भारतीय मानक

Indian Standard

IS 5887 (Part 5/Sec 2) : 2023
ISO/TS 21872-2 : 2020

खाद्य विषाक्तता उत्पन्न करने वाले जीवाणुओं के संसूचन की पद्धतियां

भाग 5 विब्रियो प्रजाति के निर्धारण के लिए क्षैतिज विधि

अनुभाग 2 न्यूक्लिक एसिड संकरण का उपयोग कर समुद्री भोजन में कुल और संभावित एंटरोपैथोजेनिक *विब्रियो पैराहामोलिटिकस* की गणना

(दूसरा पुनरीक्षण)

Methods for Detection of Bacteria Responsible for Food Poisoning

Part 5 Horizontal Method for the Determination of *Vibrio* spp.

Section 2 Enumeration of Total and Potentially Enteropathogenic *Vibrio parahaemolyticus* in Seafood Using Nucleic Acid Hybridization

(Second Revision)

ICS 07.100.30

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NATIONAL FOREWORD

This Indian standard (Part 5/Sec 2) is identical with ISO/TS 21872-2 : 2020 'Microbiology of the food chain — Horizontal method for the determination of *Vibrio* spp. — Part 2: Enumeration of total and potentially enteropathogenic *Vibrio parahaemolyticus* in seafood using nucleic acid hybridization' issued by International Organization for Standardization (ISO) was adopted by the Bureau of Indian Standards on the recommendation of the Food Hygiene, Safety Management and Other Systems Sectional Committee and approval of the Food and Agriculture Division Council.

IS 5887 was originally published in 1970. The standard was first revised in 1976 by splitting into parts with Part 5 covering the isolation, identification and enumeration of *Vibrio cholerae* and *Vibrio parahaemolyticus*. This second revision of the standard has been brought out to align it with latest version of ISO 21872, by splitting it into two sections and both the sections are identical adoptions of the two parts of ISO 21872 respectively under dual numbering. The other section in this series is:

Part 1 Detection of potentially enteropathogenic *Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio vulnificus* (Identical adoption of ISO 21872-1 : 2017)

The text of ISO Standard has been approved as suitable for publication as an Indian Standard without deviations. Certain conventions are, however, not identical to those used in Indian Standards. Attention is particularly drawn to the following:

a) Wherever the words 'International Standard' appear referring to this standard, they should be read as 'Indian Standard'.
b) Comma (,) has been used as a decimal marker while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

In this adopted standard, reference appears to the following International Standards for which Indian Standards also exist. The corresponding Indian Standards, which are to be substituted in their respective place, are listed below along with its degree of equivalence for the edition indicated:

International Standard	*Corresponding Indian Standard*	*Degree of Equivalence*
ISO 6887-1 Microbiology of the food chain — 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	IS 10232 : 2020 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — General rules for the preparation of initial suspension and decimal dilutions	Identical with ISO 6887-1 : 2017
ISO 6887-3 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products	IS 17448 : 2020 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of fish and fishery products	Identical with ISO 6887-3 : 2017

(*Continued on third cover*)

Contents

Page

Introduction

Potentially enteropathogenic strains of *Vibrio parahaemolyticus* possess thermostable direct haemolysin (TDH) and/or thermostable direct hemolysin-related hemolysin (TRH). TDH positive strains manifest Kanagawa phenomenon (KP)[1]. This characteristic is traditionally utilized in the identification of enterotoxigenic strains of *V. parahaemolyticus*. Strains possessing TRH do not share the haemolytic characteristics of TDH positive isolates and no conventional identification assay has been reported for TRH identification. Pathogenic strains in the environment are a minority[2] and differentiation between enteropathogenic and total *V. parahaemolyticus* presence is therefore useful.

This document enables the enumeration of potentially enteropathogenic *V. parahaemolyticus* and/or of total *V. parahaemolyticus*.

Indian Standard

METHODS FOR DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

PART 5 HORIZONTAL METHOD FOR THE DETERMINATION OF *VIBRIO* SPP.

SECTION 2 ENUMERATION OF TOTAL AND POTENTIALLY ENTEROPATHOGENIC *VIBRIO PARAHAEMOLYTICUS* IN SEAFOOD USING NUCLEIC ACID HYBRIDIZATION

(Second Revision)

1 Scope

This document specifies a method for the direct enumeration of potentially enteropathogenic *V. parahaemolyticus* (*tdh* and/or *trh* positive) and/or the enumeration of total *V. parahaemolyticus* in seafood.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 6887-1, *Microbiology of the food chain — 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 6887-3, *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
oligoprobe
labelled, artificially produced segment of DNA with a defined sequence used to detect target DNA by hybridization

3.2
process control
set of characterized strains analysed together with the sample throughout all the stages of the procedure

3.3
hybridization control
DNA segment with a defined sequence used to monitor the hybridization step

3.4
detection control
labelled DNA segment used to monitor the detection step

4 Principle

4.1 General

This method is based upon direct plating of sample material on a nutrient medium. The resulting colonies are transferred onto nylon membranes and hybridized with DNA digoxigenin-labelled oligoprobes to detect genes associated to pathogenicity (*tdh*[3] and *trh*[4]) and/or with a DNA digoxigenin-labelled oligoprobe detecting the *toxR* gene of *V. parahaemolyticus*[5].

As genes associated with *V. parahaemolyticus* pathogenicity have been sporadically detected in other *Vibrio* species (i.e. *V. alginolyticus*[6]), definitive confirmation of the presence of *tdh* and/or *trh* positive *V. parahaemolyticus* strains may be reached by performing isolation procedures on these strains.

The method comprises the following consecutive steps:

a) plating (see 4.2);

b) preparation of membranes (see 4.3);

c) hybridization (see 4.4);

d) detection (see 4.5).

4.2 Plating

Appropriate dilutions of the sample are plated on non-selective nutrient medium in order to allow the growth of *V. parahaemolyticus*.

4.3 Preparation of membranes

Colonies are transferred onto hybridization membranes, bacterial cells are lysed and DNA is covalently linked to the membranes.

4.4 Hybridization

Membranes are hybridized with probes specific to the target genes: *tdh* and *trh* genes for the detection and enumeration of potentially enteropathogenic *V. parahaemolyticus*, and *toxR* gene for the detection and enumeration of total *V. parahaemolyticus*.

4.5 Detection

The presence of hybridized oligoprobes is detected by colorimetric detection with a specific antibody conjugated with an enzyme.

NOTE Enzymatic activity induces the precipitation of substrate in the presence of target colonies resulting in the development of coloured dots which are counted to provide colony enumeration.

5 Culture media and reagents

5.1 General

For current laboratory practice, see ISO 7218. The composition of culture media and reagents and their preparation shall be as described in Annex B. For the preparation, production and performance testing of culture media, follow the procedures in accordance with ISO 11133.

Commercially available reagents may be used if they fulfil the requirements of this document. Follow the manufacturer's instructions for storage and preparation.

5.2 Hybridization reagents

5.2.1 Hybridization buffer

Hybridization buffer composition may vary according to the probe sequences and hybridization temperature. See Annex C for the buffer composition adopted in this procedure.

5.2.2 Digoxigenin-labelled oligoprobes

The 5′-digoxigenin-labelled oligoprobes for the determination of total and potentially enteropathogenic *Vibrio parahaemolyticus* are listed in the Annex C.

Probe concentration can vary depending on synthesis scale. Refer to the data sheet supplied by the manufacturer to calculate the concentration and for storage conditions.

Alternatives to 5′-digoxigenin-labelled oligoprobes such as DIG-labelled amplicon sequences[7] may be used to obtain higher intensity of the signal if they can be shown to provide equivalent results.

