
**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**

*Microbiologie de la chaîne alimentaire — Méthode horizontale pour
la détermination des *Vibrio* spp. —*

*Partie 2: Dénombrement de *Vibrio parahaemolyticus* total et
potentiellement entéropathogène dans les fruits de mer, par
hybridation des acides nucléiques*



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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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*.

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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

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^[2] 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/cm² 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 $15 \text{ min} \pm 1 \text{ min}$ at room temperature on a rocking platform at approximately $120 \text{ oscillations min}^{-1}$.

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 $120 \text{ oscillations min}^{-1}$ 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 $120 \text{ oscillations min}^{-1}$ 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 necessarily 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

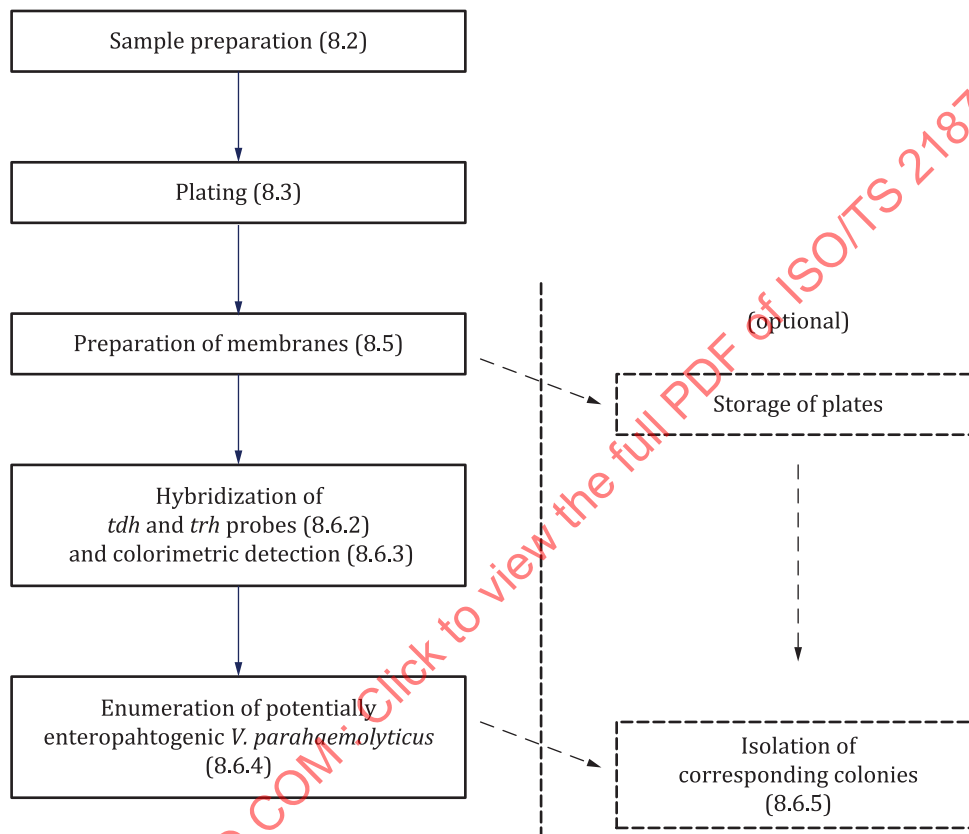


Figure A.1 — Enumeration of potentially enteropathogenic *V. parahaemolyticus*

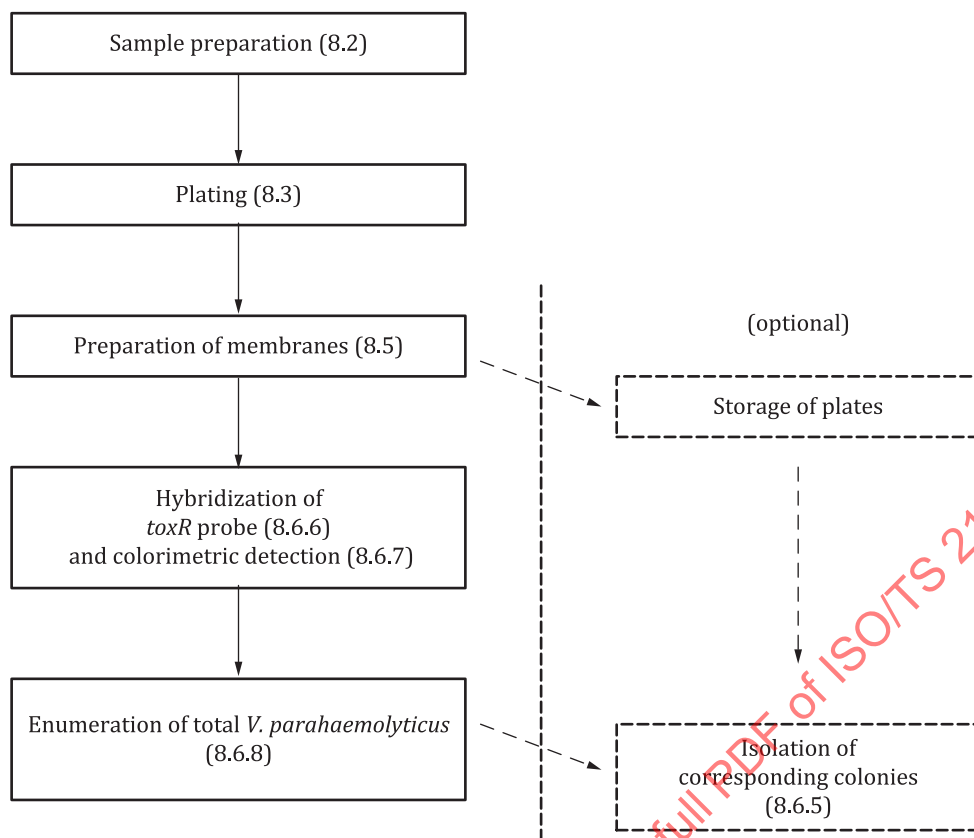


Figure A.2 — Enumeration of total *V. parahaemolyticus*

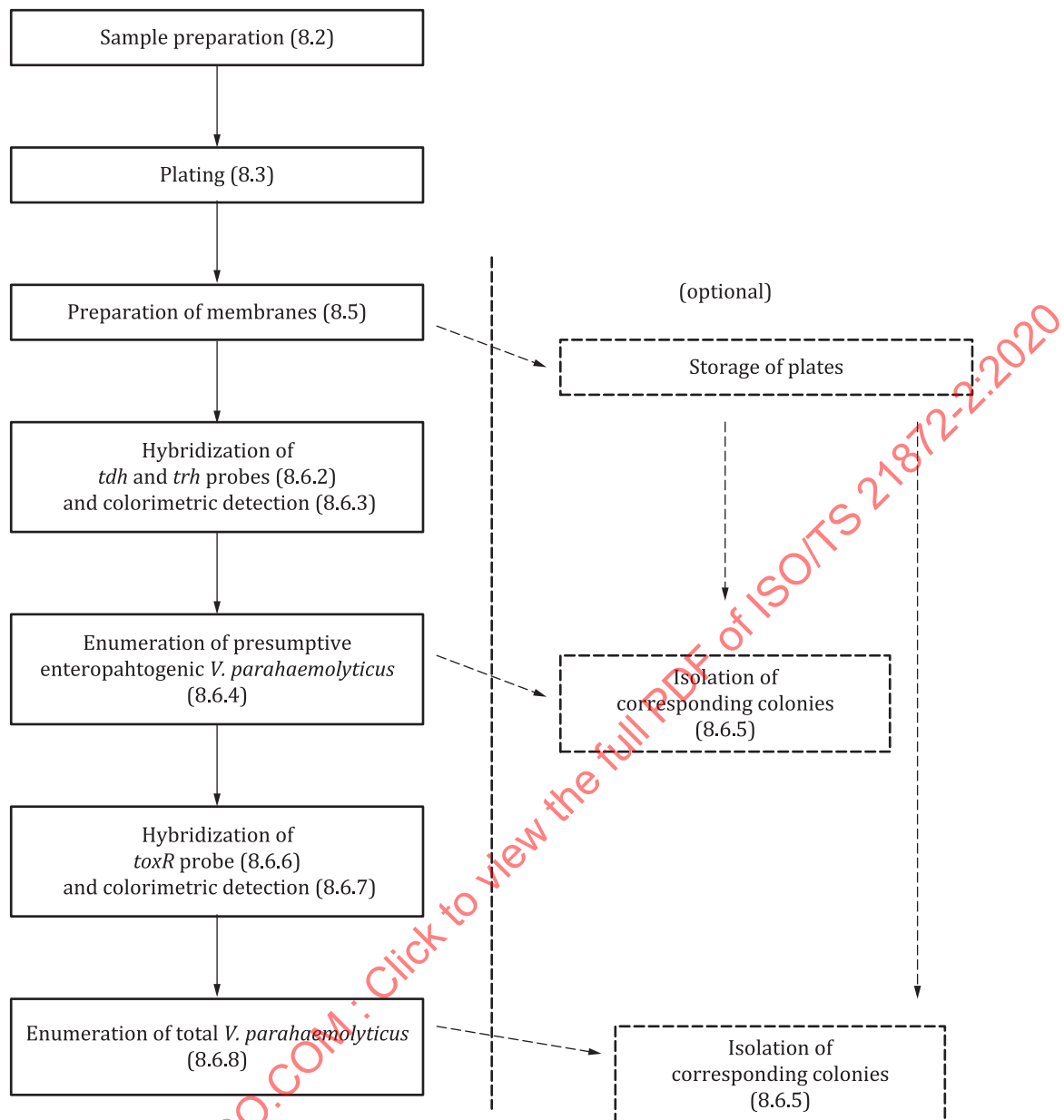


Figure A.3 — Enumeration of total and potentially enteropathogenic *V. parahaemolyticus*

