BRITISH STANDARD

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Water quality — Detection and enumeration of bacteriophages —

Part 4: Enumeration of bacteriophages infecting *Bacteriodes fragilis*

ICS 07.100.20

BSi
British Standards

National foreword

This British Standard reproduces verbatim ISO 10705-4:2001 and implements it as the UK national standard.

The UK participation in its preparation was entrusted by Technical Committee EH/3, Water quality, to Subcommittee EH/3/4, Microbiological methods, which has the responsibility to:

— aid enquirers to understand the text;

— present to the responsible international/European committee any enquiries on the interpretation, or proposals for change, and keep the UK interests informed;

— monitor related international and European developments and promulgate them in the UK.

A list of organizations represented on this subcommittee can be obtained on request to its secretary.

Cross-references

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Summary of pages

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INTERNATIONAL STANDARD

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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)

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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.

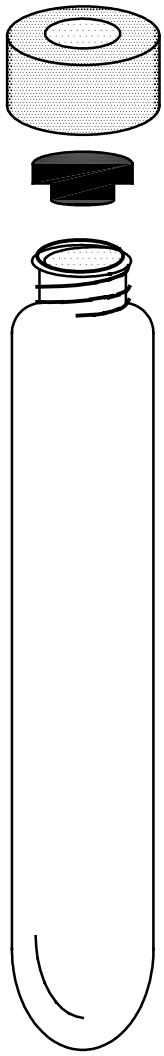

Figure 1 — Hungate glass tube with rubber stopper and screw cap

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.

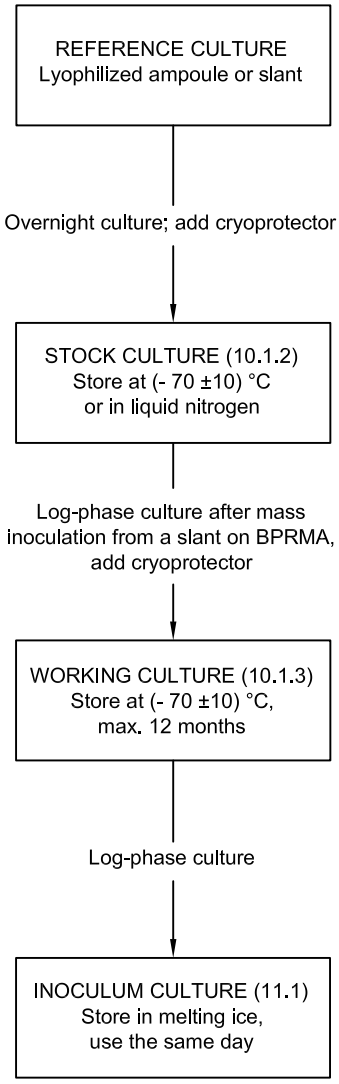

Figure 2 — Scheme for the culturing and maintenance of host strains

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.

11.3 Presence/absence test

This test can be used to examine the presence of bacteriophages in different volumes of sample. For instance, to determine the presence/absence in 100 ml, proceed as follows:

Add 100 ml of double-strength BPRMB (B.9) to a 250 ml screw-capped sterile glass bottle and prewarm at least to room temperature [faster growth will occur if the broth is prewarmed to (36 ± 2) °C]. Add 100 ml of the sample prewarmed to room temperature. Add 30 ml of an inoculum culture, in its exponential (log) growth phase, containing approximately 2×10^8 cells/ml to 5×10^8 cells/ml (11.1). To ensure anaerobic conditions it is necessary to fill the bottle completely with medium and to tighten the cap if the bottle is incubated under aerobic conditions. Incubate the culture at (36 ± 2) °C for (21 ± 3) h. Gentle magnetic stirring during incubation is recommended.

