INTERNATIONAL STANDARD

ISO 15213-2

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Microbiology of the food chain — Horizontal method for the detection and enumeration of *Clostridium* spp. —

Part 2: Enumeration of *Clostridium perfringens* by colony-count technique

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche et le dénombrement de Clostridium *spp.* —

Partie 2: Dénombrement de Clostridium perfringens *par la technique de comptage des colonies*

Reference number
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Foreword

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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).

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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,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This first edition of ISO 15213-2 cancels and replaces ISO 7937:2004, which has been technically revised.

The main changes are as follows:

— the Scope has been expanded to samples from the primary production stage;

— the heat treatment of 10 min at 80 °C has been made optional, in the case of high background flora or for the enumeration of only spores of *Clostridium (C.) perfringens* present in the sample;

— the selective medium has been re-named from sulfite-cycloserine agar (SC) to tryptose sulfite cycloserine agar (TSC agar) without changes in the formulation;

— the confirmation methods described have been modified according to ISO 14189;

— the flow diagram in normative Annex A giving a short description of the procedure has been revised;

— in Annex B, criteria for the performance testing of culture media have been added;

— in Annex C (informative), the performance characteristics have been added;

— in Annex D (informative), two molecular methods have been added for differentiation between pathogenic and non-pathogenic *C. perfringens* and one molecular method for the differentiation of *C. perfringens* type A strains carrying a chromosomally encoded *cpe* gene or a plasmid encoded *cpe* gene.

A list of all parts in the ISO 15213 series can be found on the ISO website.

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

Clostridium (C.) perfringens is a Gram-positive, anaerobic, spore-forming bacterium. As a ubiquitous bacterium, *C. perfringens* is predominantly found in soil, but also in the intestinal tract of humans and animals. Therefore, the presence of *C. perfringens* in high numbers can be an indication of inadequate preparation or handling of food.

High numbers of *C. perfringens* in ready-to-eat-food can cause human illness, mainly diarrhoea. The strains are classified into toxin types, depending on the ability to produce different so called "major" and "minor" toxins. Food poisonings are caused by *C. perfringens* isolates with the ability to produce *C. perfringens* enterotoxin (CPE).

A characteristic feature is the heat resistance of the spores; they have the ability to germinate and multiply in ready-to-eat food after the cooking process. Ingestion of contaminated food is followed by gastrointestinal disease, when enzyme-resistant *C. perfringens* enterotoxins are set free during sporulation in the small intestine. The strains are classified into different types.

This document describes the horizontal method for the enumeration of *C. perfringens* in food, feed, environmental samples and samples from the primary production stage. The method for the enumeration of sulfite-reducing *Clostridium* spp. is described in ISO 15213-1. The method for the detection of *C. perfringens* is described in ISO/TS 15213-3. These three parts are published as a series of International Standards because the methods are closely linked to each other. These methods are often conducted in association with each other in a laboratory and the media and their performance characteristics can be similar.

The main technical changes listed in the Foreword, introduced in this document compared with ISO 7937:2004, are considered as major (see ISO 17468).

These changes have a major impact on the performance characteristics of the method.

Microbiology of the food chain — Horizontal method for the detection and enumeration of *Clostridium* spp. —

Part 2: Enumeration of *Clostridium perfringens* by colony-count technique

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for enumeration of *Clostridium perfringens* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

1 Scope

This document specifies the enumeration of *Clostridium (C.) perfringens* by colony-count technique.

This document is applicable to:

— products intended for human consumption;

— products for feeding animals;

— environmental samples in the area of food and feed production and handling;

— samples from the primary production stage.

NOTE This method has been validated in an interlaboratory study for the following food categories:

— ready-to-eat, ready-to-reheat meat products;

— eggs and egg products (derivates);

— processed fruits and vegetables;

— infant formula and infant cereals;

— multi-component foods or meal components.

It has also been validated for the following other categories:

— pet food and animal feed;

— environmental samples (food or feed production).

As this method has been validated for at least five food categories, this method is applicable for a broad range of food. For detailed information on the validation, see Clause 11 and Annex C. Since the method is not commonly used for samples in the primary production stage, this category was not included in the interlaboratory study. Therefore, no performance characteristics were obtained for this category.

This horizontal method was originally developed for the examination of all samples belonging to the food chain. Based on the information available at the time of publication of this document, this method is considered to be fully suited to the examination of all samples belonging to the food chain. However, because of the large variety of products in the food chain, it is possible that this horizontal method is not

appropriate in every detail for all products. Nevertheless, it is expected that the required modifications are minimized so that they do not result in a significant deviation from this horizontal method.

This technique is suitable for, but not limited to, the enumeration of microorganisms in test samples with a minimum of 10 colonies counted on a plate. This corresponds to a level of contamination that is expected to be higher than 10 cfu/ml for liquid samples or higher than 100 cfu/g for solid samples.

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 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of the food chain — General requirements and guidance for microbiological examinations*

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

ISO 19036:2019, , *Microbiology of the food chain — Estimation of measurement uncertainty for quantitative determinations*

3 Terms and definitions

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

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

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

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

3.1
presumptive *C. perfringens*
presumptive *Clostridium perfringens*
spore-forming bacteria forming countable typical colonies in a specific selective medium under obligate anaerobic conditions

Note 1 to entry: Presumptive *C. perfringens* are spore-forming bacteria that are able to produce typical colonies under the conditions specified in this document.

3.2
confirmed *C. perfringens*
confirmed *Clostridium perfringens*
bacteria that produce characteristic colonies in the specified selective medium under obligate anaerobic conditions and possess the enzyme acid phosphatase

3.3
human pathogenic *C. perfringens*
human pathogenic *Clostridium perfringens*
confirmed *C. perfringens* strains (3.2) which possess the ability to produce *C. perfringens* enterotoxin (CPE), encoded by the *cpe* gene

Note 1 to entry: The *cpe* gene can be located either chromosomally or on plasmids. These isolates are able to produce CPE in the small intestine on sporulation and cause human illness.

3.4
enumeration of *C. perfringens*
enumeration of *Clostridium perfringens*
determination of the number of colony-forming units (cfu) of *confirmed C. perfringens* (3.2) found per gram, per millilitre, per square centimetres or per sampling device when a specified test is conducted

Note 1 to entry: Specified tests are given in Clause 9.

4 Principle

4.1 General

A specified quantity of the liquid test sample, or of an initial suspension in the case of other products, is dispensed into an empty Petri dish and mixed well with a specified molten agar culture medium to form a poured plate. Additional plates are prepared under the same conditions using decimal dilutions of the test sample. After solidification of the agar medium, an overlay is used to prevent the development of spreading colonies on the surface of the medium. If it is the intention to count only spores, a heat treatment of 10 min at 80 °C is performed before plating. Additionally, a method for molecular differentiation between human pathogenic and non-pathogenic *C. perfringens* strains is described in Annex D.

When the number of cfu is expected to be at or near the limit of determination, the use of duplicate plates is preferable. If duplicate plates are used, the minimum for the sum of colonies on both plates should be 10 colonies. In this case, the level of contamination is expected to be higher than 5 cfu/ml for liquid samples or higher than 50 cfu/g for solid samples.

A pour-plate technique with overlay is especially suited for the enumeration of products expected to contain spreading colonies that can obscure colonies of the target microorganisms.

The enumeration of *C. perfringens* requires four successive stages as specified in the normative Annex A.

4.2 Preparation of dilutions

For the preparation of decimal dilutions from the test portion, follow the procedure as specified in the ISO 6887 series.

4.3 Enumeration

Petri dishes are inoculated with a specified quantity of the test sample if the initial product is liquid, or a specified quantity of the initial suspension, in the case of other products. Additional Petri dishes are inoculated, under the same conditions, using decimal dilutions of the test sample or of the initial suspension. A selective medium is added (pour-plate technique) and then overlaid with the same medium.

The plates are incubated under anaerobic conditions at 37 °C for 20 h. After incubation, the number of typical colonies, which show black or grey to yellow-brown staining, are counted. The colour of the colonies and the surrounding zone changes due to the formation of iron(II)sulfide as a result of the reaction between sulfide ions and trivalent iron [Fe(III)] present in the medium.

4.4 Confirmation

Confirmatory tests are carried out. The result is calculated as the colony count of confirmed *C. perfringens* per sample volume. Additionally, the method described in Annex D can be used for molecular differentiation between human pathogenic and non-pathogenic *C. perfringens* strains.

5 Culture media and reagents

Follow current laboratory practices in accordance with ISO 7218. The composition of culture media and reagents and their preparation are specified in Annex B. For performance testing of culture media, follow the procedures in accordance with ISO 11133 and Annex B.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications. The usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following shall be used.

6.1 Appropriate apparatus for achieving an anaerobic atmosphere, a jar that can be hermetically sealed or any other appropriate equipment which enables anaerobic atmosphere conditions to be maintained for the total incubation time of the culture medium. Other systems of equivalent performance, such as anaerobic cabinets, may be used. Follow the manufacturer's instructions for installation and maintenance.

The composition of the atmosphere required can be achieved by means of the addition of a gas mixture (e.g. from a gas cylinder) after evacuation of air from the jar, by displacement of the atmosphere in a cabinet or by any other appropriate means (such as commercially available gas packs). In general, anaerobic incubation requires an atmosphere of less than 1 % volume fraction oxygen, 9 % volume fraction to 13 % volume fraction carbon dioxide.

6.2 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**.

