INTERNATIONAL STANDARD

ISO 10705-1

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Water quality — Detection and enumeration of bacteriophages —

Part 1: Enumeration of F-specific RNA bacteriophages

Qualité de l'eau — Détection et dénombrement des bactériophages — Partie 1: Dénombrement des bactériophages ARN F spécifiques

Reference number
ISO 10705-1:1995(E)

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.

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.

International Standard ISO 10705-1 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*

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

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Water quality — Detection and enumeration of bacteriophages —

Part 1:
Enumeration of F-specific RNA bacteriophages

1 Scope

This part of ISO 10705 specifies a method for the detection and enumeration of F-specific ribonucleic acid (RNA) bacteriophages by incubating the sample with an appropriate host strain. The method can be applied to all kinds of water, sediments and sludges, where necessary after dilution. In the case of low numbers, a preconcentration step may be necessary for which a separate part of ISO 10705 will be developed. The method can also be applied to shellfish extracts. Depending on the relative abundance of F-specific RNA bacteriophages to background organisms, additional confirmatory tests may be necessary and are also specified in this part of ISO 10705.

The presence of F-specific RNA bacteriophages in a water sample generally indicates pollution by wastewater contaminated by human or animal faeces. Their survival in the environment, removal by widely used water treatment processes and concentration or retention by shellfish resembles that of foodborne and waterborne human enteric viruses, for example the enteroviruses, hepatitis A virus and rotaviruses.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 10705. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 10705 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

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.*

3 Definition

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

3.1 F-specific RNA bacteriophages: Bacterial viruses which are capable of infecting a specified host strain with F-pili or sex-pili to produce visible plaques (clearance zones) on a confluent lawn grown under appropriate culture conditions, whereas the infectious process is inhibited in the presence of a concentration

of 40 (occasionally 400) µg/ml of RNase in the plating medium.

4 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. Where necessary, simultaneous examination of parallel plates with added RNase for confirmation by differential counts is carried out. The results are expressed as the number concentration of plaque-forming particles (C_{pfp}) per unit of volume.

5 Safety precautions

The host strain used is a *Salmonella typhimurium* mutant of low pathogenicity and should be handled in accordance with the appropriate national or international safety procedures for this bacterial species. F-specific RNA bacteriophages are non-pathogenic for man and animals, but 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 strains. Such procedures must be carried out in a biohazard cabinet or a separate area of the laboratory.

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 in accordance with ISO 3696.

6.2 Diluent

For making sample dilutions, use peptone saline solution as indicated in A.8.

6.3 Reagents

6.3.1 RNase from bovine pancreas, specific activity approximately 50 units/mg (Kunitz).

6.3.2 Antibiotic discs, for susceptibility testing with nalidixic acid (130 µg; 9 mm) and kanamycin (100 µg; 9 mm).

6.3.3 Glycerol, 870 g/litre.

6.4 Microbiological reference cultures

Salmonella typhimurium strain WG49, phage type 3 Nal^r (F′ 42 *lac*::Tn5), NCTC 12484.

Bacteriophage MS2, NCTC 12487 or ATCC 15597-B1.

Escherichia coli K-12 Hfr from appropriate culture collection, e.g. NCTC 12486 or ATCC 23631.

NOTE 1 The NCTC strains are available from the National Collection of Type Cultures, 61 Colindale Avenue, London NW9 6HT, England. The ATCC strains are available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A.

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 37 °C ± 1 °C.

7.3 Incubator or water bath, thermostatically controlled at 37 °C ± 1 °C and equipped with a rotary platform at 100 min^{-1} ± 10 min^{-1}.

7.4 Water bath, thermostatically controlled at 45 °C ± 1 °C.

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

7.6 pH-meter.

7.7 Counting apparatus, with indirect, oblique light.

7.8 Deep freezer, thermostatically controlled at − 20 °C ± 5 °C.

7.9 Deep freezer, thermostatically controlled at − 70 °C ± 10 °C.

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

Usual sterile, microbiological laboratory glassware or disposable plastics ware according to ISO 8199 and including the following.