5.2.3 Control materials

The control materials for the determination of total and potentially enteropathogenic *Vibrio parahaemolyticus* are listed in the Annex C.

6 Equipment and consumables

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 Rotary blender.

6.2 Dispenser and graduated pipettes.

6.3 Pipette and tips, capable of dispensing volumes of 100 µl.

6.4 Incubator, capable of operating at 37 °C ± 1 °C.

6.5 UV cross-linker or equivalent UV source (transilluminator) for DNA cross-linking.

6.6 Hybridization oven or water bath with shaking ability, capable of operating at the hybridization temperature.

6.7 Hybridization tubes or bags.

6.8 Hybridization meshes (optional).

6.9 Rocking platform, capable of operating at 120 oscillation min^{-1}.

6.10 Washing containers, preferably circular.

7 Sampling

Sampling is not part of the method specified in this document. Follow the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

8 Procedure

8.1 General

Perform the procedure in accordance with the diagrams given in Annex A. For current laboratory practice, see ISO 7218.

8.2 Preparation of the sample

Collect a test portion of the sample in accordance with ISO 6887-3 and prepare an initial 1:1 suspension in an appropriate diluent (B.1.1). Blend in a rotary homogenizer for approximately 90 s to 120 s and prepare sequential decimal dilutions in accordance with ISO 6887-1.

8.3 Plating

Weigh 0,2 g of the 1:1 diluted sample onto a well-dried TSA-S (B.1.2) plate; this dilution represents 0,1 g of sample. For further decimal dilutions, pipette 100 µl onto the surface of separate well-dried TSA-S plates. Immediately spread thoroughly with a sterile spreading rod.

Incubate the TSA-S plates at 37 °C ± 1 °C for 18 h to 24 h.

8.4 Preparation of process control

See Annex C for the preparation of the process control.

8.5 Preparation of membranes

8.5.1 Colony lift

Following incubation, cool down the plates of TSA-S by placing them at 5 °C ± 3 °C for at least 30 min. Record the sample information on the surface edge of a nylon membrane (B.2.1). The use of a sharpened graphite pencil to mark the membranes is recommended as ink can dissolve during the hybridization and washing steps.

Use one membrane for each plate, including the plate with the process control.

NOTE Laboratories wishing to perform isolation of *V. parahaemolyticus* strains detected by this method can mark the edge of the membranes and the corresponding plates with a reference point to allow orientation and, after colony lift, can store the plates at room temperature (up to 72 h) or at 5 °C ± 3 °C until completion of the hybridization procedure.

Place the membrane, label side upwards, onto the surface of the plate. Using a spreading rod, press the membrane onto the surface of the agar ensuring that the entire membrane is in contact with the plate surface to allow transfer of colonies.

Carefully remove the membranes from the plates and place them (colony side upwards) on absorbent paper soaked in Maas I lysis solution (B.2.2) for 30 min at room temperature.

Remove the membranes using forceps and place them (colony side upwards) on absorbent paper soaked in Maas II neutralizing solution (B.2.3) for 30 min at room temperature.

During lysis and neutralization steps, the downwards surface of the membrane should be in complete contact with the solution but the solution should not run over the upper surface and come into contact with the bacterial colonies.

Place the membranes (colony side upwards) on absorbent paper soaked in standard saline citrate 2× (SSC 2×) solution (B.2.6) and equilibrate them for 5 min at room temperature.

Move the membranes onto dry adsorbent paper to remove excess moisture and place them again on absorbent paper soaked with fresh SSC 2× solution (B.2.6) for 10 min.

Remove excess of SSC 2× buffer (B.2.6) placing the membranes on dry absorbent paper.

8.5.2 Cross-linking of DNA to the membrane

DNA adsorbed to the membranes should be covalently cross-linked by exposure to UV (120 mJ/cm^2 of membrane surface).

For 254 nm UV sources, DNA cross-linkage will require an exposure time of 1 min or less. UV sources with long wave emission could require longer exposure times (3 min to 5 min).

8.5.3 Proteinase K treatment

Fully immerse each membrane in at least 1 ml of proteinase K solution (B.2.9). Incubate at 37 °C ± 1 °C for 1 h. Avoid excessive evaporation during incubation.

Remove the membranes from the container and place them (colony side up) on absorbent paper.

Cover the membranes with absorbent paper moistened with distilled water and press gently to allow the adhesion of cell debris to the paper. Lift the absorbent paper to remove the cell debris and discard the paper. Repeat the step if cell debris are visibly still present on the membrane.

Membranes can be left to fully dry in the air and can be stored, avoiding direct light, at room temperature until performing hybridization, but no longer than 30 days. If storing membranes, care should be taken to prevent individual membranes from coming into direct contact with each other.

NOTE Separator discs provided together with nylon membranes or filter paper can be used to avoid contact between membranes during storage.

8.6 Hybridization

8.6.1 General

For the direct enumeration of potentially enteropathogenic *V. parahaemolyticus* (*tdh* and/or *trh* positive strains), follow 8.6.2. For the enumeration of total *V. parahaemolyticus*, follow 8.6.6. Enumeration of potentially enteropathogenic and of total *V. parahaemolyticus* can be performed on the same membrane by sequentially following the procedures described in 8.6.2 and 8.6.6.

Under the provisions of this document, hybridization can be carried out in a hybridization oven or shaking water bath. Buffer volumes should be adjusted to take account of the different sizes of hybridization tubes or sealable plastic bags. In each step, ensure that a sufficient buffer is used to completely cover the membranes during rotation, shaking or stationary phases.

It is advisable to use hybridization meshes to prevent membranes adhering to each other and to facilitate circulation of buffers. This can be required if hybridization is carried out in hybridization bags.

8.6.2 Hybridization of *tdh* and *trh* probes

8.6.2.1 Pre-hybridization

Pre-warm the hybridization buffer (5.2.1) to the temperature required for hybridization. See Annex C for the temperature conditions and hybridization buffer used in this procedure.

Distribute the membranes, including the membrane with the process control, into hybridization tubes or sealable plastic bags so that the surfaces of each membrane do not adhere to each other. Add to the hybridization vessels a sufficient amount of hybridization buffer to completely cover and saturate the membranes throughout the following rotation or shaking steps.

For each tube or bag, include a hybridization control. See Annex C for the preparation of the controls used in this procedure.

Incubate the membranes under hybridization conditions for at least 60 min.

NOTE This step will assist in the removal from the membranes of non-covalently linked residual material.

8.6.2.2 Hybridization with *tdh* and *trh* probes

Decant the buffer from the pre-hybridization step (see 8.6.2.1) and add the fresh pre-warmed hybridization buffer (5.2.1), ensuring that the membranes are completely covered during rotation or shaking. Add the DIG-labelled *tdh* and *trh* probes (5.2.2) in the required concentration. See Annex C for the conditions used in this procedure.

Allow the probes to hybridize at the required temperature with gentle rotation or shaking for at least 2 hours. Membranes may be left to hybridize overnight.

8.6.2.3 Stringency washes for *tdh* and *trh* detection

Transfer the membranes to vessels containing washing buffer A (B.2.10) and wash the membranes on a rocking platform at approximately 120 oscillations min^{-1} for 5 min at room temperature.

NOTE Membranes can be combined in one vessel, provided they freely float in the buffer and do not stick together. Hybridization meshes can be used to improve separation of membranes during washing steps.

Discard the buffer and repeat the washing step using fresh washing buffer A (B.2.10).

Pre-warm washing buffer B (B.2.11) to the temperature required for stringency washes. See Annex C for the conditions used in this procedure.

