INTERNATIONAL STANDARD

ISO 10705-4

First edition
2001-12-15

Water quality — Detection and enumeration of bacteriophages —

Part 4: Enumeration of bacteriophages infecting *Bacteroides fragilis*

Qualité de l'eau — Détection et dénombrement des bactériophages —

Partie 4: Dénombrement des bactériophages infectant Bacteroides fragilis

Reference number
ISO 10705-4:2001(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.ch
Web www.iso.ch

Printed 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.

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 10705-4 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: Validation of methods for concentration of bacteriophages from water*

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

Annexes A, B, C and D of this part of ISO 10705 are for information only.

Water quality — Detection and enumeration of bacteriophages —

Part 4:
Enumeration of bacteriophages infecting *Bacteroides fragilis*

1 Scope

This part of ISO 10705 specifies a method for the detection and enumeration of bacteriophages infecting *Bacteroides fragilis* 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. In the case of low phage numbers, a pre-concentration step may be necessary for which a separate International Standard has been developed. The method is also applicable to shellfish extracts.

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 3696, *Water for analytical laboratory use — Specification and test methods*

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

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

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

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

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

3 Term and definition

For the purposes of this part of ISO 10705, the following term and definition applies.

3.1
bacteriophage infecting *Bacteroides fragilis*
bacterial virus which is capable of infecting selected *Bacteroides fragilis* host strains by attachment to the bacterial cell wall as the first step of the infectious process

NOTE 1 Such bacteriophages produce visible plaques (clearance zones) in a confluent lawn of host bacteria grown under appropriate culture conditions.

NOTE 2 A general description of bacteriophages infecting *B. fragilis* is given in annex A.

4 Safety precautions

The host strain used in this part of ISO 10705 is non-pathogenic to man and animals and should be handled in accordance with the normal (national or international) safety procedures for bacteriological laboratories. Bacteriophages infecting *Bacteroides fragilis* are non-pathogenic for man and animals, but some types are very resistant to drying. Appropriate precautions shall 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 strains. 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.

It is recommended that personnel using this method have or acquire some experience in handling anaerobic bacteria.

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 plaques take place. The results are expressed as the number of plaque-forming particles (also named plaque-forming units, pfu) per unit of volume (pfp/ml, pfp/l, etc.).

6 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 B. 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.

Other grades of chemicals may be used provided they can be shown to lead to the same results.

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

6.3 Diluent, for making sample dilution, such as peptone-saline solution or another diluent complying with ISO 6887-1.

7 Apparatus

Apart from apparatus supplied sterile, sterilize any glassware and other equipment in accordance with ISO 8199.

Usual sterile, microbiological laboratory equipment and glassware or disposable plastics-ware in accordance with ISO 8199 and including the following:

7.1 Hot-air oven, for dry-heat sterilization, and an **autoclave**.

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

7.3 Incubator or **water bath**, thermostatically maintained at (36 ± 2) °C with a shaking device.

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

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

7.6 pH meter, and **pH paper**.

7.7 Counting apparatus, with indirect, oblique light.

7.8 Deep freezer, thermostatically maintained at (-20 ± 5) °C.

7.9 Deep freezer, thermostatically maintained at (-70 ± 10) °C or **liquid nitrogen storage vessel**.

7.10 Spectrophotometer, equipped with a filter for the range of 500 nm to 650 nm with a maximum bandwidth of $\pm$ 10 nm, capable of holding cuvettes (7.21) having an optical path length of 1 cm or Hungate glass tubes (7.20) with butyl rubber stopper and screw cap or screw-capped glass culture tubes.

7.11 Anaerobic cabinet, or **jars** or **bags**, as well as **anaerobiosis generators** and **anaerobiosis indicators**.

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

7.13 Petri dishes, having a diameter of 9 cm, and vented.

7.14 Graduated pipettes, having a capacity of 0,1 ml, 1 ml, 5 ml and 10 ml and **Pasteur pipettes**.

7.15 Glass bottles, of suitable volumes.

7.16 Screw-capped glass bottles, of suitable volumes.

7.17 Culture tubes, with caps or suitable alternatives.

7.18 Screw-capped glass culture tubes.

7.19 Measuring cylinders, of suitable capacity.

7.20 Hungate glass tubes, with butyl rubber stopper and screw cap or screw-capped glass culture tubes which can fit in the spectrophotometer (see Figure 1).