6.3 Freezer, capable of operating at –20 °C ± 2 °C and at –70 °C ± 3 °C.

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

6.5 pH-meter, having a maximum permissible error of calibration of ±0,1 pH unit at 25 °C.

6.6 Refrigerator, capable of operating at 5 °C ± 3 °C.

6.7 Sterile bottles, flasks or **tubes**, of appropriate capacity. Bottles, flasks or tubes with non-toxic metallic or plastic screw-caps may be used.

6.8 Sterile graduated pipettes or **automatic pipettes**, of nominal capacities 10 ml, 1 ml and 0,1 ml.

6.9 Sterile loops, of approximate diameter 3 mm (10 µl volume), and of 1 µl volume, or inoculation needle or wire.

6.10 Sterile Petri dishes, with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm).

6.11 Thermostatically controlled water bath, capable of operating at 44 °C to 47 °C and 80 °C ± 2 °C.

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 the sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

Recommended sampling techniques are given in the following documents:

— ISO/TS 17728 for food and animal feed;

— ISO 707 for milk and milk products;

— ISO 6887-3 for raw molluscs, tunicates and echinoderms from primary production areas;

— ISO 13307 for primary production stage;

— ISO 17604 for carcasses;

— ISO 18593 for surfaces.

It is important that the laboratory receives a sample that is representative of the product under consideration. The sample should not have been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned. Follow the procedures as specified in the ISO 6887 series. If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 General

The procedure as given in Annex A shall be followed.

9.2 Test portion, initial suspension and dilutions

Follow the procedures in accordance with the ISO 6887 series and the specific International Standard dealing with the product concerned. Prepare a single decimal dilution series from the test portion if the product is liquid, or from the initial suspension in the case of other products.

9.3 Heat treatment to select spores

If it is the intention to count only spores, heat the decimal dilution series to 80 °C in a water bath (6.11) for 10 min ± 1 min. Heat treatment shall be given within 15 min after preparation of the initial suspension to avoid germination of spores. If the tube is not placed in the water bath within 15 min, it should be placed immediately in melting ice for a maximum of 2 h.

The temperature during heat treatment should be monitored by placing an appropriate thermometer in a reference bottle of the same size as the sample bottle and containing the same volume of water at the same initial temperature as the sample being treated (6.7). The tubes should not be hermetically sealed during the heat treatment. The time taken to reach 80 °C shall not exceed 5 min and can be minimized by ensuring the water level to be at least 4 cm above the level of the sample and that the water bath is equipped with a circulating-water pump to maximize heat exchange.

Start the time of heating (10 min) when the temperature of the reference sample has reached 80 °C. After heat treatment, the samples should be cooled immediately till approximately 20 °C.

Heat treatment should also reduce the competitive flora in some matrices containing a high level of background flora (e.g. liquid whey, silage).

9.4 Inoculation and incubation

9.4.1 Take two sterile Petri dishes with a diameter of approximately 90 mm (6.10). Transfer to each dish, by means of a sterile pipette (6.8), 1 ml of the test sample if liquid, or 1 ml of the initial suspension (10^{-1} dilution) in the case of other products. If plates from more than one dilution are prepared, this may be reduced to one dish (see ISO 7218). When, for certain products, it is necessary to estimate low numbers of *C. perfringens*, the limit of enumeration may be lowered by a factor of 10 by examining 10 ml of the initial suspension in three large (140 mm) Petri dishes (6.10).

9.4.2 Take one other sterile Petri dish (6.10). Use another sterile pipette (6.8) to dispense 1 ml of the 10^{-1} dilution (liquid product) or 1 ml of the 10^{-2} dilution (other products).

9.4.3 If necessary, repeat the procedure with further dilutions, using a new sterile pipette (6.8) for each decimal dilution.

9.4.4 If appropriate and possible, select only the critical dilution steps (at least two consecutive decimal dilutions) for the inoculation of the Petri dishes (6.10) that will give colony counts of between 10 and 150 colonies per plate (on 90 mm Petri dishes) or between 10 and 365 colonies per plate (on 140 mm Petri dishes).

9.4.5 Pour about 12 ml to 15 ml for 90 mm Petri dishes or 30 ml to 35 ml for 140 mm Petri dishes of the tryptose sulfite cycloserine agar (TSC agar) (see B.2), molten and tempered at 44 °C to 47 °C (6.11), into each Petri dish (6.10).

9.4.6 Carefully mix the inoculum with the medium by rotating the Petri dishes and allow the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface.

9.4.7 After complete solidification, pour about 5 ml of the TSC agar (see B.2) for 90 mm Petri dishes (6.10) or 12 ml for 140 mm Petri dishes (6.10) as overlay, to prevent the development of spreading colonies on the surface of the medium. Allow to solidify as specified in 9.4.6.

9.4.8 Invert the plates obtained in 9.4.7 and incubate (6.4) the plates at 37 °C in an anaerobic atmosphere (6.1).

9.5 Enumeration of typical colonies

9.5.1 After 20 h ± 2 h of incubation, examine the plates (see 9.4.8) for presumptive *C. perfringens*. Longer incubation can result in excess blackening of the plates.

Typical colonies, which show black or grey to yellow-brown staining (even if the colour is faint) on the TSC agar, are counted.

Upon removal of the plates from the anaerobic atmosphere, plates shall be counted within 30 min as the colour of the colonies can rapidly fade and disappear upon exposure to oxygen. If anaerobic jars are used, the plates should be checked jar by jar or in small portions if the incubation was performed in an anaerobic incubator (6.1, 6.4).

NOTE Diffuse, unspecific blackening of the medium can occur. The growth of anaerobic bacteria, which produce hydrogen (not H_2S), can also reduce the sulfite present and lead to a general blackening of the medium, which makes enumeration of typical colonies difficult.

9.5.2 Select the plates (see 9.5.1) containing less than 150 presumptive colonies (for 90 mm Petri dishes) or less than 365 colonies (for 140 mm Petri dishes); count these colonies and record the number of presumptive colonies per dish. Then choose, at random, five such colonies from each dish for confirmation (see 9.6). For enumeration of plates with low or high numbers of presumptive colonies, see ISO 7218.

9.6 Confirmation of *C. perfringens*

9.6.1 Selection of colonies for confirmation

9.6.1.1 For confirmation, take five presumptive colonies from each dish retained for enumeration (see 9.5.2). If more than one morphology is present among the colonies, select one of each morphology for subculture and confirmation.

9.6.1.2 Streak each of the selected colonies with a sterile loop (6.9) onto one non-selective blood agar plates, e.g. Columbia blood agar (see B.3). If blood is not available, Columbia agar base or another nutrient-rich medium (e.g. tryptone soya agar or brain heart infusion agar) can be used.

Allow the plates to equilibrate at room temperature if they were stored at a lower temperature. If necessary, dry the surface of the plates before use (see ISO 11133).

Several isolates can be streaked onto identified sectors. Streaks should obtain well-isolated colonies.

Incubate the plates in an anaerobic atmosphere (6.1) at 37 °C (6.4) for 20 h ± 2 h. Right after incubation, select well-isolated freshly grown colonies for confirmation. Confirmation may be done either by the acid phosphatase test or by the sulfite indole motility (SIM) agar test.

NOTE Alternative procedures (see ISO 7218) can be used to confirm the isolate(s) as *C. perfringens*, provided that the suitability of the alternative procedure has been validated (see ISO 16140-4 or ISO 16140-6).

After incubation, these plates can be refrigerated at 5 °C (6.6) for a maximum of 48 h before reading. For plates which were incubated anaerobically, maintain the anaerobic atmosphere.

9.6.2 Acid phosphatase test

9.6.2.1 It is known that, beside *C. perfringens*, some other *Clostridium* strains (e.g. some strains of *C. baratii*) can produce acid phosphatase, but this ability is very limited. Therefore, only a very low percentage of false positives is expected.

9.6.2.2 Colonies grown anaerobically on blood or nutrient agar plates are spread on filter paper and 2 to 3 drops of the acid phosphatase reagent (B.4) are placed onto the colonies. If a commercially available test kit is used, follow the manufacturer's instructions.

NOTE It is possible to drip acid phosphatase reagent on colonies, if no further investigation of the colonies is needed.

9.6.2.3 A purplish colour developed within 3 min to 4 min is considered as a positive reaction.

9.6.3 Sulfite indole motility (SIM) agar test

Colonies grown anaerobically on blood agar plates or nutrient agar plates are stabbed into SIM tubes (B.5). The tubes are incubated for 22 h ± 2 h at 37 °C (6.4). After incubation the tubes are read for:

— sulfite production: tubes showing blackening are positive;

— motility: tubes showing growth outside the inoculation stab are positive;

— indole production: tubes giving a red coloured ring directly after adding Kovacs reagent (B.6) are positive.

C. perfringens is positive for sulfite production and negative for indole production and motility.

9.6.4 Differentiation between human pathogenic and non-pathogenic *C. perfringens* strains (optional)

Additionally, the method described in Annex D can be used for molecular differentiation between human pathogenic and non-pathogenic *C. perfringens* strains.

9.6.5 Interpretation

C. perfringens produces black or grey to yellow brown colonies on TSC agar, even if the colour is faint, and possesses acid phosphatase, or is positive for sulfite production, negative for indole production and motility in SIM agar.

10 Expression of results

For calculation of the results, follow the procedure(s) in accordance with ISO 7218. Calculate and report the results as the number of confirmed *C. perfringens* or, if the method of Annex D was also used for differentiation, of confirmed human pathogenic *C. perfringens,* in cfu per gram, per millilitre or per square centimetre. When the sampled area is not known, report as per sampling device, such as a cloth, sponge swab or stick.