To avoid the toxic effect of oxygen dissolved in the sample on the host cells, mainly for the presence/absence test, it is recommended to treat the sample to remove oxygen. This can be achieved by either bubbling nitrogen for 5 min at a rate of 5 l/min or by the addition of a reducing solution, such as Na_2S (final mass concentration 0,04 %). By adding a resazurine solution (0,5 ml/100 ml of a solution having a concentration of 0,025 g/100 ml), anaerobiosis is indicated by a change of colour from blue to straw.

Melt 50 ml of ssBPRMA (basal medium) (B.3) by putting the bottles in a boiling water bath (7.5). Then place them in a water bath (7.4) at (45 ± 1) °C. Aseptically add haemin, Na_2CO_3 and antibiotics and adjust pH to 6,8 ± 0,5 (B.1) as indicated in Table B.1. Distribute 2,5 ml aliquots into culture tubes with caps, which are placed in a water bath at (45 ± 1) °C. To each tube add 1 ml of inoculum culture, in its exponential (log) growth phase, containing approximately 2×10^8 bacteria/ml (11.1). Mix carefully to avoid the formation of air bubbles and pour the contents onto a layer of 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.

Transfer 1 ml of the enrichment culture to a centrifuge tube, add 0,4 ml of chloroform, mix well and centrifuge at 3 000 g for 5 min. Place one drop of the supernatant from each one of the chloroform-treated cultures onto the inoculated plates using a fine capillary or a pipette. Do not damage the top agar layer. Leave the spot to dry and incubate the plates upside down in an anaerobic cabinet, jar or bag at (36 ± 2) °C for (21 ± 3) h.

Examine the plate for a clear zone in the spotted area, which is indicative of the presence of *B. fragilis* phages in the original sample.

This procedure will result in high-titre phage suspensions. Take appropriate precautions, for example working in a biohazard cabinet or in a separate area of the laboratory, always disinfecting the work area by wiping over with 70 % alcohol or a hypochlorite solution at the end of each work session.

NOTE This procedure can also be used in an MPN format (ISO 8199). More than one spot can be placed on the surface of an inoculated plate.

11.4 Quality assurance

11.4.1 Plaque count procedure

For each series of samples examine, a procedural-blank using sterile diluent as the sample and a reference standard preparation of bacteriophage B56-3, prepared as follows:

From a high titre phage culture (e.g. as described in annex D), prepare a decimal dilution series and plate out according to 11.2. Store the dilution series in a refrigerator overnight. Count the number of plaques. From the dilution series, prepare 100 ml to 1 000 ml of a suspension with an expected concentration of plaque-forming particles of approximately 100/ml. Add 5 % (volume) of glycerol (B.7). Distribute into plastic vials in 2,4 ml aliquots and store at (-70 ± 10) °C. Thaw vials of the reference control of B56-3 before use and plate out according to the procedure used (11.2). Plot the results on a control chart. Discard the reference control sample if the mean number of pfp/ml decreases.

Optionally, use a naturally polluted reference control sample in addition, using settled sewage filtered through a 0,2 μm pore size polyvinylidene membrane (7.22) with a concentration of plaque-forming particles ranging from 20/ml to 50/ml and 5 % (volume) glycerol, and store at (-70 ± 10) °C. Discard the reference control samples if the concentration of phages decreases and remains low following a re-test.

11.4.2 Presence/absence test (11.3)

With each series of samples, examine a procedural-blank using sterile diluent as the sample and a reference standard sample, prepared as described in 11.4.1, with a concentration of plaque-forming particles of approximately 5/ml to 10/ml. Examine at least one control sample in parallel with each series of samples tested and expect to obtain a positive test result. To examine possible interfering effects from the samples, consider also adding the reference control sample to a second enrichment culture containing the actual sample.

NOTE In the absence of available standardized reference materials, any exchange programme of reference samples between laboratories or other interlaboratory tests is to be encouraged.