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 $7,3 \pm 0,2$. 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	<i>Vibrio parahaemolyticus</i>	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 [C ₄ H ₁₁ N ₃ O ₃]	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 $7,0 \pm 0,2$. 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 [C ₆ H ₅ Na ₃ O ₇ •2H ₂ O]	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 $7,0 \pm 0,2$ and bring to 1 000 ml. Sterilize for 15 min at 121 °C. Store at $5\text{ °C} \pm 3\text{ °C}$ 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 $5\text{ °C} \pm 3\text{ °C}$ 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) [C ₁₂ H ₂₅ NaO ₄ S]	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) [C ₁₂ H ₂₅ NaO ₄ S]	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 [C ₄ H ₄ O ₄]	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) [C ₅₈ H ₁₁₄ O ₂₆]	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 [C ₄ H ₁₁ N ₃ O ₃]	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) [C ₄₀ H ₃₀ N ₁₀ O ₆ Cl ₂]	3,75 mg
5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP-T) [C ₈ H ₆ NO ₄ BrCIP × C ₇ H ₉ N]	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.

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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. parahaemolyticus*-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		
	<i>toxR</i>	<i>tdh</i>	<i>trh</i>		<i>toxR</i>	<i>tdh</i>	<i>trh</i>
<i>V. parahaemolyticus</i>	+	+	+	6	6	5	6
<i>V. parahaemolyticus</i> (including ATCC 43996, CCUG 43363)	+	+	–	24	24	24	0
<i>V. parahaemolyticus</i> (including WDCM 00037, CCUG 43364)	+	–	+	39	39	0	38
<i>V. parahaemolyticus</i> (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		
		<i>toxR</i>	<i>tdh</i>	<i>trh</i>
<i>Aeromonas caviae</i> WDCM 00062	1	0	0	0
<i>Aeromonas hydrophila</i> WDCM 00063, CCUG 14551T ^a	2	0	0	0
<i>Aeromonas veronii</i> CCUG 27821	1	0	0	0
<i>Bacillus cereus</i> WDCM 00001	1	0	0	0