Transfer the membranes to vessels containing pre-warmed washing buffer B (B.2.11) and wash the membranes at the required temperature with rotation or shaking for 15 min ± 1 min.

Discard the buffer and repeat the washing step using pre-warmed washing buffer B (B.2.11).

8.6.3 Colorimetric detection

Transfer the membranes to vessels containing washing buffer C (B.2.13), add the detection control and wash the membranes on a rocking platform at approximately 120 oscillations min^{-1} for 1 min at room temperature.

Transfer each membrane colony side upwards to a separate container (e.g. Petri dishes) and add sufficient blocking solution (B.2.15) to fully cover the membrane. Incubate without shaking at room temperature for 30 min.

NOTE 1 If 90 mm Petri dishes are used, the use of at least 40 ml of blocking solution is appropriate.

Discard the blocking solution, cover the membrane with the antibody solution (B.2.17) and incubate without shaking at room temperature for 30 min.

NOTE 2 If 90 mm Petri dishes are used, the use of at least 10 ml of antibody solution is appropriate.

Transfer the membranes to vessels containing washing buffer C (B.2.13) and wash the membranes for 15 min ± 1 min at room temperature on a rocking platform at approximately 120 oscillations min^{-1}.

NOTE 3 Membranes can be combined in one vessel, provided they freely float in the buffer and do not stick together. Hybridization meshes can be used to improve separation of membranes during washing steps.

Discard the buffer and repeat the washing step using fresh washing buffer C (B.2.13).

Discard the washing buffer and add a volume of detection buffer (B.2.18) sufficient to fully cover the membranes. Incubate the membranes on a rocking platform at approximately 120 oscillations min^{-1} at room temperature for 5 min.

Transfer the membranes colony side upwards to separate containers (e.g. Petri dishes) and cover the membrane with the colorimetric substrate (B.2.19). Incubate at room temperature in the dark without shaking. Colour will start developing in 2 h. Perform overnight incubation for full colour development.

NOTE 4 If 90 mm Petri dishes are used, the use of at least 5 ml of colorimetric substrate is appropriate.

Stop the colorimetric detection with 5 sequential 5 min washes in distilled water on a rocking platform at approximately 120 oscillations min^{-1} at room temperature.

Dry the membranes on absorbent paper at room temperature.

8.6.4 Enumeration of *tdh* and/or *trh* positive colonies

Colonies positive for *tdh* and/or *trh* genes appear as violet/purple dots on the filters. Negative colonies appear colourless or can appear yellow or light brown.

Record the number of positive colonies on each membrane at each dilution.

Preferably, membranes yielding between 10 and 150 positive colony forming units (cfu) should be selected.

NOTE Colonies can appear with an irregular shape in membranes prepared from overgrown plates.

8.6.5 Isolation of colonies (optional)

Laboratories wishing to perform isolation of strains detected as positive by this method should use the mark created on the membranes and on the corresponding plates (see 8.5.1), define the approximate position of positive colonies, and perform subculture and isolation on appropriate media.

8.6.6 Hybridization of *toxR* probe

8.6.6.1 General

For enumeration of total *V. parahaemolyticus* in the sample, membranes should undergo hybridization with the species-specific *toxR* probe as described in this document.

For general requirements and advice on procedure, refer to 8.6.1.

Hybridization with *toxR* probe may also be performed on membranes already processed for *tdh* and/or *trh* enumeration in accordance with 8.6.2.

8.6.6.2 Pre-hybridization

Follow the procedure described in 8.6.2.1 for all membranes under test.

NOTE If performing pre-hybridization on membranes already processed for *tdh* and/or *trh* enumeration, a certain diffusion of colour from the membranes can be expected, with no influence on the following determination.

8.6.6.3 Hybridization with *toxR* probe

Decant the buffer from the pre-hybridization step (see 8.6.6.2). Add the pre-warmed hybridization buffer (5.2.1), ensuring that membranes are completely covered during rotation or shaking. Add the DIG-labelled *toxR* probe (5.2.2) to the required concentration. See Annex C for the conditions used in this procedure.

Allow the probe to hybridize at the required temperature with gentle rotation or shaking for at least 2 h. Membranes may be left to hybridize overnight.

8.6.6.4 Stringency washes for *toxR* detection

Following hybridization, carry out stringency washes as described in 8.6.2.3.

8.6.7 Colorimetric detection

Carry out colorimetric detection following the steps described in 8.6.3.

8.6.8 Enumeration of *toxR* positive colonies

Colonies positive for the *toxR* gene appear as violet/purple dots on the filters. Negative colonies appear colourless or can appear yellow or light brown.

Record the number of the positive colonies on each membrane at each dilution.

Preferably, membranes yielding between 10 and 150 positive colony forming units (cfu) should be selected.

NOTE 1 Colonies can appear with an irregular shape in membranes prepared from overgrown plates.

NOTE 2 Laboratories wishing to isolate *V. parahaemolyticus* strains detected by hybridization can perform a second round of isolation in accordance with the procedure described in 8.6.5.

9 Interpretation of the results

Colonies positive for the target genes *tdh*, *trh* or *toxR* appear as violet/purple dots on the membranes. Negative colonies appear colourless or can appear yellow or light brown.

Results are valid only if the process control, the hybridization control and the detection control show the expected results (see Annex C).

NOTE The detection of specific genes does not necessary imply their expression, hence the presence of *tdh* and/or *trh* genes is not always associated with toxin production.

10 Expression of results

Calculate the potentially enteropathogenic *V. parahaemolyticus* and/or the total *V. parahaemolyticus* per gram of sample as a weighted mean from two successive dilutions using Formula (1):

$$N = \frac{\sum C}{a \times 1{,}1 \times d} \quad (1)$$

where

$\sum C$ is the sum of the colonies (derived either from 8.6.4 or 8.6.8) counted on two membranes from two successive dilutions, at least one of which contains a minimum of 10 colonies;

a is the amount of inoculum plated on the first dilution retained, in grams or millilitres;

d is the dilution corresponding to the first dilution retained for counting on the membranes.

NOTE In membranes prepared from plates where the 1:1 dilution was spread, d corresponds to 0,5 and a corresponds to the amount of sample inoculated on the plate (0,2 g).

Express the results in accordance with ISO 7218.

11 Test report

The test report shall specify the following:

— the test method used, with reference to this document, i.e. ISO/TS 21872-2;

— the sampling method used, if known;

— all operating conditions not specified in this document, or regarded as optional, together with details of any incidents which can have influenced the test result(s);

— any deviations from this document;

— all information necessary for the complete identification of the sample;

— the test result(s) obtained;

— the date of the test.