12 Expression of results

12.1 Plaque count procedures (11.2)

Select plates with well-separated, and preferably more than 30, plaques, whenever present. If only counts below 30 per plate are found, select plates inoculated with the largest volume of sample. From the number of plaques counted, calculate the number of plaque-forming particles of bacteriophages infecting *Bacteroides fragilis* in 1 ml of the sample as follows.

$$n_{\text{pfp}} = \frac{N}{(n_1\, V_1\, F_1) + (n_2\, V_2\, F_2)}$$

where

n_{pfp} is the number of plaque-forming particles of bacteriophages infecting *Bacteroides fragilis* per millimetre;

N is the total number of plaques counted on plates in accordance with 11.2;

n_1, n_2 are the numbers of replicates counted for dilution F_1, F_2, respectively;

V_1, V_2 are the test volumes used with dilution F_1, F_2, respectively;

F_1, F_2 are the dilution or concentration factors used for the test portions V_1, V_2, respectively ($F = 1$ for an undiluted sample, $F =$ 0,1 for a ten-fold dilution, $F = 10$ for a ten-fold concentrate, etc.).

If only one dilution/concentrate is counted, simplify the formula to

$$n_{\text{pfp}} = \frac{N}{nVF}$$

Refer to ISO 8199 for further details.

12.2 Presence/absence test (11.3)

Express the results as bacteriophages infecting *Bacteroides fragilis* (not) detected in the volume of sample examined, expressed in millimetres.

13 Test report

The test report shall contain the following information:

a) a reference to this part of ISO 10705, i.e. ISO 10705-4;

b) all details necessary for the complete identification of the sample;

c) the inoculation procedure used;

d) the incubation time, if different from the standard time in clause 11;

e) the results, expressed in accordance with clause 12;

f) any other information relevant to the method.

Annex A
(informative)

General description of bacteriophages infecting *Bacteroides fragilis*

The most abundant bacteriophages infecting *Bacteroides fragilis*, one of the most abundant bacteria in the gut, belong to the family *Siphoviridae* with a flexible tail (dsDNA, long non-contractile tails, capsides up to 60 nm). Phages infecting *Bacteroides fragilis* are virulent phages which attach to molecules in the cell wall of the host bacteria and may lyse the host cell in 30 min to 40 min under optimal conditions. They produce clear plaques which do not differ very much in size and morphology.

The presence of phages infecting *Bacteroides fragilis* RYC 2056 in a water sample indicates pollution by human or animal faeces or by wastewaters containing these excreta. Bacteriophages infecting strain RYC 2056 have been detected in similar concentrations in raw sewage samples from different parts of the world. In the great majority of analysed sewage samples, the ratio of the numbers of somatic coliphages to the numbers of bacteriophages infecting RYC2056 ranges from 100 to 200 and the ratio of the numbers of F-specific RNA bacteriophages to the number of bacteriophages infecting RYC2056 ranges from 10 to 20. These ratios are normally one $\log_{10}$ higher than in abattoir sewage. Bacteriophages infecting *B. fragilis* have not been reported to be able to multiply under environmental conditions, and they are quite resistant to natural inactivation as well as most water disinfection procedures. Thus their behaviour resembles that of human enteric viruses more closely than faecal bacteria commonly used as quality indicators.

This method has been proven to be applicable to other strains as for example *Bacteroides fragilis* HSP40 (ATCC 51477)[2]. Bacteriophages infecting *B. fragilis* HSP40 have been said to indicate specifically faecal pollution of human origin. However the concentrations of phages infecting strain HSP40 are very low in sewages of some geographical areas.

Annex B
(informative)

Culture media and diluents

B.1 *Bacteroides* phage recovery medium broth (BPRMB)

B.1.1 Basal broth

Meat peptone	10 g
Casein peptone	10 g
Yeast extract	2 g
NaCl	5 g
Monohydrated L-cysteine	0,5 g
Glucose	1,8 g
$MgSO_4 \cdot 7H_2O$	0,12 g
$CaCl_2$ solution (0,05 g/ml, see below)	1 ml
Distilled water	1 000 ml

Dissolve the ingredients in hot water. Add the $CaCl_2$ solution, mix well and distribute the medium in bottles in volumes of e.g. 200 ml. Sterilize in an autoclave at (121 ± 3) °C for 15 min. Store in the dark at (5 ± 3) °C for no longer than 1 week once the recipient containing the broth has been opened. It can be stored for one month if the recipient is not opened. If stored longer, bring it to the boil before use.