7.11 Petri dishes, of diameter 9 cm or 15 cm.

7.12 Graduated pipettes, of capacities 1 ml, 5 ml and 10 ml.

7.13 Glass bottles, of suitable volumes.

7.14 Culture tubes, with caps.

7.15 Measuring cylinders, of suitable capacity.

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

7.17 Cuvettes, of optical path length 1 cm or **nephelometric conical flasks**, of capacity 250 ml to 300 ml, with cylindrical side-arms which can be fitted to the spectrometer (7.10) and with cotton wool plugs or suitable alternatives. (See figure 1.)

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

7.19 Plastics vials, lidded, of capacity 1,5 ml to 2 ml.

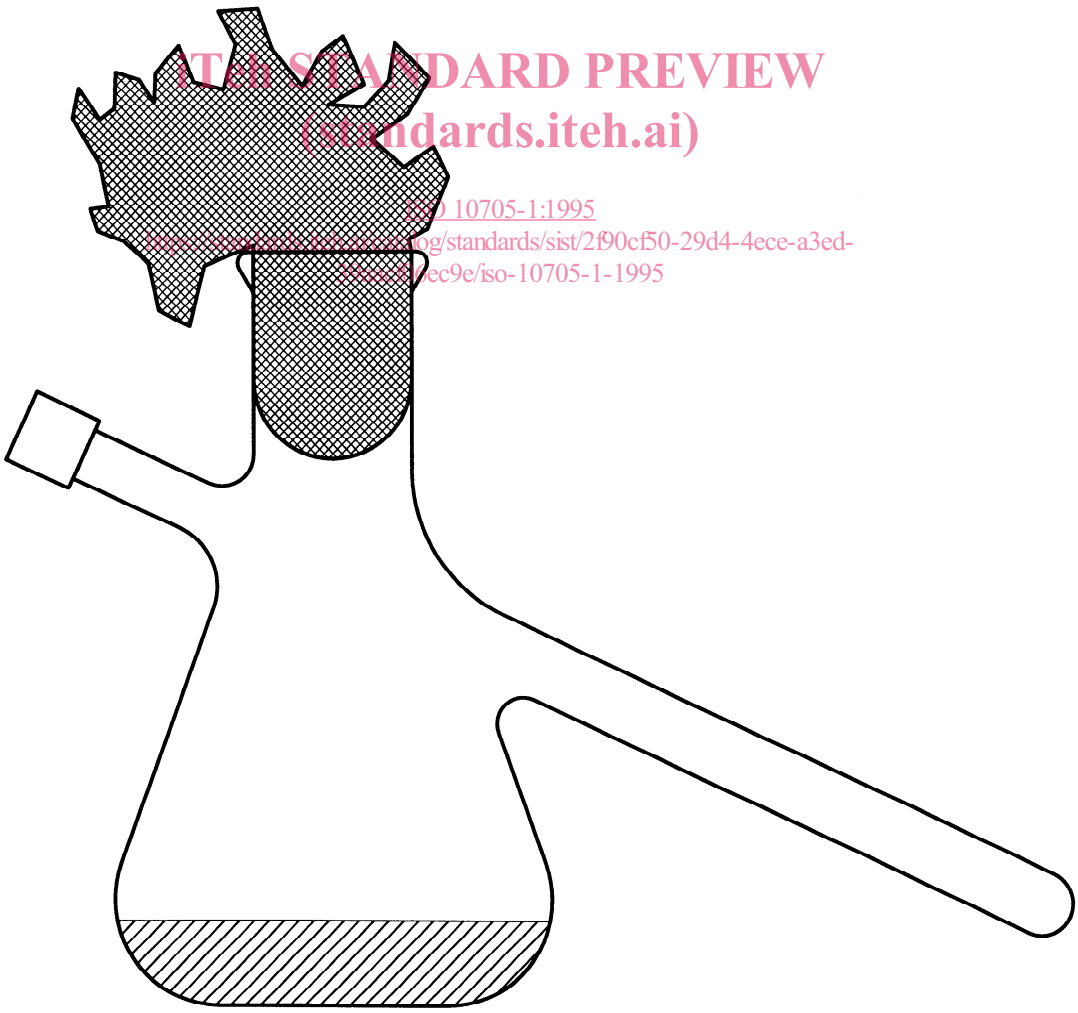

Figure 1 — Nephelometric conical flasks for culturing the host strain

8 Sampling

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

9 Preparation of test materials

9.1 Culturing and maintenance of host strains WG49 and *E. coli* K12 Hfr

The culturing and maintenance of host strains involves several stages which are summarized in figure 2. The figure also indicates the stages where quality control of the host culture is performed.

