INTERNATIONAL STANDARD

ISO 10705-2

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2000-04-01

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

Reference number
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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.

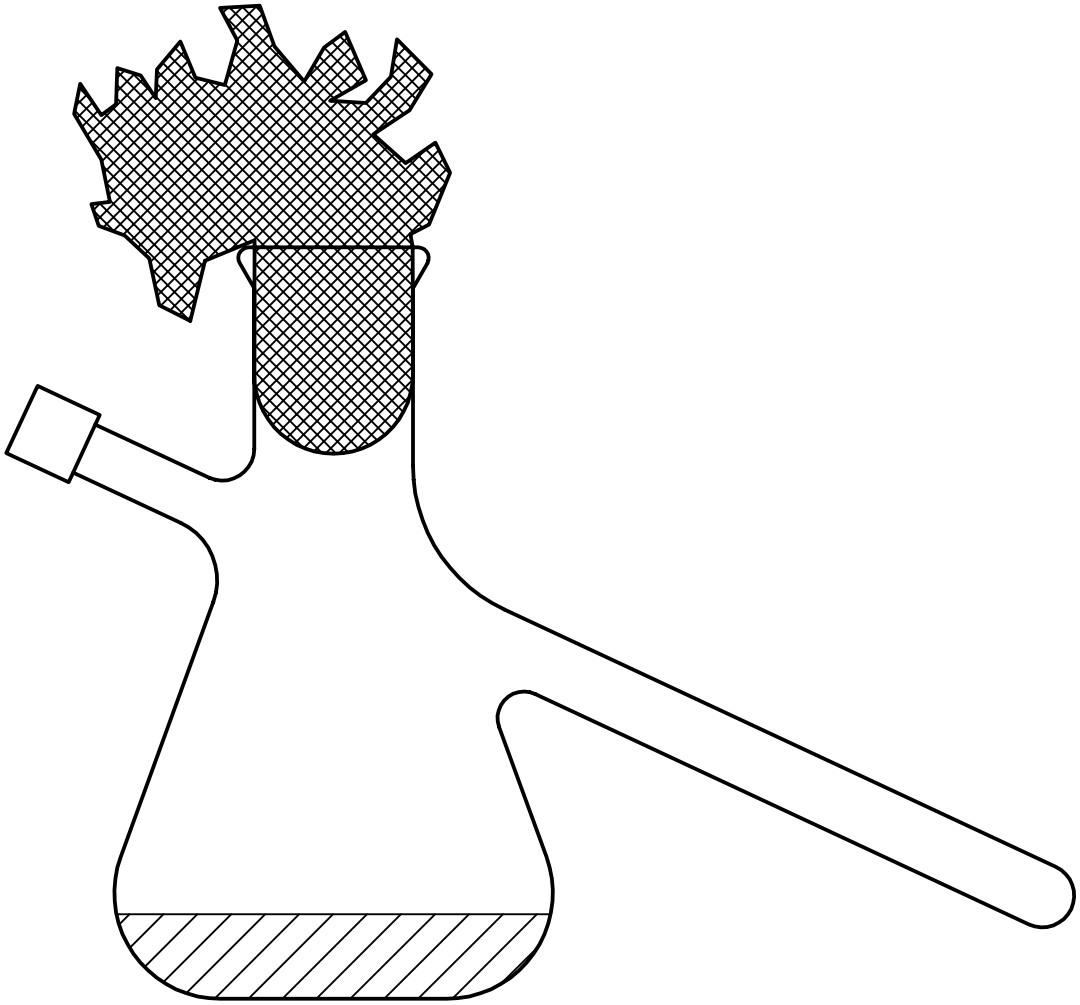

Figure 1 — Nephelometric conical flask for culturing the host strain

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.

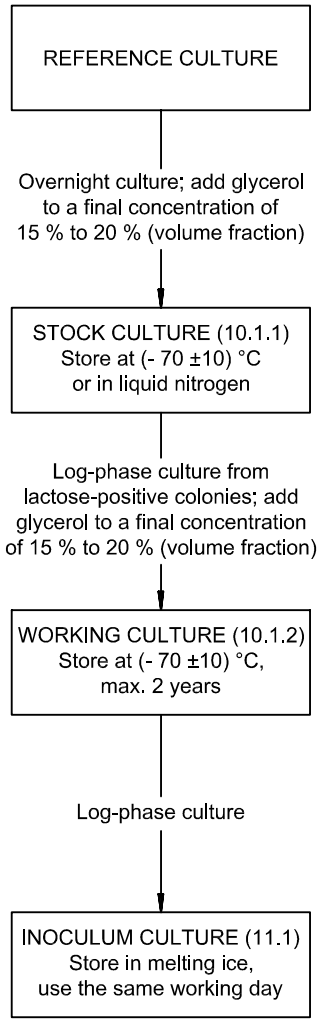

Figure 2 — Scheme for culture and maintenance of host strains

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.