Annex A
(normative)

Flow diagram for the enumeration of total and potentially enteropathogenic *Vibrio parahaemolyticus* in seafood using nucleic acid hybridization

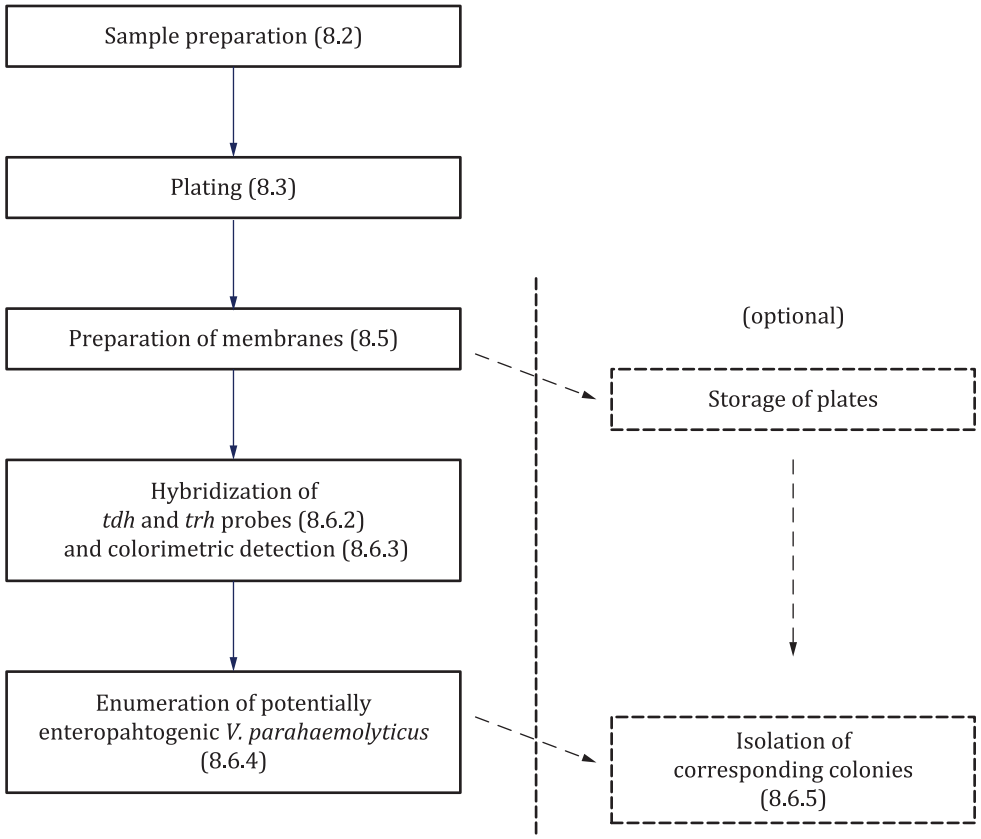

Figure A.1 — Enumeration of potentially enteropathogenic *V. parahaemolyticus*

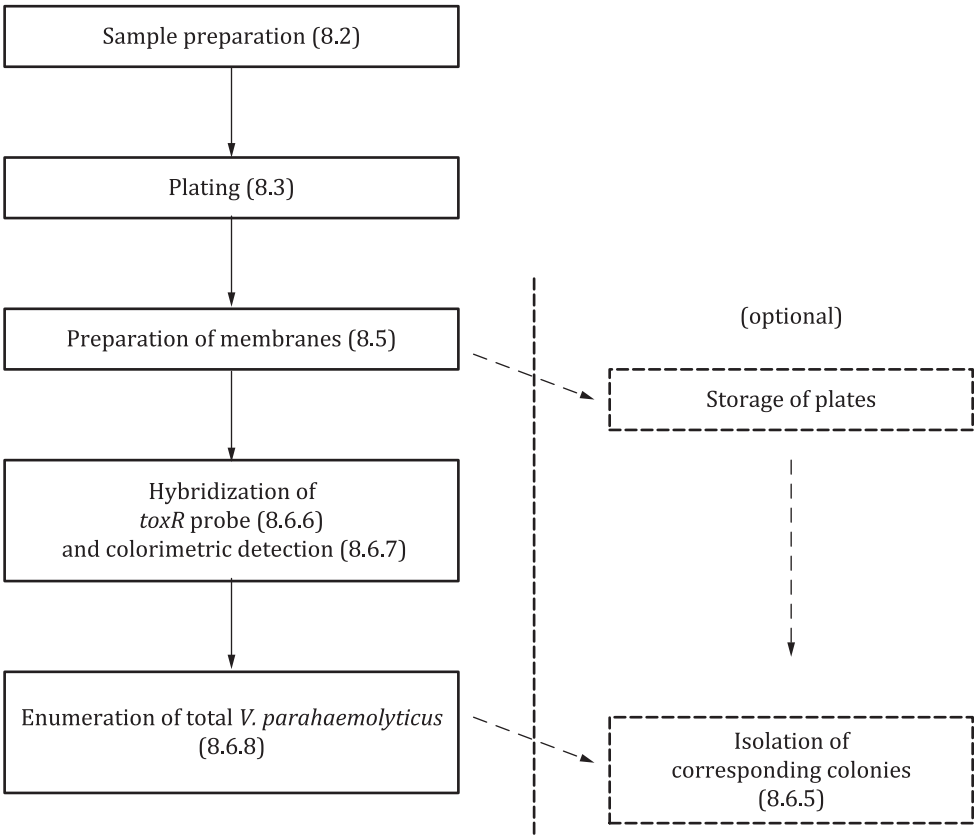

Figure A.2 — Enumeration of total *V. parahaemolyticus*

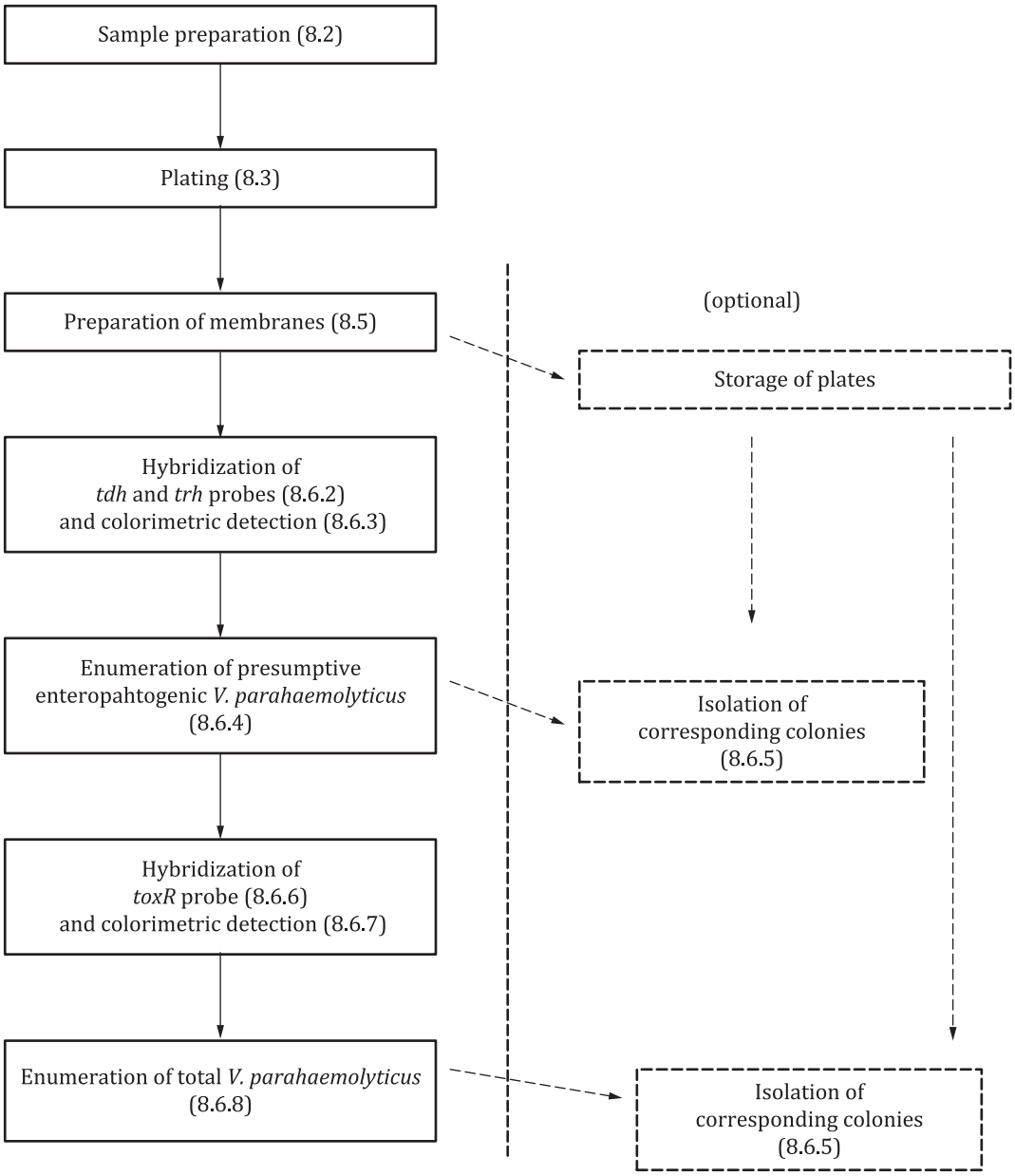

Figure A.3 — Enumeration of total and potentially enteropathogenic *V. parahaemolyticus*