B.1.2 Calcium chloride solution (0,05 g/ml)

$CaCl_2 \cdot 2H_2O$	5 g
Distilled water	100 ml

Dissolve the calcium chloride in the water while heating gently. Cool to room temperature and filter-sterilize through a 0,2 μm pore size membrane filter. Store in the dark at (5 ± 3) °C for no longer than 6 months.

B.1.3 Haemin solution

Haemin	0,1 g
NaOH solution (1 mol/l)	0,5 ml
Distilled water	99,5 ml

Dissolve the ingredients in the water by magnetic stirring (can last 30 min to 60 min). Filter-sterilize through a 0,2 μm pore size membrane filter, or sterilize in the autoclave at (121 ± 3) °C for 15 min. Store at room temperature for no longer than 6 months.

B.1.4 Disodium carbonate solution ($<$ 1 mol/l)

Na_2CO_3	10,6 g
Distilled water	100 ml

Dissolve disodium carbonate in the water. Filter-sterilize through a 0,2 μm pore size membrane filter. Store at room temperature for no longer than 6 months.

B.1.5 Complete broth

Basal broth	1 000 ml
Haemin solution	10 ml
Disodium carbonate solution	25 ml

Aseptically add the additives to the basal broth and mix by gentle shaking (to prevent oxygenation of the broth). Adjust to pH 6,8 $\pm$ 0,5 by aseptically adding 2,5 ml of HCl (35 % mass fraction). Use immediately.

To prevent contamination, it is recommended to add kanamycin monosulfate (final mass concentration of 100 μg/ml) and nalidixic acid (final mass concentration of 100 μg/ml) to the complete broth. For preparation of the antibiotic solutions see B.4 and B.5. Add 1,0 ml kanamycin monosulfate (B.4) and 4,0 ml nalidixic acid (B.5) to 1 000 ml complete medium.

B.2 *Bacteroides* phage recovery medium agar (BPRMA)

B.2.1 Basal agar

Basal broth (B.1; non-sterilized)	1 000 ml
Agar	12 g to 20 g[a]
[a] Depending on the gel strength of the agar	

Mix the basal broth and the agar while heating. Distribute the medium into bottles, in volumes of e.g. 200 ml, and sterilize in an autoclave at (121 ± 3) °C for 15 min. Cool to between 45 °C and 50 °C and add the additives (see below).

B.2.2 Complete agar

Basal agar (molten from 45 °C to 50 °C)	1 000 ml
Haemin solution (B.1.3)	10 ml
Disodium carbonate solution (B.1.4)	25 ml

Aseptically add the additives, mix well. Adjust to pH 6,8 $\pm$ 0,5 by aseptically adding 2,5 ml of HCl (35 % mass fraction). Pour into Petri dishes (20 ml in dishes of 9 cm diameter). Allow to solidify and store in the dark at (5 ± 3) °C for no longer than one month. Place the plates at room temperature for 1 h to 2 h before use to dry them.

To prevent contamination, it is recommended to always add kanamycin monosulfate (final mass concentration of 100 μg/ml) and nalidixic acid (final mass concentration of 100 μg/ml) to the complete agar. For preparation, see B.4 and B.5. Add 1,0 ml kanamycin monosulfate (B.4) and 4,0 ml nalidixic acid (B.5) to 1 000 ml complete medium.

B.3 Semi-solid *Bacteroides* phage recovery medium agar (ssBPRMA)

Prepare basal agar according to B.2 but use half of the mass of the agar (6 g to 10 g), depending on gel strength. The gel strength of ssBPRMA is critical to obtain good results and if possible different concentrations should be tested.