If heat pre-treatment for the selection of spores (9.3) was used, the result is reported as number of confirmed *C. perfringens* spores or, if the method of Annex D was also used for differentiation, of confirmed human pathogenic *C. perfringens* spores in cfu per gram, per millilitre, per square centimetre or per sampling device.

In cases when no typical colonies of *C. perfringens* have been detected, or when no typical colonies are confirmed as *C. perfringens*, follow ISO 7218 for the expression of results for special cases.

11 Validation of the method

11.1 Interlaboratory study

Results of the interlaboratory study (step 6 in ISO 17468) to determine the performance characteristics of the method are described in 11.2.

11.2 Performance characteristics

The performance characteristics of the method (repeatability and reproducibility standard deviations) were determined in an interlaboratory study. It is possible that the values derived from the interlaboratory study are not applicable to concentration ranges and (food) categories other than those used in the study. All data are given in Annex C.

A summary of the interlaboratory repeatability standard deviation (s_r) is given in Table 1.

Table 1 — Summary of s_r values from the interlaboratory study

(Food) category	(Food) item	s_r values of low inoculation level	s_r values of medium inoculation level	s_r values of high inoculation level
Ready-to-eat, ready-to-reheat meat products	Corned beef	0,292	0,272	0,100
Ready-to-eat, ready-to-reheat fishery products	Canned fish	0,204	0,193	0,035
Multi-component foods or meal components	Instant soup	0,208	0,080	0,070
Infant formula and infant cereals	Powdered infant formula	0,160	0,200	0,098
Processed fruits and vegetables	Canned pineapple	0,091	0,070	0,061
Environmental samples (food or feed production)	Environmental swab	0,160	0,117	0,065
Pet food and animal feed	Feed silage	0,227	0,098	0,134

A summary of the interlaboratory reproducibility standard deviation (s_R) is given in Table 2.

Table 2 — Summary of s_R values from the interlaboratory study

(Food) category	(Food) item	S_R values of low inoculation level	S_R values of medium inoculation level	S_R values of high inoculation level
Ready-to-eat, ready-to-reheat meat products	Corned beef	0,408	0,653	0,383
Ready-to-eat, ready-to-reheat fishery products	Canned fish	0,356	0,543	0,309
Multi-component foods or meal components	Instant soup	0,621	0,654	0,688
Infant formula and infant cereals	Powdered infant formula	0,396	0,403	0,356
Processed fruits and vegetables	Canned pineapple	0,453	0,637	0,797
Environmental samples (food or feed production)	Environmental swab	0,457	0,475	0,629
Pet food and animal feed	Feed silage	0,626	0,606	0,793

12 Test report

The test report shall specify at least the following:

— the test method used, with reference to this document, i.e. ISO 15213-2:2023;

— the sampling method used, if known;

— all operating conditions not specified in this document, or regarded as optional or informative (including informative annexes), 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;

— when necessary or if requested by the client, an estimate of the measurement uncertainty of a quantitative test result, in accordance with ISO 19036:2019, Clause 9.

13 Quality assurance

The laboratory should have a quality control system to ensure that the equipment, reagents and techniques are suitable for the method. The use of positive controls, negative controls and blanks are part of the method. Performance testing of culture media is specified in Annex B and described in ISO 11133.

Annex A
(normative)

Flow diagram of the procedure

Figure A.1 shows the diagram of procedure for the enumeration of *C. perfringens* by colony-count technique in food, animal feed, environmental and primary production stage samples.

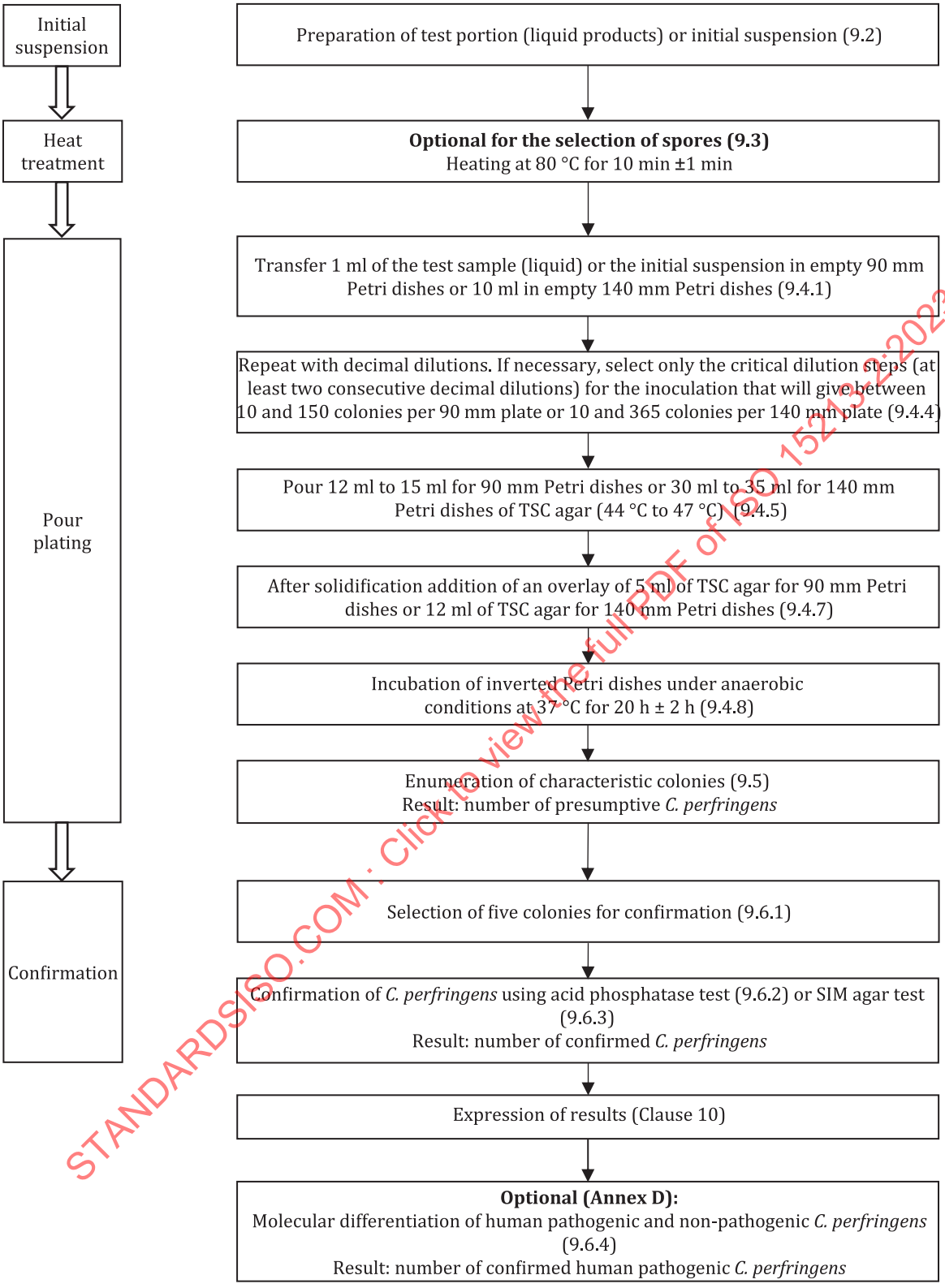

Figure A.1 — Flow diagram of the procedure for enumeration of *C. perfringens* by colony-count technique

Annex B
(normative)

Culture media and reagents

B.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf life of the media indicated in this annex has been determined in some studies. The user shall verify this under their own storage conditions (in accordance with ISO 11133).

Performance testing of culture media is described in B.7.

B.2 Tryptose sulfite cycloserine agar (TSC agar) [18]

NOTE TSC agar is also known under the name "sulfite-cycloserine agar (SC agar)".

B.2.1 Base medium

B.2.1.1 Composition

Peptone [a]		15,0 g
Enzymatic digest of soya		5,0 g
Yeast extract		5,0 g
Sodium disulfite (sodium metabisulfite), anhydrous ($Na_2S_2O_5$)	(CAS No.® [d] 7681-57-4)	1,0 g
Iron(III) ammonium citrate [b] ($C_6H_8FeNO_4$)	(CAS No.® 1185-57-5)	1,0 g
Agar [c]		9,0 g to 18,0 g
Water		1 000 ml

[a] For example, enzymatic digest of casein.

[b] This reagent should contain at least 150 g/kg of iron.

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

[d] CAS No. ® is a trademark of CAS corporation. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

B.2.1.2 Preparation

Suspend the ingredients in the water and dissolve by heating and stirring. Sterilize by autoclaving (6.2) at 121 °C for 15 min.

Allow to cool to 44 °C to 47 °C (6.11). The base medium may be stored at 5 °C (6.6) for up to four weeks in closed containers or tubes (6.7).

Prior to the preparation of the complete medium, the stored medium is melted completely and cooled down to 44 °C to 47 °C (6.11) before adding the D-cycloserine solution (see B.2.2).

B.2.2 D-cycloserine solution

B.2.2.1 Composition

D-cycloserine ($C_3H_6N_2O_2$)	(CAS No. ® 68-41-7)	4,0 g
Water		100 ml

B.2.2.2 Preparation

Dissolve the D-cycloserine in the water and filter through a membrane of 0,2 µm pore size. Dispense aseptically into suitable volumes, store at −20 °C (6.3) and use within four weeks of preparation. Alternatively, the dispensed volumes of cycloserine can be stored at −70 °C (6.3) for a maximum of 12 months.