Annex B
(normative)

Culture media and reagents

B.1 Culture media

B.1.1 Diluents

See ISO 6887-1 and the relevant section of ISO 6887-3 dealing with the product to be examined.

B.1.2 Saline tryptone soya agar (TSA-S)[8]

B.1.2.1 Composition

Tryptone	15,0 g
Soya peptone	5,0 g
Sodium chloride [NaCl]	30,0 g
Agar	15,0 g
Water	1 000 ml

B.1.2.2 Preparation

Dissolve the components in the water, if necessary, by heating. Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2. Sterilize for 15 min at 121 °C.

Cool the medium to about 45 °C and pour into sterile Petri dishes.

B.1.3 Performance testing for the quality assurance of the culture media

Information on the performance testing of the culture media indicated in this document is given in Table B.1. For the definitions of productivity and for acceptability criteria, see ISO 11133.

Table B.1 — Performance testing of culture media

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Reference medium	Criterion
TSA-S	Productivity	(18 – 24) h/ (37 ± 1) °C	*Vibrio parahaemolyticus*	00185 or 00037	Media batch TSA-S already validated	$P_R \geq 0,7$

[a] Refer to the reference strain catalogue at www.wfcc.info for information on culture collection strain numbers and contact details; WDCM: World Data Centre for Microorganisms.

B.2 Hybridization reagents

B.2.1 Nylon membranes for colony hybridization

Nylon membranes for colony hybridization (e.g. 82 mm to 85 mm of diameter for a 90 mm Petri dish).

B.2.2 Maas I solution (Lysis solution)

B.2.2.1 Composition

Sodium hydroxide [NaOH]	0,5 mol/l
Sodium chloride [NaCl]	1,5 mol/l

B.2.2.2 Preparation

Dissolve the components in distilled water. Store at room temperature for a maximum of 90 days.

B.2.3 Maas II solution (neutralizing solution)

B.2.3.1 Composition

Tris(hydroxymethyl)-aminomethane [C4H11NO3]	1,0 mol/l
Sodium chloride [NaCl]	2,0 mol/l

B.2.3.2 Preparation

Dissolve the components in distilled water. Adjust pH to 7,0 ± 0,2. Store at room temperature for a maximum of 90 days.

B.2.4 Standard saline citrate (SSC) solution 20×

B.2.4.1 Composition

Sodium citrate dihydrate [C6H5Na3O7•2H2O]	0,3 mol/l
Sodium chloride [NaCl]	3,0 mol/l

B.2.4.2 Preparation

Dissolve the components in 800 ml distilled water. Adjust pH to 7,0 ± 0,2 and bring to 1 000 ml. Sterilize for 15 min at 121 °C. Store at 5 °C ± 3 °C for a maximum of 90 days.

B.2.5 Standard saline citrate (SSC) solution 5×

B.2.5.1 Composition

Standard saline citrate (SSC) solution 20× (B.2.4)	250 ml
Sterile distilled water	750 ml

B.2.5.2 Preparation

Mix the components. Store at 5 °C ± 3°C for a maximum of 90 days.

B.2.6 Standard saline citrate (SSC) solution 2×

B.2.6.1 Composition

Standard saline citrate (SSC) solution 20× (B.2.4)	100 ml
Sterile distilled water	900 ml

B.2.6.2 Preparation

Mix the components. Store at 5 °C ± 3°C for a maximum of 90 days.

B.2.7 Standard saline citrate (SSC) solution 0,5×

B.2.7.1 Composition

Standard saline citrate (SSC) solution 20× (B.2.4)	25 ml
Sterile distilled water	975 ml

B.2.7.2 Preparation

Mix the components. Store at 5 °C ± 3 °C for a maximum of 90 days.

B.2.8 Proteinase K stock solution

B.2.8.1 Composition

Proteinase K (approximately 30 U/mg)	100 mg
Molecular grade water	5 ml

B.2.8.2 Preparation

Dissolve the proteinase K in molecular grade water by gentle mixing. Store at below –15 °C for a maximum of six months. Avoid repeated freezing and thawing.

B.2.9 Proteinase K solution

B.2.9.1 Composition

Proteinase K stock solution (B.2.8)	20 µl
Standard saline citrate (SSC) solution 2× (B.2.6)	1 ml

B.2.9.2 Preparation

Mix the components immediately before use.

B.2.10 Washing buffer A

B.2.10.1 Composition

Sodium dodecyl sulfate (SDS) [C12H25NaO4S]	1,0 g
Standard saline citrate (SSC) solution 2× (B.2.6)	1 000 ml

B.2.10.2 Preparation

Dissolve the SDS in SSC 2× by gentle mixing. Store at room temperature for a maximum of 90 days.

B.2.11 Washing buffer B

B.2.11.1 Composition

Sodium dodecyl sulfate (SDS) [C12H25NaO4S]	1,0 g
Standard saline citrate (SSC) solution 0,5× (B.2.7)	1 000 ml

B.2.11.2 Preparation

Dissolve the SDS in SSC 0,5× by gentle mixing. Store at room temperature for a maximum of 90 days.

B.2.12 Maleic acid buffer

B.2.12.1 Composition

Maleic acid [C4H4O4]	0,1 mol/l
Sodium chloride [NaCl]	0,15 mol/l

B.2.12.2 Preparation

Dissolve the components in distilled water. Adjust pH to 7,5 ± 0,2. Store at room temperature for a maximum of 90 days.

B.2.13 Washing buffer C

B.2.13.1 Composition

Polyoxyethylenesorbitan monolaurate (Tween 20) [C58H114O26]	3,0 ml
Maleic acid buffer (B.2.12)	1 000 ml

B.2.13.2 Preparation

Mix the components. Store at room temperature for a maximum of 90 days.

B.2.14 Blocking reagent

B.2.14.1 Composition

Casein from skimmed milk	10 g
Maleic acid buffer (B.2.12)	90 ml

B.2.14.2 Preparation

Dissolve the components by heating and gentle mixing. Sterilize for 15 min at 121 °C. Store at 5 °C ± 3°C for a maximum of 90 days.

B.2.15 Blocking solution

B.2.15.1 Composition

Blocking reagent (B.2.14)	10 ml
Maleic acid buffer (B.2.12)	90 ml

B.2.15.2 Preparation

Mix the components. Prepare fresh immediately before use.

B.2.16 Alkaline phosphatase (AP) conjugated anti-digoxigenin antibody

Anti-digoxigenin antibody or Fab fragments from an anti-digoxigenin antibody conjugated with alkaline phosphatase (AP) should be used. Refer to the data sheet supplied by the manufacturer for the storage conditions.