7.21 Cuvettes, having an optical path length of 1 cm.

7.22 Membrane filter units, for decontamination, having a pore size of 0,2 µm, preferably low protein-binding membranes, as for example, those composed of polyvinylidene difluoride.

7.23 Plastics vials, lidded, having a capacity of 3 ml.

7.24 Glass vials, screw-capped, having a capacity of 3 ml.

7.25 Syringes and **needles**.

7.26 Cotton swabs.

8 Microbiological reference cultures

The recommended host strain is *Bacteroides fragilis* RYC2056 (ATCC 700786).[1]

Use bacteriophage B56-3 (ATCC 700786-B1) infecting *Bacteroides fragilis* RYC2056 for the preparation of reference materials (11.4).

NOTE The ATCC strains are available from American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, USA. This information is given for the convenience of users of this part of ISO 10705 and does not constitue an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

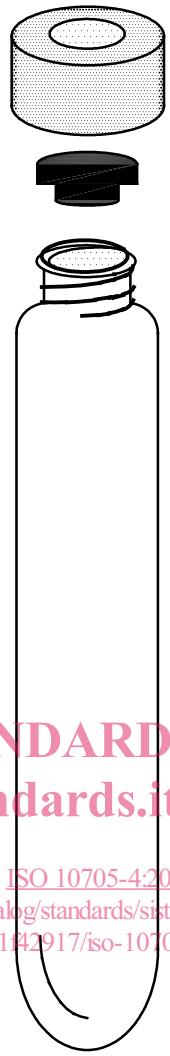

Figure 1 — Hungate glass tube with rubber stopper and screw cap

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 materials

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.

Bacteroides fragilis is an obligate anaerobe. However, it does not require handling under conditions of strict anaerobiosis. Incubation of cultures in solid media should be carried out in an anaerobic cabinet, or anaerobic jars or bags. When using liquid media it is sufficient to ensure that containers are completely filled and closed with a screw cap.

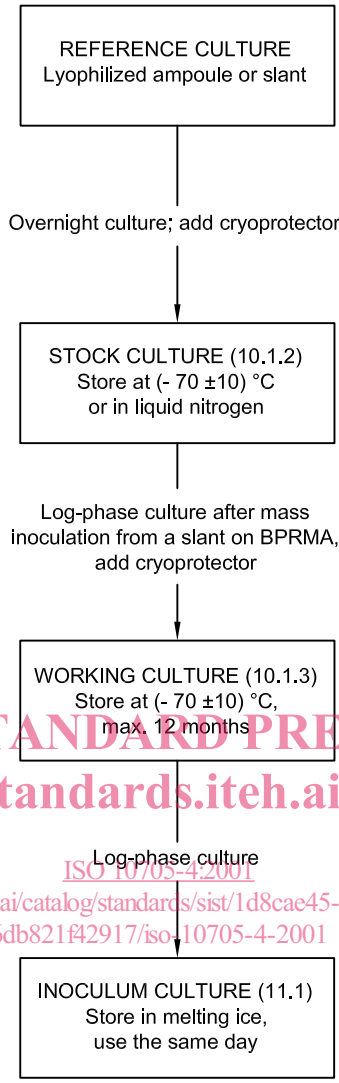

Figure 2 — Scheme for the culturing and maintenance of host strains

10.1.2 Preparation of stock cultures

Rehydrate the content of a lyophilized ampoule of the reference culture of the host strain in 1 ml of *Bacteroides* phage recovery medium broth (BPRMB) (B.1) using a Pasteur pipette (7.14). Inoculate the suspensions in 10 ml of BPRMB (B.1) in a 10 ml screw-capped glass tube (see 10.1.1) and incubate at (36 ± 2) °C for (21 ± 3) h. Aseptically soak a sterile cotton swab with the culture and streak it onto a plate of *Bacteroides* phage recovery medium agar (BPRMA) (B.2). Incubate the culture in an anaerobic cabinet, jar or bag at (36 ± 2) °C for (44 ± 4) h.

Alternatively, if a culture in slant is available, streak it with a sterile cotton swab directly onto a plate of BPRMA (B.2). Incubate the culture in an anaerobic cabinet, jar or bag at (36 ± 2) °C for (44 ± 4) h.