9.1.1 Preparation of stock cultures

Rehydrate the contents of a lyophilized ampoule of the reference culture of the host strains in a small volume of TYGB (A.1) using a Pasteur pipette. Transfer the suspension to 50 ml of TYGB in a 300 ml conical flask (7.16). Incubate for 18 h ± 2 h at 37 °C ± 1 °C while shaking at 100 min^{-1} ± 10 min^{-1}. Add 10 ml of glycerol (A.6) and mix well. Distribute into plastics vials (7.19) in 1,2 ml aliquots and store at – 70 °C ± 10 °C.

NOTE 2 This first culture of the host strain should be stored as a reference standard in the laboratory.

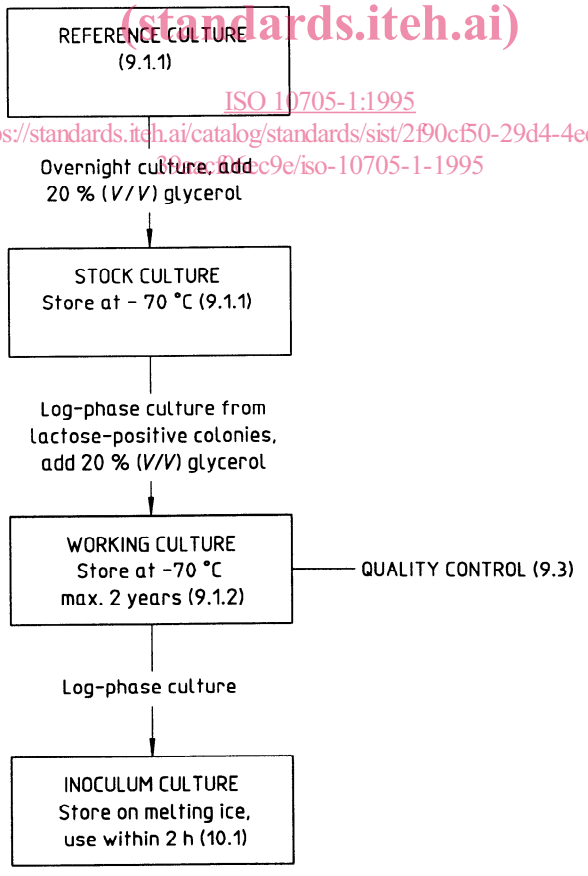

Figure 2 — Scheme for culturing, maintenance and quality control of host strain WG49

9.1.2 Preparation of working cultures

Thaw one vial of stock culture (9.1.1) at room temperature and inoculate on a plate of McConkey agar (A.7), or another lactose-containing medium, in such a way that single colonies will be obtained. Incubate at 37 °C ± 1 °C for 18 h ± 2 h. Add 50 ml of TYGB to (A.1) a 300 ml conical flask (7.16) and warm to room temperature. Select three to five lactose-positive colonies from the McConkey agar and inoculate material from each of these colonies in the flask with TYGB. Incubate for 5 h ± 1 h at 37 °C ± 1 °C while shaking at 100 min^{-1} ± 10 min^{-1}. Add 10 ml of glycerol (A.6) and mix well. Distribute into plastics vials (7.19) in 1,2 ml aliquots and store at − 70 °C ± 10 °C for a maximum of 2 years. Control the quality of the working culture according to 9.3.

NOTES

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

4 If quality control fails, prepare new inocula from the stock culture. After repeated failures, or if the stock culture is depleted, obtain a new lyophilized ampoule of the reference culture. Do not subculture repeatedly in the laboratory.