B.2.17 Antibody solution — Preparation

Following the manufacturer's instructions, dilute the alkaline phosphatase (AP) conjugated anti-digoxigenin antibody (B.2.16) in the blocking solution (B.2.15) to a concentration suitable for colony hybridization applications. Prepare fresh immediately before use.

NOTE Depending on the manufacturer's instructions, a dilution of 1:2 000 to 1:5 000 is acceptable.

B.2.18 Detection buffer

B.2.18.1 Composition

Tris(hydroxymethyl)-aminomethane [C4H11NO3]	0,1 mol/l
Sodium chloride [NaCl]	0,1 mol/l

B.2.18.2 Preparation

Dissolve the components in distilled water. Adjust pH to 9,5 ± 0,2. Store at room temperature for a maximum of 90 days.

B.2.19 Colorimetric substrate

B.2.19.1 Composition

Nitro blue tetrazolium chloride (NBT) [C40H30N10O6Cl2]	3,75 mg
5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP-T) [C8H6NO4BrCIP × C7H9N]	1,88 mg
Detection buffer (B.2.18)	10 ml

B.2.19.2 Preparation

Dissolve the components in the detection buffer (B.2.18). Prepare fresh before use and protect from light while waiting for use.

Annex C
(informative)

Hybridization of *tdh*, *trh* and *toxR* genes of *V. parahaemolyticus*

C.1 General

This annex describes a method for the detection by means of nucleic acid hybridization of the genes encoding the thermostable direct hemolysin (*tdh*) and the thermostable direct hemolysin-related hemolysin (*trh*), and for the detection of the *V. parahemolyticus*-specific sequence of the *toxR* gene.

Two oligoprobes are used for the detection of the variants *tdh1* and *tdh2* of the *tdh* gene. Two oligoprobes are used for the detection of the variants *trh1* and *trh2* of the *trh* gene. One oligoprobe is used for the detection of *toxR* gene. All oligoprobes are labelled with digoxigenin at 5′ end.

For limitations, see C.6.

C.2 Performance characteristics

C.2.1 General

The method is applicable for the enumeration of *V. parahaemolyticus* from naturally contaminated samples[8].

C.2.2 Selectivity

C.2.2.1 Inclusivity test

An inclusivity test of the oligoprobes was performed on 175 *V. parahaemolyticus* strains. An inclusivity of 99,4 % was obtained for the detection of *toxR* gene, 96,7 % for *tdh* gene and 97,8 % for *trh* gene.

The strains tested included reference strains and isolates from samples of human, environmental and food origin (see Table C.1).

Table C.1 — Inclusivity of the oligoprobes using target strains

Species	Gene profile			Number of strains	Gene detected		
	toxR	*tdh*	*trh*		*toxR*	*tdh*	*trh*
V. parahaemolyticus	+	+	+	6	6	5	6
V. parahaemolyticus (including ATCC 43996, CCUG 43363)	+	+	–	24	24	24	0
V. parahaemolyticus (including WDCM 00037, CCUG 43364)	+	–	+	39	39	0	38
V. parahaemolyticus (including WDCM 00185, ATCC 27519)	+	–	–	106	105	0	0

C.2.2.2 Exclusivity test

An exclusivity test of the oligoprobes was performed on 233 non-*V. parahaemolyticus* strains. An exclusivity of 100 % was obtained for the *tdh*, *trh* and *toxR* genes.

The strains tested included reference strains and isolates from samples of human, environmental and food origin (see Table C.2).

Table C.2 — Exclusivity of the oligoprobes using non-target strains

Species	Number of strains	Gene detected		
		toxR	*tdh*	*trh*
Aeromonas caviae WDCM 00062	1	0	0	0
Aeromonas hydrophila WDCM 00063, CCUG 14551T	2	0	0	0
Aeromonas veronii CCUG 27821	1	0	0	0
Bacillus cereus WDCM 00001	1	0	0	0
Bacillus subtilis WDCM 00003	1	0	0	0
Citrobacter freundii ATCC 8090	1	0	0	0
Clostridium perfringens WDCM 00007	1	0	0	0
Enterobacter aerogenes WDCM 00175	1	0	0	0
Enterobacter cloacae WDCM 00082	1	0	0	0
Enterococcus faecalis WDCM 00087	1	0	0	0
Escherichia coli WDCM 00013, ATCC 15766	2	0	0	0
Kocuria rhizophila ATCC 9341	1	0	0	0
Listeria innocua WDCM 00017	1	0	0	0
Listeria ivanovii WDCM 00018	1	0	0	0
Listeria monocytogenes ATCC 19115	1	0	0	0
Photobacterium angustum ATCC 25915	1	0	0	0
Photobacterium damselae BCCM/LMG 7892	1	0	0	0
Photobacterium phosphoreum BCCM/LMG 4233	1	0	0	0
Plesiomonas shigelloides ATCC 14029	1	0	0	0
Proteus hauseri ATCC 13315	1	0	0	0
Pseudomonas aeruginosa WDCM 00025, WDCM 00026	2	0	0	0
Saccaromices cerevisiae WDCM 00058	1	0	0	0
Salmonella enterica ser. Enteriditis WDCM 00030	1	0	0	0
Salmonella enterica ser. Typhimurium WDCM 00031	1	0	0	0
Shigella sonnei ATCC 25931	1	0	0	0
Staphylococcus aureus WDCM 00034, WDCM 00211, WDCM 00131, ATCC 13565, ATCC 29971	5	0	0	0
Staphylococcus epidermidis WDCM 00036	1	0	0	0
Staphylococcus xylosus ATCC 29971	1	0	0	0
Streptococcus pyogenes ATCC 19615	1	0	0	0
Vibrio alginolyticus (including ATCC 17749, NCTC 12160)	87	0	0	0

Key

n.d. = not done

[a] This strain represents polymerase chain reaction (PCR) amplified DNA from a *trh*+ *V. alginolyticus* isolate[6]. The probes used in this method has been designed to exclude hybridization with this *trh* sequence as well as *tdh* and *trh* sequences currently identified in *Vibrio* species other than *V. parahaemolyticus*.

[b] Environmental isolates identified by partial sequencing of the *rpoA* gene[9].

Table C.2 *(continued)*

Species	Number of strains	Gene detected		
		toxR	***tdh***	***trh***
Vibrio alginolyticus[a]	1	n.d.	n.d.	0
Vibrio anguillarum/ordalii[b]	1	0	0	0
Vibrio campbellii ATCC 25920	1	0	0	0
Vibrio cholerae (including WDCM 00203, ATCC 14103, ATCC 14035, CCUG 33379, CCUG 45388)	54	0	0	0
Vibrio cincinnatensis ATCC 33912	1	0	0	0
Vibrio diabolicus[b]	1	0	0	0
Vibrio fluvialis WDCM 00137	1	0	0	0
Vibrio fisheri BCCM/LMG 7891	1	0	0	0
Vibrio harveyi ATCC 14126	2	0	0	0
Vibrio hollisae ATCC 35912, NCTC 11645	2	0	0	0
Vibrio metschnikovii[b]	2	0	0	0
Vibrio mimicus ATCC 33653, ATCC 33654, NCTC 11435	3	0	0	0
Vibrio natriegens BCCM/LMG 10935	1	0	0	0
Vibrio navarrensis BCCM/LMG 15976	1	0	0	0
Vibrio nereis BCCM/LMG 3895	1	0	0	0
Vibrio penaecida BCCM/LMG 19663	1	0	0	0
Vibrio proteolyticus ATCC 15338	1	0	0	0
Vibrio rotiferanus[b]	1	0	0	0
Vibrio scophtalmi BCCM/LMG 19158	1	0	0	0
Vibrio tubiashii CCUG 38428T	1	0	0	0
Vibrio vulnificus (including WDCM 00139, ATCC 33149, ATCC 33815, NCTC 11067)	32	0	0	0

Key

n.d. = not done

[a] This strain represents polymerase chain reaction (PCR) amplified DNA from a *trh*+ *V. alginolyticus* isolate[6]. The probes used in this method has been designed to exclude hybridization with this *trh* sequence as well as *tdh* and *trh* sequences currently identified in *Vibrio* species other than *V. parahaemolyticus*.