Inoculate cells (mass inoculation with a sterile cotton swab) from the plate into 10 ml of BPRMB (B.1) in a 10 ml screw-capped glass tube (7.18). Be sure that the tube is completely filled (see 10.1.1). If dense growth occurs, inoculate $1/8$ of the growth onto the BPRMA plate; if poor growth occurs, use $1/2$ of the growth onto the plate. Incubate the culture at (36 ± 2) °C for (21 ± 3) h.

Mix culture and cryoprotector (B.6) in a ratio 1:1 (volume). Mix well avoiding bubble formation. Distribute into screw-capped, preferably glass, vials (7.24) in aliquots of approximately 1,0 ml and store at (-70 ± 10) °C or in liquid nitrogen for up to five years.

This first passage of the host strain should be stored as a reference in the laboratory. Purity of the culture should be checked before storage by Gram staining, by testing absence of growth under aerobic conditions and by testing sensitivity to a reference bacteriophage (i.e. B56-3).

10.1.3 Preparation of working cultures

Remove a vial of stock culture (10.1.2) from frozen storage, allow the vial to equilibrate to room temperature (i.e. 15 °C to 30 °C) and streak the culture with a sterile cotton swab onto a plate of BPRMA (B.2). Incubate the culture in an anaerobic cabinet, jar or bag at (36 ± 2) °C for (44 ± 4) h. Inoculate cell material (mass inoculation with a sterile cotton swab) from the plate into 10 ml of prewarmed BPRMB (B.1) kept in a 10 ml screw-capped glass tube (see 10.1.1). If dense growth occurs, inoculate 1/8 of the growth onto the BPRMA plate; if poor growth occurs, use 1/2 of the growth onto the plate. Incubate the culture at (36 ± 2) °C for (21 ± 3) h.

Add BPRMB (B.1) to a tube for anaerobic cultures (7.18) and warm to at least room temperature [faster growth will occur if the broth is prewarmed to (36 ± 2) °C]. Transfer an aliquot of the above-mentioned culture, without shaking the tube and taking the aliquot from the middle part of the tube, to the tube containing prewarmed BPRMB in a ratio of culture-to-BPRM of 1,5:10 (volume). Be sure that the inoculated tube is completely filled (see 10.1.1). Incubate the culture at (36 ± 2) °C to reach approximately 2×10^9 cfu/ml.

Mix working culture and cryoprotector (B.6) in a ratio of 1:1 (volume) avoiding bubble formation. Distribute into screw-capped, preferably glass, vials (7.24) in aliquots of approximately 1,5 ml and store at (70 ± 10) °C for a maximum of 12 months.

Ensure that the culture does not reach the stationary phase before mixing it with the cryoprotector. Absorbance stabilization will indicate the end of the log phase, which may last 5 h to 8 h.

10.2 Calibration of absorbance measurements for counts of viable host bacteria

Remove a vial of working culture (10.1.3) from the deep freeze (7.9) and allow the vial to equilibrate to room temperature (i.e. 15 °C to 30 °C). Add BPRMB (B.1) to a tube for anaerobic cultures (7.18) and warm to at least room temperature [faster growth will occur if the broth is prewarmed to (36 ± 2) °C]. Before inoculation, adjust the spectrophotometer to zero. Transfer the working culture into BPRMB (B.1) in a ratio of respectively 1:10 (volume), completely filling the tube. Tubes for anaerobic cultures may be inoculated/sampled by puncture with sterile syringes and needles (7.25). Incubate the culture at (36 ± 2) °C. Every 30 min, measure the absorbance (using 7.10) and withdraw, by puncture, a 0,3 ml sample for viable cell counts. Ensure that the tube is out of the incubator for as short a time as possible.

Melt 50 ml of semi-solid BPRMA (ssBPRMA) (B.3) (basal agar) by putting bottles in a boiling water bath. Then place the bottles in a water bath at (45 ± 1) °C. Aseptically add haemin solution, Na_2CO_3 and antibiotics and adjust pH to 6,8 ± 0,5 (B.1) according to Table B.1. Distribute 2,5 ml aliquots into culture tubes with caps, placed in a water bath at (45 ± 1) °C.