B.2.3 Complete medium

B.2.3.1 Composition

Base medium (B.2.1)	100 ml
D-cycloserine solution (B.2.2)	1 ml

B.2.3.2 Preparation

Immediately before use in the pour-plate method (see 9.4.5), to each 100 ml of sterile molten base (B.2.1) cooled to 44 °C to 47 °C (6.11), add 1 ml of D-cycloserine solution (B.2.2) to obtain a final D-cycloserine concentration of 0,4 g per litre TSC agar. The final pH of the medium should correspond to 7,6 ± 0,2 at 25 °C (6.5).

B.3 Columbia blood agar (CBA)

B.3.1 Columbia blood agar base

B.3.1.1 Composition

Enzymatic digest of animal tissue		23,0 g
Starch soluble ($C_{12}H_{22}O_{11}$)	(CAS No. ® 9005-84-9)	1,0 g
Sodium chloride (NaCL)	(CAS No. ® 7647-14-5)	5,0 g
Agar [a]		8,0 to 18,0 g
Water		1 000 ml

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

B.3.1.2 Preparation

Dissolve the ingredients in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C (6.5).

Dispense the medium in flasks (6.7) of suitable capacity to obtain portions appropriate for the test.

Sterilize for 15 min in the autoclave (6.2) set at 121 °C.

B.3.2 Defibrinated blood (horse or sheep blood)

B.3.3 Complete base

B.3.3.1 Composition

Base (B.3.1)	100 ml
Defibrinated blood (B.3.2)	5 ml

B.3.3.2 Preparation

Add the blood to the base previously cooled to 44 °C to 47 °C (6.11). Mix well.

B.3.3.3 Preparation of blood agar plates

Dispense the medium (B.3.3.1) into sterile Petri dishes (6.10) in portions appropriate for the test. Allow to solidify.

Immediately before use, dry the agar plates following the procedure as given by ISO 11133.

Store the poured plates, protected from drying, at 5 °C (6.6) for up to four weeks.

B.4 Acid phosphatase reagent

WARNING — Fast Blue B Salt is toxic and can cause cancer. Appropriate precautions shall be taken when weighing out, preparing and discarding the reagent.

B.4.1 Composition

1-naphthylphosphate disodium salt ($C_{10}H_7Na_2O_4P$) [a]	(CAS No. ® 2183-17-7)	0,4 g
Fast Blue B Salt (o-Dianisidine bis(diazotized) zinc double salt)	(CAS No. ® 14263-94-6)	0,8 g
Acetate buffer (pH 4,6 ± 0,2)		20 ml

[a] Instead of 1-naphthylphosphate disodium salt, 1-naphthylphosphate monosodium salt (CAS No. ® 81012-89-7) can be used.

B.4.2 Preparation

Prepare the acetate buffer by dissolving 0,3 ml glacial acetic acid (CAS No. ® 64-19-7) and 0,4 g sodium acetate (CAS No. ® 127-09-3) in deionized water and make up to 1 000 ml. Alternatively use a commercially available product.

Dissolve the ingredients in the acetate buffer and allow to stand for 60 min ± 5 min at 5 °C (6.6) to allow precipitation.

Then filter the solution through a fluted filter to remove the precipitate. Store the prepared solution at 5 °C (6.6) for no longer than two weeks. If precipitation occurs again, filter again once more before use.

NOTE Acid phosphatase composition and preparation is the same as in ISO 14189, but different from the original description in the literature.

B.5 Sulfite indole motility agar (SIM agar)

B.5.1 Composition

Peptone [a]		6,0 g
Enzymatic digest of soya		20 g
Ferrous ammonium sulfate (anhydrous) ($FeH_8N_2O_8S_2$)	(CAS No. ® 10045-89-3)	0,2 g
Sodium thiosulfate ($Na_2O_3S_2$)	(CAS No. ® 7772-98-7)	0,2 g
Agar [b]		2,5 to 4,5 g
Water		1 000 ml

[a] For example, enzymatic digest of casein.

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

B.5.2 Preparation

Dissolve the ingredients in the water, by heating if necessary. Fill 10 ml into tubes (6.7) and sterilize for 15 min in the autoclave set at 121 °C (6.2). Allow to cool in upright position.

B.6 Kovacs reagent

B.6.1 Composition

4-(methylamino)benzaldehyde (C_8H_9NO)	(CAS No. ® 556-21-8)	5,0 g
Hydrochloric acid, ρ – 1,18 g/ml to 1,19 g/ml		25 ml
2-Methyl-2-butanol ($C_5H_{12}O$)	(CAS No. ® 75-85-4)	75 ml

B.6.2 Preparation

Mix the components and store the reagent in a closed flask (6.7) in the dark at 5 °C for up to six months.

B.7 Performance testing

For the definition of selectivity and productivity, refer to ISO 11133. In general, follow the procedures for performance testing described in ISO 11133. Table B.1 provides performance testing information for quality assurance of the culture media and reagents.

Table B.1 — Performance testing for the quality assurance of the culture media and reagents

Medium/ reagent	Function	Incubation	Control strains	WDCM numbers[a]	Reference medium	Method of control	Criteria and characteristic reactions[c, e]
TSC	Productivity	(20 ± 2) h / (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080 00174	A suitable nonselective medium for anaerobes	Quantitative	$P_R \geq 0,5$ ($P_R \geq 0,7$ when compared to TSC batch already validated), black colonies
	Selectivity		*Bacillus subtilis* subsp. *spizizenii*	00003	-	Qualitative	Total inhibition (0)
CBA	Productivity	(20 ± 2) h / (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b]	-	Qualitative	Good growth (2), colonies with beta-haemolysis
Acid phosphatase	Confirmation	In 3 min to 4 min reaction time	*Clostridium perfringens*[d]	00007 00080 00174	-	Qualitative	Positive reaction: Purplish colour
			Bacillus subtilis subsp. *spizizenii*[d]	00003	-	Qualitative	Negative reaction: No colour change
SIM agar in combination with Kovacs reagent	Confirmation	(22 ± 2) h / (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*[d]	00007 00080 00174	-	Qualitative	Positive reaction: Good growth (2), blackening of the tube, no growth outside the inoculation stab and no red coloured ring after adding Kovacs reagent
			Escherichia coli[d]	00012 or 00013	-	Qualitative	Negative reaction: Good growth (2), no blackening of the tubes, possible growth outside the inoculation stab and red coloured ring after adding Kovacs reagent

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

[b] Strain to be used as a minimum.

[c] P_R = productivity ratio.

[d] Strain free of choice; one of the strains has to be used as a minimum.

[e] Growth is categorized as 0: no growth; 1: weak growth (partial inhibition); 2: good growth (see ISO 11133).

Annex C
(informative)

Method validation studies and performance characteristics

An interlaboratory study (see References [17] and [24]) involving 17 laboratories in 15 countries was organized by the Dutch National Institute of Public Health (RIVM) in January 2000, and was carried out on cheese, meat, dried animal feed and a reference material for the validation of ISO 7937. The food/feed samples were each tested at three different levels of contamination with *C. perfringens*. Because the composition of TSC agar was changed, a new interlaboratory study for this document was carried out in two parts in 2019 and 2021.

The first part of the interlaboratory study involving 11 laboratories in 5 countries was carried out on instant soup, canned pineapple, environmental swabs and feed silage. The (food) samples were each tested at a low, medium and high level of contamination. The study was organized in 2019 by Merck KGaA, Darmstadt, Germany, FrieslandCampina and the Bavarian Health and Food Safety Authority.

The second part of the interlaboratory study involving 10 laboratories in 5 countries was carried out on canned fish, canned meat and powdered infant formula. The food samples were each tested at a low, medium and high level of contamination. The study was organized in 2021 by Merck KGaA, Darmstadt, Germany, FrieslandCampina and the Bavarian Health and Food Safety Authority.

The method submitted to both parts of the interlaboratory study was that of this document.

The values of the performance characteristics, for each (food) item and category, derived from this interlaboratory study, are shown in Tables C.1 to C.7 and were calculated in accordance with ISO 17468.

Table C.1 — Results of data analysis obtained with instant soup (category: multi-component foods or meal components)

Parameter	Low level	Medium level	High level
Number of participating collaborators	11	11	11
Number of collaborators retained after evaluation of the data	11	11	11
Number of samples	22	22	22
Number of sample results retained after evaluation of the data	22	22	22
Mean value Σa ($\log_{10}$ cfu/g)	0,647	0,683	0,718
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,208	0,080	0,070
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,621	0,654	0,688
NOTE Strain used for inoculation: *Clostridium perfringens* WDCM 00201.			

Table C.2 — Results of data analysis obtained with canned pineapple (category: processed fruits and vegetables)

Parameter	Low level	Medium level	High level
Number of participating collaborators	11	11	11
Number of collaborators retained after evaluation of the data	9	10	11
Number of samples	18	20	22
Number of sample results retained after evaluation of the data	18	20	22
NOTE Strain used for inoculation: *Clostridium perfringens* WDCM 00201.			

Table C.2 *(continued)*

Parameter	Low level	Medium level	High level
Mean value Σa ($\log_{10}$ cfu/g)	0,477	0,668	0,832
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,091	0,070	0,061
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,453	0,637	0,797
NOTE Strain used for inoculation: *Clostridium perfringens* WDCM 00201.			