— Pour over 50 ml of complete MSA in a 14 cm to 15 cm diameter Petri dish (or use two 9 cm diameter Petri dishes, each containing 20 ml of MSA).

NOTE This procedure will allow detection of one plaque-forming particle in 50 ml or 100 ml, if 10 or 20 plates are inoculated in parallel. Due to the high consumption of culture media, it may be advisable to use concentration methods which will also be necessary for even lower counts.

11.5 Presence/absence test

For presence/absence test in 1 ml sample proceed as follows:

Remove a vial of working culture from the deep freeze (7.9) and allow to equilibrate to room temperature (15 to 30) °C. Add (25 ± 2,5) ml of MSB (A.1) to a plain conical flask (7.16) and prewarm to at least room temperature (faster growth will occur if the broth is prewarmed to 37 °C). Aseptically add 150 µl of a calcium chloride solution (A.2.2) prewarmed at room temperature and 0,25 ml of working culture (10.1.3). Incubate at (36 ± 2) °C, while gently shaking for approximately 3 h. Add 1 ml of sample or a dilution thereof (prewarmed to room temperature) and continue incubation for (18 ± 2) h.

Transfer 1 ml of the culture to a centrifuge tube, add 0,4 ml of chloroform, mix well and centrifuge at 3 000 *g* for 5 min.

Prepare an inoculum culture as described in 11.1.

Melt bottles of 50 ml 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) 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 tube, add 1 ml of inoculum culture, mix carefully avoiding the formation of air bubbles and pour the contents onto a layer of MSA (A.2) in a 9 cm Petri dish prewarmed at room temperature. Distribute evenly, allow to solidify on a horizontal, cool surface and dry in a laminar flow cabinet or in a (36 ± 2) °C incubator for 30 min, while the plates are inverted with the lids off.

Place one drop of the chloroform-treated culture on the inoculated plate using a fine capillary or pipette. Do not damage the top agar layer. Leave the spot to dry and incubate the plates upside-down at (36 ± 2) °C for (18 ± 2) h.

Examine the plate for a clear zone in the spotted area, which is indicative of the presence of somatic coliphages in the original sample.

NOTE 1 This procedure can also be used in an MPN format (ISO 8199) or to examine larger samples. In the latter case, use double-strength MSB (double the amounts of ingredients in the same amount of water as used for single-strength MSB; add a proportional volume of calcium chloride solution) in equal volumes as the sample. To obtain sufficient aeration during enrichment, make sure that the volume of sample and broth is not greater than 20 % of the nominal capacity of the conical flask.

NOTE 2 More than one spot can be placed on the surface of an inoculated plate.

This procedure produces phage suspensions with a high titre. Take appropriate precautions, such as working in a biohazard cabinet or in a separate area of the laboratory, to prevent cross-contamination of samples or stock cultures.

11.6 Quality assurance

11.6.1 Plaque count procedures (11.2 to 11.4)

With each series of samples, examine a procedural blank using sterile diluent as the sample and a reference control of ϕX174, prepared as follows.

From a high titre phage culture (e.g. as described in annex C), 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, and prepare 100 ml to 1 000 ml of a suspension with an expected concentration of plaque-forming particles of ϕX174 of approx. 100 ml^{-1}. Add 5 % (volume fraction) of glycerol (A5). Distribute into plastics vials in 2,4 ml aliquots and store at (−70 ± 10) °C. Thaw vials of the reference control of ϕX174 before use and plate out according to the

procedure used (11.2 or 11.4). Plot the results on a control chart. Discard the reference control samples if the mean number of pfp/ml decreases.

Optionally, use in addition a naturally polluted reference control sample, using sewage or surface water, diluted to a concentration of plaque-forming particles of approx. 100 ml^{-1} in peptone-saline and 5 % (volume fraction) glycerol and stored at (−70 ± 10) °C. Discard the reference control samples if the concentration of somatic coliphages decreases and is still decreased following a re-test.

11.6.2 Presence/absence test (11.5)

Prepare a control sample as described in 11.6.1 with a concentration of plaque-forming particles of ϕX174 of approx. 5 ml^{-1}. Examine at least one control sample in parallel with each series of samples tested, expecting to obtain a positive test result. To examine possible interfering effects from the samples, consider also adding the control sample to a second enrichment culture containing the actual sample.