Choose the agar concentration that produces the highest plaque counts but also controls plaque-size to reduce confluence. Distribute into bottles in volumes of 50 ml, allow the agar to solidify and store it at (5 ± 3) °C.

Before use, melt the ssBPRMA by putting the bottles in a boiling water bath, then cool them to a temperature between 45 °C and 50 °C. Aseptically add haemin, Na_2CO_3, antibiotics and adjust the pH to 6,8 ± 0,5 (see B.2).

To prevent contamination, it is recommended to always add kanamycin monosulfate (final mass concentration of 100 μg/ml) and nalidixic acid (final mass concentration of 100 μg/ml) to the complete agar. For preparation, see B.4 and B.5. Add 1,0 ml kanamycin monosulfate (B.4) and 4,0 ml nalidixic acid (B.5) to 1 000 ml complete medium.

B.4 Kanamycin monosulfate

Kanamycin monosulfate	1,25 g
Distilled water	10 ml

Many supplies of kanamycin monosulfate contain less than 100 % active kanamycin. Make the necessary corrections to reach 1,25 g of active kanamycin monosulfate to 10 ml of water. Dissolve the ingredient in the water and mix well. Filter-sterilize through a 0,2 μm pore size filter. Store at (5 ± 3) °C for no longer than 8 h or at (-20 ± 5) °C for no longer than six months.

B.5 Nalidixic acid solution

Nalidixic acid	250 mg
NaOH solution (1 mol/l)	2 ml
Distilled water	8 ml

Dissolve the nalidixic acid in the NaOH solution, then add distilled water and mix well. Filter-sterilize through a 0,2 μm pore size filter. Store at (5 ± 3) °C for no longer than 8 h or at (-20 ± 5) °C for no longer than six months.

B.6 Cryoprotector (carrier) - BSA + sucrose

Bovine serum albumin, fraction V (BSA)	10 g
Sucrose	20 g
Distilled water	100 ml

Dissolve the ingredients in the water using a magnetic stirrer for approximately 1 h. Filter-sterilize through a 0,2 μm pore size filter. Filtration can be difficult. Use immediately.

B.7 Glycerol (sterile)

Glycerol (870 g/l)	100 ml

Distribute in 20 ml volumes and sterilize in an autoclave at (121 ± 3) °C for 15 min. Store in the dark for no longer than 1 year.

B.8 Peptone saline solution

Peptone	1,0 g
Sodium chloride	8,5 g
Distilled water	1 000 ml

Dissolve the ingredients in hot water. Adjust the pH to 7,0 $\pm$ 0,5 at (45 ± 3) °C so that after sterilization it will be 7,2 $\pm$ 0,5. Dispense in convenient volumes and autoclave at (121 ± 3) °C for 15 min. Store in the dark for no longer than 6 months.

B.9 Double-strength BPRMB (dsBPRMB)

B.9.1 Basal double-strength broth

Meat peptone	20 g
Casein peptone	20 g
Yeast extract	4 g
NaCl	10 g
Monohydrated L-cystein	1 g
Glucose	3,6 g
$MgSO_4 \cdot 7H_2O$	0,24 g
$CaCl_2$ solution (0,05 g/ml, see below)	2 ml
Distilled water	1 000 ml

Dissolve the ingredients in hot water. Add the $CaCl_2$ solution, mix well and distribute the medium into bottles in volumes of e.g. 200 ml. Sterilize in an autoclave at (121 ± 3) °C for 15 min. Store in the dark at (5 ± 3) °C for no longer than 1 week once the recipient containing the broth has been opened. It can be stored for one month if the recipient is not opened. If stored longer bring it to a boil before use.

B.9.2 Complete double-strength broth

Basal broth	1 000 ml
Haemin solution	20 ml
Disodium carbonate solution	50 ml

Before use, aseptically add haemin, Na_2CO_3, antibiotics and adjust the pH to 6,8 $\pm$ 0,5 (see B.2). Add double the amount of antibiotics added to the single-strength broth. Use immediately.