Table C.3 — Results of data analysis obtained with environmental swab (category: environmental samples (food or feed production))

Parameter	Low level	Medium level	High level
Number of participating collaborators	10	10	11
Number of collaborators retained after evaluation of the data	10	10	11
Number of samples	20	20	22
Number of sample results retained after evaluation of the data	20	20	22
Mean value Σa ($\log_{10}$ cfu/g)	0,477	0,498	0,657
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,160	0,117	0,065
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,457	0,475	0,629
NOTE Strain used for inoculation: *Clostridium perfringens* WDCM 00201.			

Table C.4 — Results of data analysis obtained with feed silage (category: pet food and animal feed)

Parameter	Low level	Medium level	High level
Number of participating collaborators	8	8	10
Number of collaborators retained after evaluation of the data	8	8	10
Number of samples	16	16	20
Number of sample results retained after evaluation of the data	16	16	20
Mean value Σa ($\log_{10}$ cfu/g)	0,662	0,642	0,831
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,227	0,098	0,134
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,626	0,606	0,793
NOTE Strain used for inoculation: *Clostridium perfringens* WDCM 00201.			

Table C.5 — Results of data analysis obtained with canned fish (category: ready-to-eat, ready-to-re-heat fishery products)

Parameter	Low level	Medium level	High level
Number of participating collaborators	9	9	9
Number of collaborators retained after evaluation of the data	9	9	9
Number of samples	18	18	18
Number of sample results retained after evaluation of the data	18	18	18
Mean value Σa ($\log_{10}$ cfu/g)	0,226	0,429	0,566
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,204	0,193	0,035
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,356	0,543	0,309
NOTE Strain used for inoculation: *Clostridium perfringens* WDCM 00201.			

Table C.6 — Results of data analysis obtained with canned corned beef (category: ready-to-eat, ready-to-reheat meat products)

Parameter	Low level	Medium level	High level
Number of participating collaborators	9	9	9
Number of collaborators retained after evaluation of the data	8	8	8
Number of samples	16	16	16
Number of sample results retained after evaluation of the data	16	16	16
Mean value Σa ($\log_{10}$ cfu/g)	0,223	0,427	0,565
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,292	0,272	0,100
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,408	0,653	0,383
NOTE Strain used for inoculation: *Clostridium perfringens* WDCM 00201.			

Table C.7 — Results of data analysis obtained with powdered infant formula (category: infant formula and infant cereals)

Parameter	Low level	Medium level	High level
Number of participating collaborators	12	12	12
Number of collaborators retained after evaluation of the data	8	8	8
Number of samples	16	16	16
Number of sample results retained after evaluation of the data	16	16	16
Mean value Σa ($\log_{10}$ cfu/g)	0,251	0,393	0,556
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,160	0,200	0,098
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,396	0,403	0,356
NOTE Strain used for inoculation: *Clostridium perfringens* WDCM 00201.			

Annex D
(informative)

Molecular differentiation between pathogenic and non-pathogenic *C. perfringens*

D.1 General

This annex describes two methods, one gel-based multiplex PCR assay and one real-time-PCR assay, for the amplification and detection of genes specific for the different major and minor toxins produced by *C. perfringens*. On basis of the major toxin genes detected it is possible to differentiate the *C. perfringens* toxinotypes A, B, C, D and E (see Table D.1). Additionally, the ability for producing the minor toxins *C. perfringens* enterotoxin (CPE) and *C. perfringens* beta2 (CPB2) can be detected. Normally human disease is caused by *C. perfringens* type A strains, in rare cases also by *C. perfringens* type D strains, which carry the *cpe* gene.

Clause D.4 describes additionally a gel-based multiplex PCR assay for the differentiation of *C. perfringens* type A strains carrying a chromosomally encoded *cpe* gene or a plasmid encoded *cpe* gene. Strains carrying a chromosomally encoded *cpe* gene produce high heat resistant spores, while strains carrying a plasmid encoded *cpe* gene produce less heat resistant spores and grow better at low temperatures.

Both methods cover only the toxinotypes described in Table D.1[22]. The new nomenclature, proposed in 2018[23], and the new toxin gene *netB* are not part of the PCR methods described in this annex.

Table D.1 — Toxino- and corresponding genotypes of *C. perfringens*[22]

Toxino-type	Major toxins				Genotype
	alpha	beta	epsilon	iota	
A	X				*cpa, (cpe), (cpb2)*
B	X	X	X		*cpa, cpb1, etx, (cpe), (cpb2)*
C	X	X			*cpa, cpb1, (cpe), (cpb2)*
D	X		X		*cpa, etx, (cpe), (cpb2)*
E	X			X	*cpa, iap, (cpe), (cpb2)*

D.2 Gel-based multiplex PCR assay for differentiation of *C. perfringens*

D.2.1 Performance characteristics (see ISO 22118)

D.2.1.1 General

The method has been validated for DNA extracted from *C. perfringens* reference strains. The method has been published, see References [19] and [22].

D.2.1.2 Theoretical evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database (NCBI Blast®[1)] search, EMBL database, 22 September 2015). The result of the search confirmed a 100 % sequence identity only with the expected target sequences.

D.2.1.3 Selectivity

The inclusivity of the method was tested with 124 *C. perfringens* reference and field strains, see Table D.2.

Table D.2 — Inclusivity of the multiplex PCR using target strains

Strain, type and sub-type	Number of strains	Toxin genes					
		cpa	*cpe*	*cpb1*	*cpb2*	*etx*	*iap*
C. perfringens type A	92	92	39	0	28	0	0
C. perfringens type B	2	2	0	2	0	2	0
C. perfringens type C	1	1	0	1	0	0	7
C. perfringens type D	25	25	1	0	1	25	6
C. perfringens type E	4	4	0	0	0	0	4

The exclusivity of the method was tested with 30 non-target organisms, see Table D.3. No cross-reactivity was observed with the non-target bacteria.

Table D.3 — Exclusivity of the multiplex PCR using non-target strains

Strains	Number of strains	Toxin genes detected					
		cpa	*cpe*	*cpb1*	*cpb2*	*etx*	*iap*
Clostridium sporogenes WDCM 00008	1	0	0	0	0	0	0
Clostridium carnis NCTC 13036	1	0	0	0	0	0	0
Clostridium histolyticum NCTC 503	1	0	0	0	0	0	0
Clostridium butyricum NCTC 7423	1	0	0	0	0	0	0
Clostridium barati NCTC 10986	1	0	0	0	0	0	0
Bacillus subtilis subsp. *spizizenii* WDCM 00003	1	0	0	0	0	0	0
Bacillus cereus (WDCM 00219 and field strains)	3	0	0	0	0	0	0
Escherichia coli (WDCM 00013 and field strains)	3	0	0	0	0	0	0
Salmonella spp. (WDCM 00030 and field strain)	2	0	0	0	0	0	0
Listeria spp. (WDCM 00017 and WDCM 00109)	2	0	0	0	0	0	0
Brochotrix thermospacta WDCM 00071	1	0	0	0	0	0	0
Enterococcus faecalis WDCM 00087	1	0	0	0	0	0	0
Citrobacter freundii WDCM 00078	1	0	0	0	0	0	0
Pseudomonas spp. (field strains)	3	0	0	0	0	0	0
Yersinia enterocolitica (field strains)	3	0	0	0	0	0	0
Lactobacillus fermentum (field strain)	1	0	0	0	0	0	0
Aspergillus spp. (field strains)	2	0	0	0	0	0	0
Saccharomyces cerevisiae (field strain)	1	0	0	0	0	0	0

1) NCBI Blast® is a trademark of the National Library of Medicine. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

All tests were performed using positive and negative process controls. Validation was carried out with an Eppendorf 5331 MasterCycler Gradient Thermal Cycler[2)] and the MasterMix according to Table D.5.

D.2.2 Instruments and reagents

D.2.2.1 Principle

Specific DNA fragments of the genes specific for the toxin genes of *C. perfringens* are amplified by multiplex-PCR using 12 primers. The detection of the PCR products is done using agarose gel-electrophoresis.

D.2.2.2 Reagents

Follow ISO 22174 for the quality of reagents used.

D.2.2.2.1 Reagents for thermal lysis

D.2.2.2.1.1 Tris/borate (TBE) buffer solution (0,5×), c(Tris) = 0,055 mol/l, c(boric acid) = 0,055 mol/l, c(Na_2-EDTA) = 0,001 mol/l.

NOTE 0,5× means 0,5-fold, i.e. the concentration of the PCR buffer.

Adjust the pH to 8,0 ± 0,2 at 25 °C with HCl or NaOH. It is advisable to prepare the TBE buffer solution as a concentrated stock solution (maximum 10-fold concentrated). Discard it if precipitation is visible. Dilution of the concentrated buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water.

D.2.2.2.2 Reagents for PCR

D.2.2.2.2.1 Nuclease-free water.

D.2.2.2.2.2 PCR buffer solution, 10×.

NOTE 10× means 10-fold, i.e. the concentration of the PCR buffer.

The PCR buffer solution is usually delivered with the DNA polymerase, which can sometimes include $MgCl_2$ in a concentration specified by the manufacturer. The final $MgCl_2$ concentrations are method-specific and are therefore listed in Table D.5. Ready-to-use reagents can be commercially available. The manufacturer's instructions for use should be considered.

D.2.2.2.2.3 $MgCl_2$ solution.

D.2.2.2.2.4 Thermostable DNA polymerase (for hot-start PCR).

D.2.2.2.2.5 dNTP solution.

D.2.2.2.2.6 Oligonucleotides.