[b] Environmental isolates identified by partial sequencing of the *rpoA* gene[9].

C.2.3 Sensitivity

C.2.3.1 Reaction sensitivity

The limit of detection of hybridization assays was assessed on the target sequences amplified by polymerase chain reaction (PCR) (see Table C.3).

Table C.3 — Sensitivity of the hybridization assay

Probe	Detection limit (gene copies)
toxR	$1{,}13 \times 10^7$
tdh1 and *tdh2*[a]	$5{,}18 \times 10^7$
trh1	$4{,}17 \times 10^6$
trh2	$1{,}35 \times 10^7$

[a] The majority of the strains tested carry both *tdh1* and *tdh2* gene variants. The test was performed using a PCR amplified template containing both variants of the target gene.

C.2.3.2 Method sensitivity

In accordance with ISO 7218, the limit of detection (LOD) of the method is 10 cfu/g and the limit of quantification (LOQ) is 100 cfu/g.

C.2.4 Performance parameters: accuracy, linearity, repeatability

Accuracy and linearity of the method was evaluated on experimentally contaminated seafood matrices (shellfish, finfish, cephalopods and crustaceans), with a contamination ranging from 10^1 cfu/g to 10^4 cfu/g for enteropathogenic *V. parahaemolyticus* and from 10^1 cfu/g to 10^5 cfu/g for total *V. parahaemolyticus*. The results obtained for the linear regression of the log transformed data, for the Pearson correlation coefficient (r^2) and for the standard deviation of repeatability (s_r log) in the different matrices and, overall, in the seafood category, are reported in Table C.4.

Table C.4 — Accuracy, linearity and repeatability of the method[a]

Matrix	Enumeration of potentially enteropathogenic *V. parahaemolyticus*		Enumeration of total *V. parahaemolyticus*	
Shellfish	y = 0,9122x + 0,0020		y = 1,0251x + 0,2631	
	r^2 = 0,965	s_r = 0,210	r^2 = 0,981	s_r = 0,087
Finfish	y = 0,8953x + 0,0376		y = 1,0380x + 0,1852	
	r^2 = 0,981	s_r = 0,201	r^2 = 0,998	s_r = 0,098
Cephalopods	y = 0,9866x + 0,2455		y = 0,9647x + 0,6430	
	r^2 = 0,981	s_r = 0,162	r^2 = 0,994	s_r = 0,049
Crustaceans	y = 0,9256x + 0,0238		y = 1,0733x − 0,0821	
	r^2 = 0,986	s_r = 0,225	r^2 = 0,992	s_r = 0,056
Seafood	y = 0,9299x + 0,0772		y = 1,0253x + 0,2523	
	r^2 = 0,981	s_r = 0,204	r^2 = 0,994	s_r = 0,073

[a] For the calculation of the standard deviation of repeatability (s_r), only results with counts equal or above 100 cfu/g were taken into account.

C.2.5 Robustness

The robustness of the colony hybridization procedure was tested by applying the following modifications:

— increasing the pH of the Maas II solution (B.2.3) to 8,0;

— changing the UV exposure for cross-linking (see 8.5.2) by decreasing it by 25 % and increasing it by 100 %;

— changing the UV source for cross-linking (see 8.5.2) by using different instruments (Stratalinker 1800, transilluminator ETX-20.M)[1)];

— increasing the rotation speed of the rocking platform in washing steps (see 8.6.2.3, 8.6.3, 8.6.6.4, 8.6.7) to 360 oscillations min^{-1};

— increasing and decreasing the antibody solution volume (B.2.17) by 25 %.

The modifications did not influence the results on process control (see C.5.1).

1) Stratalinker 1800 and ETX-20.M are trade names of products supplied by Stratagene and Vilber Lourmat, respectively. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

The robustness of the hybridization reaction was tested by applying the following modifications:

— increasing and decreasing probes concentration (see Tables C.6 and C.7) by 25 %;

— increasing and decreasing the hybridization temperature (see C.4.3) by 1 °C;

— hybridization using different hybridization ovens (Hybaid Shake'n'Stake™, Labexchange GFL 7601, HL-2000 HybriLinker System, Amersham RPN2511E) and a shaking water bath (Julabo SW23)[2].

The modifications did not influence the results on the hybridization control for the *toxR* gene (see C.5.2).

C.3 Procedure

C.3.1 Principle

Specific fragments of the *tdh1, tdh2, trh1, trh2 and toxR* genes of *Vibrio parahaemolyticus* are detected by DNA-DNA hybridization. The detection is based on specific oligoprobes labelled with digoxigenin (DIG).

C.3.2 Reagents

C.3.2.1 General

For current laboratory practice, see ISO 7218.

The reagents given in C.3.2.2 should be used.

C.3.2.2 Reagents for hybridization

C.3.2.2.1 Hybridization buffer

C.3.2.2.1.1 Composition

Bovine serum albumin	0,5 g
Sodium dodecyl sulfate (SDS) [C12H25NaO4S]	1,0 g
Polyvinylpyrrolidone [(C6H9NO)n]	0,5 g
Urea [CO(NH2)2]	36,04 g

C.3.2.2.1.2 Preparation

Dissolve the components in standard saline citrate (SSC) solution 5× (B.2.5) to a final volume of 100 ml. Heating at approximately to 50 °C in a water bath will improve dissolving of the components. Store at 5 °C ± 3 °C for up to one week.

C.3.2.2.2 Oligoprobes

See Table C.5.

2) Hybaid Shake'n'Stake, Labexchange GFL 7601, HL-2000 HybriLinker System, Amersham RPN2511E and Julabo SW23 are trade names of products supplied by Thermo Scientific, Labexchange, UVP, GE Healthcare Bioscience and Julabo, respectively. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

Table C.5 — Sequences of the oligoprobes

Target gene	Probe	Sequence[a]	Reference
tdh	*tdh1*	5′-DIG-CAACTTTTAATACCCAAGCTCCGGTCAATGTAAAGG-3’	[8]
	tdh2	5′-DIG-CAACTTTTAATACCAATGCACCGGTCAATGTAGAGG-3’	[8]
trh	*trh1*	5′-DIG-AGGCTCAAAATGGTTAAGCGCCTATATGACGGTAAA-3’	[8]
	trh2	5′-DIG-CAAAGATGTATACGGTCAATCGGTTTTCACAACWGC-3’	[8]
toxR	*toxR*	5′-DIG-ATTACTACCGATTTGCGTACTGCTGTTTACAAACCC-3’	[8]

[a] DIG: Digoxigenin.

C.4 Molecular procedure

C.4.1 Hybridization reaction with *tdh* and *trh* probes

Different volumes may be used according to the type (tube or bag) and the size of the hybridization vessel used. A hybridization volume of 20 ml per hybridization reaction is used for tubes ranging from 35 mm × 150 mm to 80 mm × 300 mm. The reagents are listed in Table C.6.