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

12 Expression of results

12.1 Plaque count procedures (11.2 to 11.4)

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 X of plaque-forming particles of somatic coliphages in 1 ml of the sample as follows:

$$X = \frac{N}{(n_1 V_1 F_1) + (n_2 V_2 F_2)}$$

where

X is the number of plaque-forming particles of somatic coliphages per millilitre (pfp/ml);

N is the total number of plaques counted on plates according to 11.2, 11.3 or 11.4;

n_1, n_2 is the number of replicates counted for dilution F_1, F_2;

V_1, V_2 is the test volume, in millilitres, used with dilution F_1, F_2;

F_1, F_2 is the dilution or concentration factor used for the test portion V_1, V_2 (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:

$$X = \frac{N}{nVF}$$

Refer to ISO 8199 for further details.

12.2 Presence/absence test (11.5)

Express the results as "somatic coliphages (not) detected in V ml", V being the volume of sample examined.

13 Test report

The test report shall contain the following information:

a) a reference to this part of ISO 10705;

b) all details necessary for 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
(normative)

Culture media, reagents and diluent

A.1 Modified Scholtens' Broth (MSB)

Peptone	10 g
Yeast extract	3 g
Meat extract	12 g
NaCl	3 g
Na_2CO_3 solution (150 g/l)	5 ml
$MgCl_2$ solution (100 g of $MgCl_2 \cdot 6H_2O$ in 50 ml water)	0,3 ml
Distilled water	1 000 ml

a) Preparation of broth

Dissolve the ingredients in hot water. Adjust the pH to $7,2 \pm 0,2$ at (45 ± 3) °C so that after sterilization it will be $7,2 \pm 0,5$. Distribute the medium in bottles in volumes of 200 ml and sterilize in the autoclave at (121 ± 3) °C for 15 min. Store in the dark at (5 ± 3) °C for not longer than 6 months.

b) Preparation of $MgCl_2$ solution for broth

$MgCl_2 \cdot 6H_2O$ is very hygroscopic and shall not be stored in the crystalline form once the container has been opened. Therefore, use the total contents of a container and dissolve in the appropriate amount of water, e.g. add 100 g of $MgCl_2 \cdot 6H_2O$ to 50 ml water. The final concentration of Mg^{2+} in this solution will be 4,14 mol/l. Sterilize by autoclaving and store at room temperature in the dark.

A.2 Modified Scholtens' Agar (MSA)

A.2.1 Basal medium

Peptone	10 g
Yeast extract	3 g
Meat extract	12 g
NaCl	3 g
Na_2CO_3 solution (150 g/l)	5 ml
Agar	10 g to 20 g [a]
$MgCl_2$ solution (100 g of $MgCl_2 \cdot 6H_2O$ in 50 ml water)	0,3 ml
Distilled water	1 000 ml

[a] Depending on the gel strength of the agar.

Dissolve the ingredients in boiling water. Adjust the pH to 7,2 ± 0,2 at (55 ± 3) °C so that after sterilization it will be 7,2 ± 0,5. Distribute the medium in bottles in volumes of 200 ml and sterilize in the autoclave at (121 ± 3) °C for 15 min. Store in the dark at (5 ± 3) °C for not longer than 6 months.

Prepare the $MgCl_2$ solution according to A.1 b).

A.2.2 Calcium chloride solution (c = 1 mol/l)

$CaCl_2 \cdot 2H_2O$	14,6 g
Distilled water	100 ml

Dissolve the calcium chloride in the water while heating gently. Cool to room temperature and filter-sterilize through a membrane filter of 0,2 µm pore size. Store in the dark at (5 ± 3) °C for not longer than 6 months.

A.2.3 Complete medium

Basal medium	200 ml
Calcium chloride solution	1,2 ml

Melt the basal medium and cool to between 45 °C and 50 °C. Aseptically add the calcium chloride solution, mix well and pour into Petri dishes as follows:

— 20 ml in dishes of 9 cm diameter;

— 50 ml in dishes of 14 cm to 15 cm diameter.

Allow to solidify and store in the dark at (5 ± 3) °C for not longer than 1 month if well protected against desiccation.

A.3 Semi-solid Modified Scholtens' Agar (ssMSA)

Prepare basal medium according to A.2 but use half of the mass of the agar (6 g to 10 g), depending on gel strength; the gel strength of ssMSA is critical to obtain good results, and if possible different concentrations should be tested. Choose the agar concentration that produces highest plaque counts but also controls plaque size to reduce confluence. Distribute into bottles in volumes of 50 ml.