Sequences of the oligonucleotides are listed in Table D.4.

2) Eppendorf 5331 MasterCycler Gradient Thermal Cycler is the trade name of a product supplied by Eppendorf, Germany. The information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to give the same results.

Table D.4 — Sequences of oligonucleotides

Toxin gene	Primer	Sequence (5′ — 3′)	Amplicon size (bp)
cpa	cpa_fw	GCT AAT GTT ACT GCC GTT GA	324
	cpa_re	CCT CTG ATA CAT CGT GTA AG	
cpb1	cpb_fw	GCG AAT ATG CTG AAT CAT CTA	126
	cpb_re	GCA GGA ACA TTA GTA TAT CTT C	
etx	etx_fw	GCG GTG ATA TCC ATC TAT TC	655
	etx_re	CCA CTT ACT TGT CCT ACT AAC	
iap	ia_fw	ACT ACT CTC AGA CAA GAC AG	446
	ia_re	CTT TCC TTC TAT TAC TAT ACG	
cpb2	cpb2_fw	AGA TTT TAA ATA TGA TCC TAA CC	657
	cpb2_re	CAA TAC CCT TCA CCA AAT ACT C	
cpe	cpe_fw	GGA GAT GGT TGG ATA TTA GG	233
	cpe_re	GGA CCA GCA GTT GTA GAT A	

D.2.2.2.3 Reagents for gel electrophoresis

The agarose gel electrophoresis may be carried out with TAE buffer or TBE buffer. Solutions as described in this method do not usually need to be autoclaved.

D.2.2.2.3.1 Agarose, suitable for DNA electrophoresis and for the intended size separation of the DNA molecules.

D.2.2.2.3.2 Boric acid (H_3BO_3), for the TBE buffer system only.

D.2.2.2.3.3 Bromophenol blue ($C_{19}H_9Br_4O_5SNa$) and/or xylene cyanole FF ($C_{25}H_{27}N_2O_6S_2Na$).

D.2.2.2.3.4 DNA molecular mass standard, e.g. a commercial preparation containing DNA fragments (e.g. 100 bp to 1 000 bp).

D.2.2.2.3.5 Glacial acetic acid (CH_3COOH), for the TAE buffer system only.

D.2.2.2.3.6 Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) ($C_{10}H_{14}N_2O_8Na_2$).

D.2.2.2.3.7 Ethidium bromide (EtBr) ($C_{21}H_{20}N_3Br$).

WARNING — Ethidium bromide is toxic and can cause cancer; appropriate precautions shall be taken when weighing out, preparing and discarding the reagent.

D.2.2.2.3.8 Glycerol ($C_3H_8O_3$).

D.2.2.2.3.9 Sodium acetate ($C_2H_3O_2Na$), for the TAE buffer system only.

D.2.2.2.3.10 Hydrochloric acid, *c*(HCl) = 37 g/l.

D.2.2.2.3.11 Sodium hydroxide (NaOH).

D.2.2.2.3.12 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$).

D.2.2.2.3.13 TAE buffer solution (1×), c(Tris) = 0,050 mol/l, $c(C_2H_3O_2Na)$ = 20 mmol/l, $c(Na_2\text{-EDTA})$ = 0,001 mol/l.

Adjust the pH to 8,0 ± 0,2 at 25 °C with glacial acetic acid or NaOH. It is advisable to prepare the TAE buffer solution as a concentrated stock solution (maximum 50-fold concentrated). Discard it if a precipitate is visible. Dilution of the concentrated electrophoresis buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water.

D.2.2.2.3.14 Tris/borate (TBE) buffer solution (0,5×), c(Tris) = 0,055 mol/l, c(boric acid) = 0,055 mol/l, $c(Na_2EDTA)$ = 0,001 mol/l.

Adjust the pH to 8,0 ± 0,2 at 25 °C with HCl or NaOH. It is advisable to prepare the TBE buffer solution as a concentrated stock solution (maximum 10-fold concentrated). Discard it if precipitation is visible. Dilution of the concentrated electrophoresis buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water.

D.2.2.2.3.15 Sample loading buffer solution (5×), c(glycerol) = 500 ml/l, ρ(bromophenol blue) = 2,5 g/l and/or ρ(xylene cyanol) = 2,5 g/l, dissolved in electrophoresis buffer solution (D.2.2.2.3.13 or D.2.2.2.3.14).

NOTE Other concentrations of loading buffer solution can also be used.

D.2.2.2.3.16 Ethidium bromide solution, c(EtBr) = 0,5 mg/l.

It is advisable to store the ethidium bromide solution as a concentrate (e.g. 10 mg/ml) at 5 °C in the dark (EtBr is light-sensitive). It is also advisable to avoid weighing EtBr. The stock solution should be prepared by filling an appropriate amount of water in the vessel already containing the EtBr powder, or alternatively, by employing pre-weighed EtBr tablets. Solubilization of EtBr should be carried out protected from light, under agitation at room temperature. This usually takes approximately 1 h.

NOTE Ethidium bromide solution is mutagenic/teratogenic. Appropriate alternative DNA intercalating dye can be used.

D.2.2.3 Apparatus

The appropriate equipment according to the method and, in particular, the following shall be used.

D.2.2.3.1 Equipment used for thermal lysis

D.2.2.3.1.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

D.2.2.3.1.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

D.2.2.3.1.3 Thermo block, with a mixing frequency between 300 r/min and 1 400 r/min.

D.2.2.3.1.4 Centrifuge, for reaction tubes having a capacity of 1,5 ml and 2,0 ml, e.g. microcentrifuge, capable of achieving an acceleration of up to 12 000 *g.* In some steps, a refrigerated centrifuge is required.

D.2.2.3.1.5 Mixer, e.g. type Vortex.

D.2.2.3.2 Equipment used for PCR

D.2.2.3.2.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

D.2.2.3.2.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

D.2.2.3.2.3 Thin-walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

D.2.2.3.2.4 Thermal cycler.

D.2.2.3.3 Equipment used for the detection of the PCR product

D.2.2.3.3.1 Microwave oven or boiling water bath.

D.2.2.3.3.2 Horizontal gel system.

D.2.2.3.3.3 Power supply.

D.2.2.3.3.4 Ultraviolet (UV) transilluminator or UV light box.

D.2.2.3.3.5 Gel documentation system.

D.2.2.4 Procedure

D.2.2.4.1 Thermal lysis

Transfer 500 µl of the TBE buffer (D.2.2.2.1) into a microcentrifuge tube (D.2.2.3.1.2). Resuspend a single colony (confirmed as *C. perfringens)* from solid medium in the TBE buffer. Incubate the suspension at 95 °C for 15 min in a thermo block under agitation with a mixing frequency of 600 r/min (D.2.2.3.1.3). Centrifuge for 3 min at approximately 12 000 *g* (D.2.2.3.1.4). Transfer the upper phase (aqueous) to a new tube (D.2.2.3.1.2).

After incubation, the isolates should normally be examined immediately. They may, however, be stored for up to 48 h in the refrigerator.

D.2.2.4.2 PCR set-up

The method is described for a total PCR volume of 25 µl per reaction, containing 2,5 µl of template DNA (D.2.2.4.1) with the reagents as listed in Table D.5. The PCR can also be carried out in a larger volume if the solutions are adjusted accordingly. The final concentrations of reagents as outlined in Table D.5 have proven to be suitable.

Table D.5 — MasterMix

Reagent	Final concentration	Volume per sample (µl)
Template DNA	—	5
Nuclease-free water	—	As required
PCR-buffer (without $MgCl_2$)[a]	10×	
$MgCl_2$ solution	15 mmol/l	
dNTP solution	0,12 mmol/l	
PCR primers *cpa* (according to Table D.4)	0,5 µmol/l each	
PCR primers *cpe* (according to Table D.4)	0,34 µmol/l each	
PCR primers *cpb1* and *cpb2* (according to Table D.4)	0,36 µmol/l each	
PCR primers *etx* (according to Table D.4)	0,44 µmol/l each	
PCR primers iap (according to Table D.4)	0,52 µmol/l each	
Thermostable DNA polymerase	5 IU	
Total volume	—	25

[a] If the PCR buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture is adjusted to 5,0 mmol/l.

D.2.2.5 PCR controls

D.2.2.5.1 General

In accordance with ISO 22174 the following controls are necessary.

D.2.2.5.2 Negative PCR control

DNA-free water is used as a negative control.

D.2.2.5.3 Positive PCR control

A mixture of DNA from *C. perfringens*, positive for all six target specific sequences (*cpa, cpe, cpb1, cpb2, etx, iap*), approximately 1 000 copies each.

D.2.2.6 Temperature-time-programme

The temperature-time programme as outlined in Table D.6 has been used for the validation study with an Eppendorf 5331 MasterCycler Gradient Thermal Cycler using a FastStart® Taq DNA polymerase (Roche Diagnostics)[3)] and the MasterMix according to Table D.5. The use of other thermal cyclers can make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used. If using a hot-start polymerase, the recommendation of the manufacturer shall be followed unless the protocol states otherwise.

3) Eppendorf 5331 MasterCycler Gradient Thermal Cycler and FastStart® TaqDNA polymerase are examples of suitable products available commercially from Eppendorf and Roche Diagnostics. 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 give the same results.