B.10 Triphenyltetrazolium chloride solution

Triphenyltetrazolium chloride	1 g
Ethanol (96 %)	100 ml

Add 1 ml to 100 ml of ssBPRMA.

B.11 Final composition of liquid and solid culture media

Table B.1 summarizes the amounts of additives, prepared as indicated in this annex, to be added to liquid and solid culture media.

Table B.1 — Final composition of liquid and solid culture media

	BPRMB	ssBPRMA	BPRMA	dsBPRMB
Basal broth (B.1)	1 l	1 l	1 l	—
Double-strength basal broth (B.9)	—	—	—	1 l
Calcium chloride solution (B.1)	1 ml	1 ml	1 ml	2 ml
Haemin solution (B.1)	10 ml	10 ml	10 ml	20 ml
Disodium carbonate solution (B.1)	25 ml	25 ml	25 ml	50 ml
Agar-agar	—	6 g to 10 g	12 g to 20 g	—
HCl (35 % mass fraction) to bring pH to 6,8 $\pm$ 0,5	2,5 ml	2,5 ml	2,5 ml	5 ml
Kanamycin (B.4) (100 μg/ml) [a]	1 ml	1 ml	1 ml	2 ml
Kanamycin (B.4) (300 μg/ml) [a, b]	—	3 ml	3 ml	6 ml
Nalidixic acid (100 μg/ml)	4 ml	4 ml	4 ml	8 ml

[a] Mass concentration in the final medium.

[b] Used when high background flora is expected.

Annex C
(informative)

Alternative method for preparing inoculum cultures

An alternative way to prepare an inoculum culture is the following. Remove a vial of working culture (10.1.3) from frozen storage, allow to equilibrate to room temperature (i.e. 15 °C to 30 °C) and streak with a sterile cotton swab on a plate of BPRMA (B.2). Incubate the culture in an anaerobic cabinet, jar or bag at (36 ± 2) °C for (21 ± 3) h. Inoculate cell material (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 (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. Transfer an aliquot of this culture into BPRMB (B.1) in a screw-capped glass tube 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. Measure the absorbance of the culture (using 7.10) 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.

Annex D
(informative)

Culturing of Bacteriophage B56-3

Use normal procedures for phage propagation as described in the open literature. The following is an example of a procedure which has given good results.

Incubate a culture of *Bacteroides fragilis* as for an inoculum culture as described in 11.1. When the culture reaches approximately 2×10^8 cell/ml, add B56-3 from a stock to give a final concentration of plaque-forming particles of approximately 10^8/ml (ratio cfu:pfp = 1:1).

Incubate the culture overnight at (36 ± 2) °C. Mix with chloroform in a ratio culture-to-chloroform of 2,5:1 (volume). Shake thoroughly on a vortex mixer for 3 min and centrifuge at a minimum of 3 000 g for 10 min.

Pipette the supernatant carefully into a sterile tube and store at (5 ± 3) °C.

It is recommended to filter the supernatant through a 0,2 μm pore size polyvinylidene difluoride membrane (7.22), so as to exclude the remaining cells of *B. fragilis*.

The titre of the phage suspensions should be above 10^9 pfp/ml, and frequently reaches values over 10^{10} pfp/ml. In some cases, it may be necessary to repeat the cycle to obtain sufficiently high titres, higher phage inputs may then be used.

NOTE The titre of the phage stock suspension decreases very slowly with time.

Bibliography

[1] PUIG a., JOFRE J. and ARAUJO R. *Water Sci. Tech.*, **35**, 1997, pp. 11-12

[2] TARTERA C. and JOFRE J. *Appl. Environ. Microbiol.*, **53**, 1987, pp. 1632-1637

[3] ISO 31-0:1992, *Quantities and units — Part 0: General principles*

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