<i>Bacillus subtilis</i> WDCM 00003	1	0	0	0
<i>Citrobacter freundii</i> ATCC 8090	1	0	0	0
<i>Clostridium perfringens</i> WDCM 00007	1	0	0	0
<i>Enterobacter aerogenes</i> WDCM 00175	1	0	0	0
<i>Enterobacter cloacae</i> WDCM 00082	1	0	0	0
<i>Enterococcus faecalis</i> WDCM 00087	1	0	0	0
<i>Escherichia coli</i> WDCM 00013, ATCC 15766	2	0	0	0
<i>Kocuria rhizophila</i> ATCC 9341	1	0	0	0
<i>Listeria innocua</i> WDCM 00017	1	0	0	0
<i>Listeria ivanovii</i> WDCM 00018	1	0	0	0
<i>Listeria monocytogenes</i> ATCC 19115	1	0	0	0
<i>Photobacterium angustum</i> ATCC 25915	1	0	0	0
<i>Photobacterium damsela</i> BCCM/LMG 7892	1	0	0	0
<i>Photobacterium phosphoreum</i> BCCM/LMG 4233	1	0	0	0
<i>Plesiomonas shigelloides</i> ATCC 14029	1	0	0	0
<i>Proteus hauseri</i> ATCC 13315	1	0	0	0
<i>Pseudomonas aeruginosa</i> WDCM 00025, WDCM 00026	2	0	0	0
<i>Saccaromices cerevisiae</i> WDCM 00058	1	0	0	0
<i>Salmonella enterica</i> ser. Enteritidis WDCM 00030	1	0	0	0
<i>Salmonella enterica</i> ser. Typhimurium WDCM 00031	1	0	0	0
<i>Shigella sonnei</i> ATCC 25931	1	0	0	0
<i>Staphylococcus aureus</i> WDCM 00034, WDCM 00211, WDCM 00131, ATCC 13565, ATCC 29971	5	0	0	0
<i>Staphylococcus epidermidis</i> WDCM 00036	1	0	0	0
<i>Staphylococcus xylosus</i> ATCC 29971	1	0	0	0
<i>Streptococcus pyogenes</i> ATCC 19615	1	0	0	0
<i>Vibrio alginolyticus</i> (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 <i>trh</i> + <i>V. alginolyticus</i> isolate ^[6] . The probes used in this method has been designed to exclude hybridization with this <i>trh</i> sequence as well as <i>tdh</i> and <i>trh</i> sequences currently identified in <i>Vibrio</i> species other than <i>V. parahaemolyticus</i> .				
^b Environmental isolates identified by partial sequencing of the <i>rpoA</i> gene ^[9] .				