Table D.6 — Temperature-time programme

Step	Temperature-time combination
Activation/initial denaturation	6 min/95 °C
Amplification	60 s/95 °C
	60 s/55 °C
	60 s/72 °C
Number of cycles (amplification)	35
Final extension	7 min/72 °C

D.2.2.7 Detection of PCR products (gel electrophoresis)

D.2.2.7.1 General

The agarose gel electrophoresis may be carried out with TAE buffer or with TBE buffer. Use the same buffer to dissolve the agarose and to fill the electrophoresis tank.

D.2.2.7.2 Agarose gel preparation

The amplified PCR products should be detected using a 1,5 % agarose gel. Weigh an appropriate amount of agarose (D.2.2.2.3.1) and add it to the electrophoresis buffer solution (D.2.2.2.3.13 or D.2.2.2.3.14). Allow the solution to boil in a microwave oven or in a water bath (D.2.2.3.3.1) until the agarose is completely dissolved, cool down the solution to about 60 °C and keep it at this temperature until usage. Prepare a gel support (gel tray) with a suitable sample comb placed in right position. Pour the agarose solution onto the gel tray and allow the gel to solidify at room temperature (1 h is usually recommended).

D.2.2.7.3 Agarose gel electrophoresis

Following the amplification step, add the loading buffer (D.2.2.2.3.15) to 10 µl reaction mix in the ratio 1:5 (e.g. add 2,5 µl of loading buffer to 10 µl of reaction mix) and mix well. If the unknown samples are suspected to be too concentrated, also provide some dilutions of them to be loaded onto the gel.

NOTE Other concentrations of loading buffer solution can also be used.

To determine the size of the PCR products, add the loading buffer (D.2.2.2.3.15) and DNA molecular mass standard (D.2.2.2.3.4) in the proportion of 1:5. The DNA molecular mass standard is loaded on the gel at least before the first and after the last sample well. Carefully remove the sample comb from the gel. Transfer the gel (with its gel tray) to the electrophoresis cell, so that the wells reside close to the cathode (negative electrode). Fill the cell with the electrophoresis buffer (D.2.2.2.3.13 or D.2.2.2.3.14). Load the samples using a micropipette. Carry out the electrophoresis at room temperature at the appropriate voltage and power intensity (generally a maximum constant voltage of 5 V/cm, with respect to the distance between the electrodes, is recommended). Under the described conditions, DNA is negatively charged, so it migrates from the cathode to the anode. The electrophoresis time depends on the migration distance required, on the current generated by the power supply, the buffer used, the electro-endosmosis and the concentration of the agarose in the gel.

D.2.2.7.4 Staining

After completing the electrophoresis, incubate the gel for 15 min to 50 min in the ethidium bromide solution (D.2.2.2.3.16) at room temperature, preferably in the dark with gentle shaking. If necessary, reduce the background staining by de-staining the gel in water for 10 min to 30 min.

As an alternative to post-electrophoresis staining, EtBr can be added to the gel before pouring it. In this case, EtBr is added to the gel to a final concentration of 0,01 mg/ml of gel when the gel has been cooled to a temperature of about 60 °C. To minimize the problems of EtBr movement in the gel, some EtBr can also be added to the electrophoresis (tank) buffer. After the gel electrophoresis, no de-staining step is usually required.

NOTE The staining can be performed using other DNA intercalating dye.

D.2.2.7.5 Gel recording

Transfer the gel to the transilluminator surface, switch on the UV light and record the DNA fluorescence by photography. The sizes of the amplification products are given in Table D.7.

Table D.7 — Size of amplification products

Type	Primer	Product size (bp)
cpa	cpa_fw/cpa_re	324
cpe	cpe_fw/cpe_re	233
cpb1	cpb1_fw/ cpb1_re	126
cpb2	cpb2_fw/cbp2_re	567
etx	etx_fw/etx_re	655
iap	iap_fw/iap_re	446

The target sequences are considered to be detected if the size of the PCR product corresponds to the expected length of the target DNA sequences. Follow ISO 22174 for the interpretation of the results.

D.3 Real-time-multiplex PCR assay for differentiation of *C. perfringens*

D.3.1 Performance characteristics (see ISO 22118)

D.3.1.1 General

The method has been validated for DNA extracted from *C. perfringens* reference strains. The method has been published, see Reference [20].

D.3.1.2 Theoretical evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database (NCBI Blast® search, EMBL database, 22 September 2015). The result of the search confirmed a 100 % sequence identity only with the expected target sequences.

D.3.1.3 Selectivity

The inclusivity of the method was tested with 124 *C. perfringens* reference and field strains, see Table D.8.

Table D.8 — Inclusivity of the multiplex PCR using target strains

Strain, type and subtype	Number of strains	Toxin genes					
		cpa	*cpe*	*cpb1*	*cpb2*	*etx*	*iap*
C. perfringens type A	92	92	39	0	28	0	0
C. perfringens type B	2	2	0	2	0	2	0
C. perfringens type C	1	1	0	1	0	0	7
C. perfringens type D	25	25	1	0	1	25	6
C. perfringens type E	4	4	0	0	0	0	4

The exclusivity of the method was tested with 30 non-target organisms, see Table D.9. No cross-reactivity was observed with the non-target bacteria.

Table D.9 — Exclusivity of the multiplex PCR using non-target strains

Strains	Number of strains	Toxin genes detected					
		cpa	*cpe*	*cpb1*	*cpb2*	*etx*	*iap*
Clostridium sporogenes WDCM 00008	1	0	0	0	0	0	0
Clostridium carnis NCTC 13036	1	0	0	0	0	0	0
Clostridium histolyticum NCTC 503	1	0	0	0	0	0	0
Clostridium butyricum NCTC 7423	1	0	0	0	0	0	0
Clostridium barati NCTC 10986	1	0	0	0	0	0	0
Bacillus subtilis subsp. *spizizenii* WDCM 00003	1	0	0	0	0	0	0
Bacillus cereus (WDCM 00219 and field strains)	3	0	0	0	0	0	0
Escherichia coli (WDCM 00013 and field strains)	3	0	0	0	0	0	0
Salmonella spp. (WDCM 00030 and field strain)	2	0	0	0	0	0	0
Listeria spp. (WDCM 00017 and WDCM 00109))	2	0	0	0	0	0	0
Brochotrix thermospacta WDCM 00071	1	0	0	0	0	0	0
Enterococcus faecalis WDCM 00087	1	0	0	0	0	0	0
Citrobacter freundii WDCM 00078	1	0	0	0	0	0	0
Pseudomonas spp. (field strains)	3	0	0	0	0	0	0
Yersinia enterocolitica (field strains)	3	0	0	0	0	0	0
Lactobacillus fermentum (field strain)	1	0	0	0	0	0	0
Aspergillus spp. (field strains)	2	0	0	0	0	0	0
Saccharomyces cerevisiae (field strain)	1	0	0	0	0	0	0

All tests were performed using positive and negative process controls and, additionally for PCR, a heterologous internal amplification control (IAC). Validation was carried out with a Stratagene MXP3000 thermal cycler using Stratagene QPCR Multiplex MasterMix[4].

D.3.2 Instruments and reagents

D.3.2.1 Principle

Specific DNA fragments of the genes specific for the different toxin genes of *C. perfringens* are amplified by multiplex-real-time-PCR. The PCR products are detected by measuring the fluorescence of the probes.

4) The information on the thermal cycler and the MasterMix used is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products named. Equivalent products may be used if they can be shown to give the same results.

D.3.2.2 Reagents

Follow ISO 22174 for the quality of reagents used.

D.3.2.2.1 Reagents for thermal lysis

D.3.2.2.1.1 Tris/borate (TBE) buffer solution (0,5×), c(Tris) = 0,055 mol/l, c(boric acid) = 0,055 mol/l, $c(Na_2EDTA)$ = 0,001 mol/l.

Adjust the pH to 8,0 ± 0,2 at 25 °C with HCl or NaOH. It is advisable to prepare the TBE buffer solution as a concentrated stock solution (maximum 10-fold concentrated). Discard it if precipitation is visible. Dilution of the concentrated buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water.

D.3.2.2.2 Reagents for PCR

D.3.2.2.2.1 Nuclease-free water.

D.3.2.2.2.2 A ready-to-use MasterMix containing PCR-buffer solution, $MgCl_2$-solution, dNTP-solution, (optional) a decontamination system (dUTP included uracil-N-glycosylase) and Taq-Polymerase that is mostly adapted to the thermal cycler used. The manufacturer's instructions for use should be considered.

D.3.2.2.2.3 Oligonucleotides.

Sequences of the oligonucleotides are listed in Table D.10.

Table D.10 — Sequences of oligonucleotides

Toxin gene	Primer	Sequence (5′ — 3′)	Gene accession number
cpa	cpa_fw	GCT AAT GTT ACT GCC GTT GA	DQ184176
	cpa_re	CCT CAT TAG TTT TGC AAC C	
	cpa_P	[a]FAM-GCG CAG GAC ATG TTA AGT TTG-TAMRA[b]	
cpe	cpe_fw	GAA ATC CTT GAT TTA GCT GCT G	X81849
	cpe_re	TGA GTT AGA AGA ACG CCA ATC A	
	cpe_P	[c]HEX-GAT GCA TTA AAC TCA AAT CCA GC-TAMRA[b]	
cpb2	cpb2_fw	TTT AAA TAT GAT CCT AAC C	AY730632
	cpb2_re	GCA CCA TTC ATA AAT TCA GT	
	cpb2_p	[a]FAM-TGA AAT ACT TAA TTC ACA AAA GA-TAMRA[b]	
cpb1	cpb_fw	TCA ATT GAA AGC GAA TAT GCT G	X73562
	cpb_re	CTG TAA ATT TTG TAT CCC ATG AAG C	
	cpb_p	[c]HEX- GAA TAT GTC CAA CCT GAT TTT TCT-TAMRA[b]	
etx	etx_fw	AGC TTT TCC TAG GGA TGG TTA	M95206
	etx_re	AAC TGC ACT ATA ATT TCC TTT TCC	
	etx_p	[a]FAM-AAG AGT GAT TTA AAT GAA GAT GG-TAMRA[b]	

[a] FAM: 6-carboxyfluorescein.

