inoculum culture should ideally have a count of approximately 10^8 cfu per ml.

11.2 Standard procedure

Prepare an inoculum culture as described in 11.1.

Melt bottles of 50 ml semi-solid Modified Scholtens' Agar (ssMSA) (A.3) in a boiling water bath (7.5) and place in a water bath at (45 ± 1) °C. Aseptically add 300 µl of a calcium chloride solution (A.2.2) prewarmed at room temperature and distribute 2,5 ml aliquots into culture tubes (7.14) with caps, placed in a water bath at (45 ± 1) °C.

To each culture tube, add 1 ml of the original sample (or diluted or concentrated sample) prewarmed at room temperature. Examine each aliquot at least in duplicate.

Add 1 ml of inoculum culture to each culture tube containing the aliquots of sample and ssMSA, mix carefully avoiding the formation of air bubbles and pour the contents on a layer of complete MSA (A.2.3) in a 9 cm Petri dish prewarmed at room temperature. Distribute evenly and allow to solidify on a horizontal, cool surface. Dry the plates by incubating with partially opened lids, then cover and incubate the plates upside-down at (36 ± 2) °C for (18 ± 2) h. Do not stack more than 6 plates.

Count the number of plaques on each plate within 4 h after finishing incubation, using indirect oblique light.

NOTE 1 If a great number of tests is anticipated, several conical flasks can be inoculated in parallel. In this case the contents of the different flasks should be mixed together and homogenized before analysis or, as an alternative, a reference control of ϕX174 should be carried out for each flask or inoculum culture.

NOTE 2 If necessary, plates can be read after 6 h of incubation. This may be useful if a preliminary count is required and also if high background of contaminating bacterial colonies is expected. If a reading is taken after 6 h, this should be noted when expressing the results in clause 12.

NOTE 3 Freshly prepared triphenyltetrazolium chloride solution (A.3) can be added to ssMSA to enhance contrast for counting plaques.

NOTE 4 The addition of sample and ice-cold host culture to the semi-solid agar may lead to a sharp drop in temperature and solidification of the medium. Ensure a sufficient time interval between these two steps to allow reheating. However, make sure that inoculated tubes remain in the water bath (45 ± 1) °C for not more than 10 min.

11.3 Method for samples with high bacterial background flora

Proceed according to 11.2.

Add nalidixic acid to ssMSA (A.3) to give a final concentration of 250 µg/ml. Use *E. coli* CN as the inoculum culture.

NOTE Nalidixic acid is heat-stable. It can either be added from a filter-sterilized solution after melting of soft agar, or can be added before autoclaving.

11.4 Samples with low phage counts

Proceed according to 11.2 but use the following modifications:

— 10 ml of ssMSA, 60 µl of calcium chloride solution, 1 ml of host culture and 5 ml of sample in duplicate.