Table C.2 (continued)

Species	Number of strains	Gene detected		
		<i>toxR</i>	<i>tdh</i>	<i>trh</i>
<i>Vibrio alginolyticus</i> ^a	1	n.d.	n.d.	0
<i>Vibrio anguillarum/ordalii</i> ^b	1	0	0	0
<i>Vibrio campbellii</i> ATCC 25920	1	0	0	0
<i>Vibrio cholerae</i> (including WDCM 00203, ATCC 14103, ATCC 14035, CCUG 33379, CCUG 45388)	54	0	0	0
<i>Vibrio cincinnatiensis</i> ATCC 33912	1	0	0	0
<i>Vibrio diabolicus</i> ^b	1	0	0	0
<i>Vibrio fluvialis</i> WDCM 00137	1	0	0	0
<i>Vibrio fisheri</i> BCCM/LMG 7891	1	0	0	0
<i>Vibrio harveyi</i> ATCC 14126	2	0	0	0
<i>Vibrio hollisae</i> ATCC 35912, NCTC 11645	2	0	0	0
<i>Vibrio metschnikovii</i> ^b	2	0	0	0
<i>Vibrio mimicus</i> ATCC 33653, ATCC 33654, NCTC 11435	3	0	0	0
<i>Vibrio natriegens</i> BCCM/LMG 10935	1	0	0	0
<i>Vibrio navarrensis</i> BCCM/LMG 15976	1	0	0	0
<i>Vibrio nereis</i> BCCM/LMG 3895	1	0	0	0
<i>Vibrio penaeicida</i> BCCM/LMG 19663	1	0	0	0
<i>Vibrio proteolyticus</i> ATCC 15338	1	0	0	0
<i>Vibrio rotiferanus</i> ^b	1	0	0	0
<i>Vibrio scophtalmi</i> BCCM/LMG 19158	1	0	0	0
<i>Vibrio tubiashii</i> CCUG 38428T	1	0	0	0
<i>Vibrio vulnificus</i> (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 <i>trh</i> + <i>V. alginolyticus</i> isolate ^[6] . The probes used in this method has been designed to exclude hybridization with this <i>trh</i> sequence as well as <i>tdh</i> and <i>trh</i> sequences currently identified in <i>Vibrio</i> species other than <i>V. parahaemolyticus</i> . ^b Environmental isolates identified by partial sequencing of the <i>rpoA</i> 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)
<i>toxR</i>	$1,13 \times 10^7$
<i>tdh1</i> and <i>tdh2</i> ^a	$5,18 \times 10^7$
<i>trh1</i>	$4,17 \times 10^6$
<i>trh2</i>	$1,35 \times 10^7$
^a The majority of the strains tested carry both <i>tdh1</i> and <i>tdh2</i> gene variants. The test was performed using a PCR amplified template containing both variants of the target gene.	