9.2 Calibration of turbidity measurements

Take one vial of working culture of host strain WG49 from the freezer and thaw at room temperature. Add 50 ml of TYGB (A.1) to a nephelometric conical flask (7.17), warm to room temperature, adjust the spectrometer reading to 0 on the filled side-arm. Alternatively, use a plain conical flask (7.16) and adjust the spectrometer reading to 0 on broth transferred to a cuvette (7.17). Inoculate 0,5 ml of working culture. Incubate at 37 °C ± 1 °C while shaking at 100 min^{-1} ± 10 min^{-1} for up to 3 h. Every 30 min, measure the turbidity and withdraw a 1 ml sample for viable counts, assuring that the flask is taken from the incubator for as short a time as possible.

Dilute samples to 10^{-6} and spread 0,1 ml volumes of the 10^{-4}, 10^{-5} and 10^{-6} dilutions on TYGA plates in duplicate (A.2); incubate at 37 °C ± 1 °C for 24 h ± 2 h. Count the total number of colonies on each plate yielding between 30 and 300 colonies and calculate the number of cfp/ml (consult ISO 8199 if necessary).

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

9.3 Quality control of host strain WG49

Use a culture as prepared in 9.2.

At times $t = 0$ h and $t = 3$ h, also inoculate two plates of McConkey agar (A.7), or another lactose-containing medium with the same dilution series, and incubate at 37 °C ± 1 °C for 24 h ± 2 h. From plates yielding between 30 and 300 colonies, count the number of lactose-positive and lactose-negative colonies and calculate the percentage of lactose-negative colonies.

At times $t = 0$ h and $t = 3$ h, spread 0,1 ml of the 10^{-2} dilution on a plate of McConkey agar or alternative, place one disk with nalidixic acid (Nal) and one disk with kanamycin (Km) on the plates and incubate for 24 h ± 2 h at 37 °C ± 1 °C.

Measure inhibition zones around the antibiotic disks.

The host strain is acceptable if the following criteria are met:

plate count on TYGA (9.2) at 0 h: 0,5 to 3×10^7 cfp/ml;

plate count on TYGA (9.2) at 3 h: 7 to 40×10^7 cfp/ml;

lactose-negative colonies (plasmid segregation) < 8 %;

inhibition zone around Nal disk: absent;

Km disk: < 20 mm diameter.

NOTE 6 Antibiotic disks with a different diameter or concentration can be used; another criterion for the maximum inhibition zone around the Km disk should be set.

Check the host strain for sensitivity for F-specific RNA bacteriophages as follows.

Prepare a stock culture of bacteriophage MS2 as described in annex C and store at 4 °C ± 2 °C. Prepare a decimal dilution series and plate out according to 10.1; but use the *E. coli* K-12 Hfr host strain. Store the dilution series at 4 °C ± 2 °C overnight. Count the number of plaques, from the dilution series, and prepare 100 ml to 1 000 ml of a suspension of MS2 in peptone-saline solution (A.8) which is expected to contain approximately 100 pfp/ml. Add glycerol (5 g/l).

Distribute over plastics vials (7.19) in 1,2 ml aliquots and store at − 20 °C ± 5 °C or − 70 °C ± 5 °C.

Thaw four vials at room temperature, combine them in one tube and plate out 1 ml volumes in duplicate

on the *E. coli* K-12 Hfr strain and on WG49 according to 10.1. Count the number of plaques on each plate and calculate the recovery on WG49 relative to the *E. coli* strain. Accept WG49 if the recovery is > 80 %.

10 Procedure

10.1 Standard procedure

Take one vial of working culture from the freezer and thaw it at room temperature. Add 50 ml of TYGB (A.1) to a nephelometric conical flask (7.17), or plain conical flask (7.16). Adjust the spectrometer reading to 0 as described in 9.2 and prewarm to room temperature. Inoculate 0,5 ml of working culture. Incubate at 37 °C ± 1 °C while shaking at 100 min^{-1} ± 10 min^{-1}. Measure turbidity every 30 min. At a turbidity corresponding to a cell density of approximately 10^8 cfu/ml (based on data obtained in 9.2), take the inoculum culture from the incubator and quickly cool on melting ice. Use within 2 h.

NOTE 7 It is essential that the culture is quickly cooled to prevent loss of F-pili by the cells, which will negatively influence recovery.