NOTE Triphenyltetrazolium chloride (1 ml of a solution of 1 g in 100 ml 96 % ethanol per 100 ml of ssMSA) can be added to improve contrast for counting plaques.

A.4 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, add distilled water and mix well. Filter through a membrane filter of 0,2 µm pore size, or sterilize in the autoclave at (121 ± 3) °C for 15 min. Store at (5 ± 3) °C for not longer than 8 h or at (–20 ± 3) °C for not longer than six months.

A.5 Glycerol (sterile)

Glycerol (870 g/l)	100 ml

Distribute into bottles in 20 ml volumes and sterilize in the autoclave at (121 ± 3) °C for 15 min. Store in the dark for not longer than 1 year.

A.6 McConkey agar

Peptone	20 g
Lactose	10 g
Bile salts	5 g
Neutral red	75 mg
Agar	12 g to 20 g
Distilled water	1 000 ml

Dissolve the ingredients in boiling water. Adjust pH so that after sterilization it will be 7,4 ± 0,1 at (25 ± 3) °C. Distribute the medium in bottles in volumes of 200 ml and sterilize in the autoclave at (121 ± 3) °C for 15 min. Cool to between 45 °C and 50 °C and pour 20 ml in Petri dishes of 9 cm diameter. Allow to solidify and store in the dark at (5 ± 3) °C for not longer than 6 months.

A.7 Peptone saline solution

Peptone	1,0 g
NaCl	8,5 g
Distilled water	1 000 ml

Dissolve the ingredients in hot water. Adjust the pH to 7,2 ± 0,2 at (45 ± 3) °C so that after sterilization it will be 7,2 ± 0,5. Dispense in convenient volumes and autoclave at (121 ± 3) °C for 15 min. Store in the dark for not longer than 6 months.

Annex B
(informative)

General description of somatic bacteriophages

Somatic coliphages are bacteriophages (bacterial viruses) which consist of a capsid containing single- or double-stranded DNA as the genome. The capsids may be of simple cubic symmetry or complex structures with heads, tails, tail-fibres etc. They belong to the morphological groups A to D and are classified into the following families: *Myoviridae* (ds DNA, long contractile tails, capsids up to 100 nm), *Siphoviridae* (ds DNA, long non-contractile tails, capsids 50 nm), *Podoviridae* (ds DNA, short non-contractile tails, capsids 50 nm) and *Microviridae* (ss DNA, no tail, capsid 30 nm). Somatic coliphages are virulent phages which attach to lipopolysaccharide or protein receptors in the bacterial cell wall and may lyse the host cell in 20 min to 30 min under optimal conditions. They produce plaques of widely different size and morphology.

The presence of somatic coliphages in a water sample usually indicates pollution by human or animal faeces or by wastewaters containing these excreta. They thus provide a relatively rapid and simple method for detection of faecal pollution, and their resistance in water and food tends to resemble that of human enteric viruses more closely than faecal bacteria commonly used as quality indicators. Natural host strains of somatic coliphages include, in addition to *Escherichia coli*, other closely related bacterial species, some of which may occur in pristine waters, so that exceptionally somatic coliphages may also multiply in these environments.

Annex C
(informative)

Culturing of bacteriophage ϕX174

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.

Introduce 25 ml of MSB into a conical flask of 300 ml volume and inoculate with *E. coli* or a nalidixic acid resistant mutant (see clause 8). Incubate for (20 ± 4) h at (36 ± 2) °C while shaking at (100 ± 10) r/min.

Prewarm 25 ml of MSB in a conical flask of 300 ml volume to room temperature and inoculate with 0,25 ml of the host culture.

Incubate as above for 90 min. Add ϕX174 from a stock solution to give a final concentration of plaque-forming particles of approx. 10^7 ml^{-1}.

Incubate as above for 4 h to 5 h. Add 2,5 ml of chloroform ($CHCl_3$), mix well and place overnight at (5 ± 3) °C.

Decant the aqueous phase in centrifuge tubes and centrifuge at a minimum of 3 000 *g* for 20 min.

Pipette the supernatant carefully and store at (5 ± 3) °C.

NOTE 1 The titre of the phage suspension should be above 10^9 ml^{-1}. In some cases it may be necessary to repeat the cycle to obtain sufficiently high titres; higher phage inputs may then be used.

NOTE 2 The titre of the phage stock suspension will slowly decrease with time.

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