[b] TAMRA: 6-carboxytetramethylrhodamine.

[c] HEX: 5'-Hexachloro-Fluorescein.

[d] ROX = carboxy-X-rhodamine.

Table D.10 *(continued)*

Toxin gene	Primer	Sequence (5´ — 3´)	Gene accession number
iap	iap_fw	CGT GGA GGA TAT ACC GCA AT	X73562
	iap_re	GGT GTG AGC TTT AAT GCG	
	iap_p	[c]HEX-TGG TCC TTT AAA TAA TCC TAA TCC A-TAM-RA[b]	
Internal amplification control (IAC)	IAC_pUC_fw	TGT GAA ATA CCG CAC AGA TG	pUC 19
	IAC_pUC_re	AGC TGG CGT AAT AGC GAA G	
	IAC_pUC_S	[d]ROX-GAG AAA ATA CCG CAT CAG GC-TAMRA[b]	

[a] FAM: 6-carboxyfluorescein.

[b] TAMRA: 6-carboxytetramethylrhodamine.

[c] HEX: 5'-Hexachloro-Fluorescein.

[d] ROX = carboxy-X-rhodamine.

D.3.2.3 Apparatus

The appropriate equipment according to the method and, in particular, the following shall be used.

D.3.2.3.1 Equipment used for thermal lysis

D.3.2.3.1.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

D.3.2.3.1.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

D.3.2.3.1.3 Thermo block, with a mixing frequency between 300 r/min and 1 400 r/min.

D.3.2.3.1.4 Centrifuge, for reaction tubes having a capacity of 1,5 ml and 2,0 ml, e.g. microcentrifuge, capable of achieving an acceleration of up to 12 000 *g*. In some steps, a refrigerated centrifuge is required.

D.3.2.3.1.5 Mixer, e.g. type Vortex.

D.3.2.3.2 Equipment used for PCR

D.3.2.3.2.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

D.3.2.3.2.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

D.3.2.3.2.3 Thin-walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

D.3.2.3.2.4 Real-time PCR thermal cycler.

D.3.2.4 Procedure

D.3.2.4.1 Thermal lysis

Transfer 500 µl of the TBE buffer (D.3.2.2.1.1) into a microcentrifuge tube (D.3.2.3.1.2). Resuspend a single colony (confirmed as *C. perfringens)* from solid medium in the TBE buffer. Incubate the suspension at 95 °C for 15 min in a thermo block under agitation with a mixing frequency of 600 r/min (D.3.2.3.1.3).

Centrifuge for 3 min at approximately 12 000 *g* (D.3.2.3.1.4). Transfer the upper phase (aqueous) to a new tube (D.3.2.3.1.2).

D.3.2.4.2 PCR set-up

The method is described for a total PCR volume of 25 µl per reaction with the reagents as listed in Tables D.11 and D.12. The PCR can also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Tables D.11 and D.12 have proven to be suitable.

Three multiplex-systems are used, one system for the detection of the *cpa* gene and the *cpe* gene in combination with the IAC, and two systems for the detection of the *cbp1* gene and *cpb2* gene and the *etx* gene and the *iap* gene, respectively.

Table D.11 — MasterMix (triplex real-time-PCR-system, *cpa* gene, *cpe* gene and IAC)

Reagent		Final concentration	Volume per sample (µl)
Template DNA		maximum 250 ng	5
TaqMan DNA polymerase	2x Brilliant Multiplex qPCR MasterMix[a]	1×	12,5
PCR-buffer			
$MgCl_2$ solution			
dNTP solution			
PCR primers for *cpa, cpe* and IAC (according to Table D.10), 10 µmol/l		0,3 µmol/l each	0,75 each
PCR probes for *cpa, cpe* and IAC (according to Table D.10), 10 µmol/l		0,2 µmol/l each	0,5 each
Nuclease-free water			0,5
pUC 19-plasmid, 1 fg		1 fg	1

[a] The information on the MasterMix used 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 give the same results.

Table D.12 — MasterMix (duplex real-time-PCR-system, *cpb1* and *cpb2* gene or *etx* gene and *iap* gene)

Reagent		Final concentration	Volume per sample (µl)
Template DNA		maximum 250 ng	5
TaqMan DNA polymerase	2x Brilliant Multiplex qPCR MasterMix[a]	1×	12,5
PCR-buffer			
$MgCl_2$ solution			
dNTP solution			
PCR primers for *cpb1* and *cpb2* or *etx* and *iap* (according to Table D.10) 10 µmol/l		0,5 µmol/l each	1,25 each
PCR probes for *cpb1* and *cpb2* or *etx* and *iap* (according to Table D.10), 10 µmol/l		0,2 µmol/l each	0,5 each
Nuclease-free water			1,5

[a] The information on the MasterMix used 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 give the same results.

D.3.2.5 PCR controls

D.3.2.5.1 General

In accordance with ISO 22174, the following controls are necessary.

D.3.2.5.2 Negative PCR control

DNA-free water is used as a negative control.

D.3.2.5.3 Positive PCR control

A mixture of DNA from *C. perfringens*, positive for all six target specific sequences (*cpa, cpe, cpb1, cpb2, etx, iap*), approximately 1 000 copies each.

D.3.2.5.4 Amplification control

An example for a heterologous IAC is given in Table D.10.

D.3.2.6 Temperature-time-programme

The temperature-time programme as outlined in Table D.13 has been used for the validation study using the MX 3000P and the MX 3005P system in combination with the 2x Brilliant Multiplex QPCR MasterMix®[8]. The use of other thermal cyclers can make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used. If using a hot-start polymerase, the recommendation of the manufacturer shall be followed unless the protocol states otherwise.

Table D.13 — Temperature-time programme

Step	Temperature-time combination
Activation/initial denaturation	10 min/95 °C
Number of cycles (amplification)	45
Amplification	15 s/95 °C
	60 s/55 °C

D.3.2.7 Interpretation of the results

The threshold value to determine the cycle of threshold (C_q) shall be specified by the analyst or by the cycler-specific software. A positive sample generates an amplification plot with at least the exponential phase of a typical amplification curve see ISO 22119. The amplification curve of these samples crosses the defined threshold setting after a certain number of cycles. A sample with a fluorescence signal above the threshold is considered positive.

D.4 Multiplex PCR assay for differentiation of chromosomally encoded *cpe* genes and plasmid encoded *cpe* genes

D.4.1 Performance characteristics (see ISO 22118)

D.4.1.1 General

The method has been validated for DNA extracted from *C. perfringens* reference strains. The method has been published, see Reference [21].

D.4.1.2 Theoretical evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database (NCBI Blast® search, EMBL database, 22 September 2015). The result of the search confirmed a 100 % sequence identity only with the expected target sequences.

D.4.1.3 Selectivity

The inclusivity of the method was tested with 86 *C. perfringens* reference and field strains, see Table D.14[21].

Table D.14 — Inclusivity of the multiplex PCR using target strains

Strain, type and sub-type	Number of strains	genes			
		internal *cpe*	chromosomal *cpe* locus	plasmid *cpe* locus with an IS1470-like sequence	plasmid *cpe* locus with an IS1151 sequence
C. perfringens type A, chromosomal *cpe* locus	29	29	29	0	0
C. perfringens type A, plasmid *cpe* locus with an IS1470-like sequence	41	41	0	41	0
C. perfringens type A plasmid *cpe* locus with an IS1151 sequence	16	16	0	0	16

The exclusivity of the method was tested with 46 non-target organisms, see Table D.15. No cross-reactivity was observed with the non-target bacteria.

Table D.15 — Exclusivity of the multiplex PCR using non-target strains

Strains	Number of strains	genes detected			
		internal *cpe*	chro-mo-so-mal *cpe* locus	plasmid *cpe* locus with an IS1470-like se-quence	plasmid *cpe* locus with an IS1151 sequence
Clostridium perfringens type A without *cpe* gene	17	0	0	0	0
Clostridium sporogenes WDCM 00008	1	0	0	0	0
Clostridium carnis NCTC 13036	1	0	0	0	0
Clostridium histolyticum NCTC 503	1	0	0	0	0
Clostridium butyricum NCTC 7423	1	0	0	0	0
Clostridium barati NCTC 10986	1	0	0	0	0
Bacillus subtilis subsp. *spizizenii* WDCM 00003	1	0	0	0	0
Bacillus cereus (WDCM 00219 and field strains)	3	0	0	0	0
Escherichia coli (WDCM 00013 and field strains)	3	0	0	0	0
Salmonella spp. (WDCM 00030 and field strain)	2	0	0	0	0
Listeria spp. (WDCM 00017and WDCM 00109)	2	0	0	0	0
Brochotrix thermospacta WDCM 00071	1	0	0	0	0
Enterococcus faecalis WDCM 00087	1	0	0	0	0
Citrobacter freundii WDCM 00078	1	0	0	0	0
Pseudomonas spp. (field strains)	3	0	0	0	0
Yersinia enterocolitica (field strains)	3	0	0	0	0