Melt bottles of ssTYGA (A.3), cool to 44 °C to 50 °C, aseptically add calcium-glucose solution (A.1) (0,5 ml/50 ml) and distribute 2,5 ml aliquots into culture tubes with caps, placed in a water bath at 45 °C ± 1 °C. To each tube, add 1 ml of sample (or dilution or concentrate). Examine each volume or dilution step at least in duplicate.

Add 1 ml of inoculum culture, mix carefully and pour the contents over the surface of a 9 cm TYGA plate (A.2). Distribute evenly, allow to solidify on a perfectly horizontal, cool surface and incubate the plates upside-down at 37 °C ± 1 °C for 18 h ± 2 h.

NOTES

8 Do not stack more than 6 (preferably 4) plates.

9 The addition of ice-cold sample and host culture to the top-agar may lead to a sharp drop in temperature and solidification of the medium. Assure a sufficient time interval between these two steps to allow reheating. However, make sure that inoculated tubes remain in the water bath for not more than 10 min.

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

10.2 Method for samples with high bacterial background flora

Add nalidixic acid to ssTYGA (A.3) until a final concentration of 100 µg/ml is obtained.

NOTE 10 Nalidixic acid is stable when heated. It can either be added from a filter-sterilized solution (A.4) (0,2 ml/50 ml) after melting of ssTYGA or can be added to TYGA before autoclaving.

10.3 Confirmatory test

In parallel with the series of plates described under 10.1, prepare a similar series with RNase-solution (A.5) added to the tubes of ssTYGA until a final concentration of 40 µg/ml is obtained (i.e. 100 µl of RNase solution to 2,5 ml of ssTYGA in a tube).

NOTES

11 Confirmatory tests should at least be carried out

a) when examining new sampling points;

b) regularly at fixed sampling points when N_{RNase}/N (see clause 11) is usually less than 10 %;

c) always at fixed sampling points when N_{RNase}/N is usually > 10 %;

d) if large, circular, clear plaques with smooth edges (probably somatic *Salmonella* phages) are regularly seen.

12 In rare cases, RNA-phages may not be inhibited by RNase at 40 µg/ml and it may be necessary to increase the concentration of RNase to 400 µg/ml.

10.4 Samples with low phage counts

Proceed according to 10.1 but with the following modifications:

— 10 ml of ssTYGA, 1 ml of host culture and 5 ml of sample in duplicate per dilution step;

— pour over 50 ml of TYGA in a 14 cm Petri dish.

NOTE 13 This procedure will be able to detect up to 1 pfu/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.

10.5 Quality assurance

With each series of samples, examine a procedural blank using sterile diluent as the sample and a stan-

dard preparation of MS2 (see 9.3). Plot the results on a control chart.

Optionally, also use a naturally polluted standard sample, taken from sewage or surface water, diluted to approximately 100 pfp/ml in peptone saline solution and glycerol (5 g/l) and stored at − 20 °C ± 5 °C or − 70 °C ± 5 °C. Discard the standard samples if the concentration of RNA-phages decreases.

NOTE 14 In the absence of easily available standardized reference materials, any programme for the exchange of standard samples between laboratories should be encouraged.

If sensitivity to phages is lost (this is unusual but it may happen very suddenly and completely), prepare a new set of inocula according to 9.1.2.

11 Expression of results

Select plates with 30 to 300 plaques. From the number of plaques counted, and taking into account the results of previous confirmatory tests, calculate the number concentration of (plaque-forming particles of) F-specific RNA bacteriophages in 1 ml of the sample as follows:

$$C_{pfp} = \frac{N - N_{RNase}}{n} \times F$$

where

C_{pfp} is the confirmed number concentration of F-specific RNA bacteriophages per millilitre;

N is the total number of plaques counted on WG49 plates according to 10.1, 10.2 or 10.4;

N_{RNase} is the total number of plaques counted on WG49 plates with RNase according to 10.3;

n is the number of replicates;

F is the dilution (or concentration) factor (1/5 in the case of 10.4).

12 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) if a confirmatory test was used and the ratio of N_{RNase} to N, as a percentage;

d) the results expressed in accordance with clause 11;

e) any other information relevant to the method.