Dilute the aliquots sampled from the culture to 10^{-8} and add 1 ml volumes of the 10^{-6}, 10^{-7} and 10^{-8} dilutions to each tube of 2,5 ml of melted ssBPRMA, in duplicate. Pour onto a layer of BPRMA in a 90 mm Petri dish (B.2) prewarmed at room temperature. Distribute evenly, allow to solidify on an horizontal, cool surface and incubate the plates upside down in an anaerobic cabinet, jar or bag at (36 ± 2) °C for (44 ± 4) h. Ensure that the process is performed in a period of time as short as possible and that the diluents had been autoclaved immediately just before use (to have them free of oxygen). Be sure that the diluents have cooled to room temperature before use. Count the total number of colonies in each plate yielding between 30 colonies and 300 colonies and calculate the number of cfu/ml (consult ISO 8199 if necessary).

This procedure should be carried out several times (approximately 4 to 5 times) to establish the relationship between absorbance measurements and colony counts. If sufficient data have been obtained, further work can then be based only on absorbance measurements.

11 Procedure

11.1 Preparation of inoculum cultures

Remove a vial of working culture (10.1.3) from the deep freeze (7.9) and allow the vial to equilibrate to room temperature, (i.e. 15 °C to 30 °C). Add BPRMB (B.1) to a screw-capped tube and warm to at least room temperature [faster growth will occur if the broth is prewarmed to (36 ± 2) °C]. Before inoculation, adjust the spectrophotometer to zero for the tube (10.2). Transfer the working culture into the tube filled with BPRMB (B.1) in a ratio of 1:10 (volume). Be sure that the tube is completely filled (see 10.1.1). Incubate the culture at (36 ± 2) °C. After 2 h, measure the absorbance of the culture every 30 min. At an absorbance corresponding to a cell density of approximately 2×10^8 cfu/ml (based on data obtained in 10.2) take the inoculum culture from the incubator and either use it immediately or quickly cool the culture by placing it in melting ice. Use the culture within 6 h. Cell densities ranging from 1×10^8 cfu/ml to 4×10^8 cfu/ml give similar plaque counts, however cell densities of 1×10^8 cfu/ml or 2×10^8 cfu/ml give larger plaques.

An alternative method for preparing inoculum cultures is given in annex C.

NOTE If the cell density of approximately 2×10^8 cfu/ml is not reached within 3 h, it is possible to increase the amount of working culture transferred into the BPRMB to a ratio of respectively 1,5:10 (volume).

11.2 Standard procedure

Prepare an inoculum culture as described in 11.1. Allow sample to equilibrate to room temperature (15 °C to 30 °C). Melt 50 ml of ssBPRMA (B.3) (basal agar) by putting the bottles in a boiling water bath (7.5) then place them in a water bath at (45 ± 1)° C. Aseptically add haemin, Na_2CO_3 and antibiotics and adjust the pH to 6,8 ± 0,5 (B.1) according to Table B.1. Distribute 2,5 ml aliquots into culture tubes with caps (7.17), which are placed in a water bath at (45 ± 1) °C.

To each culture tube (7.17), add 1 ml of sample (or diluted or concentrated sample) prewarmed to 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 ssBPRMA, then mix carefully avoiding the formation of air bubbles and pour the contents on a layer of complete BPRMA (B.2) in a 90 mm Petri dish prewarmed to room temperature. Distribute the contents evenly allowing the agar to solidify on a horizontal, cool surface. *B. fragilis* is an anaerobic bacterium. Therefore, once dried, distribute inoculated tubes as fast as possible and place the plates into the anaerobic jars as soon as possible. Incubate the plates upside down in an anaerobic cabinet, jar or bag at (36 ± 2) °C for (21 ± 3) h.

After incubation, count the number of plaques on each plate. If it is not possible to count the plates after finishing incubation, keep the plates at (5 ± 3) °C until reading.

For samples containing high background flora, it is recommended to decontaminate the samples by filtration through low protein-binding membranes, as for example, those composed of polyvinylidene difluoride 0,2 μm pore size (7.22), or to increase the amount of ssBPRMA kanamycine monosulfate to obtain a final concentration in the final medium of 300 μg ml^{-1} instead of 100 μg ml^{-1} (the concentration recommended for growing *B. fragilis*).

NOTE 1 Freshly prepared triphenyltetrazoliumchloride solution (B.10) can be added to enhance contrast for counting plaques. If this procedure is used, plates should remain under aerobic conditions for 1 h to 2 h before counting plaques.

NOTE 2 The addition of ice-cold inoculum culture (11.1) to the ssBPRMA may lead to a sharp drop in temperature and solidification of the medium. To avoid this, prewarm the ice-cold inoculum culture to room temperature before adding it to the tubes containing ssBPRMA and the aliquot of the sample.