Table C.6 — Reagents for hybridization with *tdh* and *trh* probes

Reagent	Final concentration	Volume per vessel
Probe *tdh1* (100 µM)	5 nmol/l	1 µl
Probe *tdh2* (100 µM)	5 nmol/l	1 µl
Probe *trh1* (100 µM)	5 nmol/l	1 µl
Probe *trh2* (100 µM)	5 nmol/l	1 µl
Hybridization buffer (C.3.2.2.1)	1×	20 ml

C.4.2 Hybridization reaction with *toxR* probe

Different volumes may be used according to the type (tube or bag) and the size of the hybridization vessel used. A hybridization volume of 20 ml per hybridization reaction is used for tubes ranging from 35 × 150 mm to 80 × 300 mm. The reagents are listed in Table C.7.

Table C.7 — Hybridization reaction with *toxR* probe

Reagent	Final concentration	Volume per vessel
Probe *toxR* (100 µM)	5 nmol/l	1 µl
Hybridization buffer (C.3.2.2.1)	1×	20 ml

C.4.3 Hybridization conditions

Pre-hybridization (see 8.6.2.1 and 8.6.6.2) and hybridization (see 8.6.2.2 and 8.6.6.3) reactions should be performed at a temperature of 54 °C ± 1 °C.

C.4.4 Stringency washes conditions

Stringency washes steps (see 8.6.2.3 and 8.6.6.4) should be performed at a temperature of 65 °C ± 1 °C.

C.5 Controls

C.5.1 Process controls

C.5.1.1 Composition

See Table C.8.

Table C.8 — Strains for process control

Strains[a]	Expected result				
	tdh1	***tdh2***	***trh1***	***trh2***	***toxR***
V. parahaemolyticus[b]	+	+	−	−	+
	+	−	−	−	+
	−	+	−	−	+
V. parahaemolyticus	−	−	+	−	+
V. parahaemolyticus	−	−	−	+	+
V. parahaemolyticus	−	−	−	−	+
V. cholerae nonO1-nonO139	−	−	−	−	−

[a] The following strains may be used as process controls: *V. parahaemolyticus* ATCC 43996 (*tdh1*+, *tdh2*+, *trh1*-, *trh2*-, *toxR*+), *V. parahaemolyticus* CCUG 43364 (*tdh1*-, *tdh2*-, *trh1*+, *trh2*-, *toxR*+), *V. parahaemolyticus* WDCM 00037 (*tdh1*-, *tdh2*-, *trh1*-, *trh2*+, *toxR*+), *V. parahaemolyticus* WDCM 00185 (*tdh1*-, *tdh2*-, *trh1*-, *trh2*-, *toxR*+) and *V. cholerae* WDCM 00203 (*tdh1*-, *tdh2*-, *trh1*-, *trh2*-, *toxR*-). Strains with equivalent characteristics may be used.

[b] This subset of the process control may be represented by a single strain carrying simultaneously the *tdh1* and *tdh2* genes or by two separate strains carrying either the *tdh1* or the *tdh2* gene.

C.5.1.2 Preparation

Inoculate by means of a loop the surface of one TSA-S plate (B.1.2) with a culture of the appropriate microorganism (C.5.1.1). Incubate the plate together with the plates containing the samples under analysis (see 8.3). Proceed with the preparation of the membranes (see 8.5) and the hybridization as described in the procedure (see 8.6).

NOTE The set of control strains can be inoculated on a single plate previously divided into sections, taking care to indicate the position of each control on the membrane.

C.5.2 Hybridization controls

C.5.2.1 General

Hybridization controls may be prepared by different techniques, e.g. plasmid cloning of DNA sequences targeted by the probes, *in vitro* synthesis of probes' reverse complement sequences, polymerase chain reaction (PCR) amplification of targeted genes, etc.

Subclause C.5.2.2 describes the preparation of hybridization controls by PCR amplification of targeted genes, but other approaches are acceptable if they can be shown to provide equivalent results.

C.5.2.2 Preparation by PCR amplification

Perform polymerase chain reaction (PCR) amplification of *tdh1* and *tdh2*, *trh1*, *trh2* and *toxR* genes of *Vibrio parahaemolyticus*, including the region targeted by the DIG-labelled oligoprobes. Amplification can be achieved using the PCR protocols described in Table C.9.

Table C.9 — PCR protocols for hybridization control production

DNA sequence[a]	Protocol (reference)	Amplicon size (bp)
tdh1 and *tdh2*	[10]	424
trh1 and *trh2*	[11]	500
toxR	[12]	368

[a] The following strains may be used for production of hybridization controls by PCR: *V. parahaemolyticus* ATCC 43996 (*tdh1+*, *tdh2+*, *trh1-*, *trh2-*, *toxR+*), *V. parahaemolyticus* CCUG 43364 (*tdh1-*, *tdh2-*, *trh1+*, *trh2-*, *toxR+*) and *V. parahaemolyticus* WDCM 00037 (*tdh1-*, *tdh2-*, *trh1-*, *trh2+*, *toxR+*). Strains with equivalent characteristics may be used.

Purify the PCR products using DNA purification reagents.

Determine the absorbance at 260 nm of the DNA hybridization controls using spectrophotometry. Multiply the absorbance reading by 5×10^{-8} to give the concentration of DNA in grams per microlitre. Divide this number by the mass in grams of a single amplicon to calculate the concentration of the PCR products in copies per microlitre.

NOTE 1 The mass of an individual amplicon can be calculated by multiplying the amplicon length in the base pair by 607,4 (the relative molecular mass of an average base pair) and dividing by the Avogadro constant ($6{,}02 \times 10^{23}$).

Dilute the PCR products to a concentration of 1×10^{8} to 1×10^{10} copies per microlitre and prepare the hybridization control by spotting 1 µl of each suspension on a membrane strip. Link covalently the DNA to the membrane by UV cross-linking (see 8.5.2).

NOTE 2 The hybridization controls can be spotted on a single membrane or membrane strip, taking care to indicate the relative position of each control sequence.

Purified PCR products can be prepared in advance and can be stored at below –15 °C for up to one year. Membranes containing the hybridization control can be prepared in advance and can be stored and maintained dried up to 30 days at room temperature in the dark.

C.5.3 Detection control

A DIG-labelled DNA sequence may be synthetized by PCR with any sequence of choice or can be purchased from commercial suppliers.

If using a DIG-labelled DNA sequence synthetized by PCR, purify it using DNA purification reagents and determine the absorbance at 260 nm using spectrophotometry. Multiply the absorbance reading by 5×10^{-8} to give the concentration of DNA in grams per microlitre.

Dilute the DIG-labelled DNA to a concentration of 10 pg/µl and prepare the detection controls by spotting 1 µl of the DNA suspension on a membrane strip. Link covalently the DNA to the membrane by UV cross-linking (see 8.5.2).

The DIG-labelled DNA can be prepared in advance and can be stored at below –15 °C for up to one year. Membranes containing the detection control can be prepared in advance and can be stored and maintained dried up to 30 days at room temperature in the dark.

C.6 Limitations of the assay

Genes associated with *V. parahaemolyticus* pathogenicity have been sporadically detected in other *Vibrio* species (i.e. *V. alginolyticus*[6]) and genes analogous to the *Vibrio parahaemolyticus* virulence-related genes may occur in other Vibrionaceae species[13]. *V. parahaemolyticus* strains lacking the *tdh* and *trh* pathogenicity markers have been isolated from clinical cases[14].

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(*Continued from second cover*)

International Standard	*Corresponding Indian Standard*	*Degree of Equivalence*
ISO 7218 Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations	IS 16122 : 2013 Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations	Identical with ISO 7218 : 2007
ISO 11133 Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media	IS 17383 : 2020 Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media	Identical with ISO 11133 : 2014

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 2022 ‘Rules for rounding off numerical values (*second revision*)’.

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