TECHNICAL SPECIFICATION

ISO/TS 17919

First edition
2013-11-01

Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Detection of botulinum type A, B, E and F neurotoxin-producing clostridia

Microbiologie de la chaîne alimentaire — Réaction de polymérisation en chaîne (PCR) pour la détection de micro-organismes pathogènes dans les aliments — Détection des clostridies productrices de neurotoxine botulique de type A, B, E et F

Reference number
ISO/TS 17919:2013(E)

COPYRIGHT PROTECTED DOCUMENT

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

ISO/TS 17919 was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275 *Food analysis — Horizontal methods*, in collaboration with Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Introduction

Botulinum neurotoxin-producing clostridia are ubiquitous in the environment. Botulism is a severe neuroparalytic disease resulting from the action of botulinum neurotoxins (BoNTs). Seven different serotypes of BoNTs (type A to G) and a number of subtypes have been identified to date.

BoNT type A (BoNT/A), type B (BoNT/B), type E (BoNT/E) and type F (BoNT/F) are mainly responsible for botulism in humans and the genes encoding these toxins are the targets of this Technical Specification. BoNT type A, B, E, and F-producing clostridia exist in four physiologically distinct groups (Group I *Clostridium botulinum*, Group II *C. botulinum*, *C. baratii*, *C. butyricum*).

The International Organization for Standardization (ISO) draws attention to the fact that it is claimed that compliance with this document may involve the use of patents.

ISO take no position concerning the evidence, validity and scope of this patent right.

The holder of this patent right has assured ISO that they are willing to negotiate licences under reasonable and non-discriminatory terms and conditions with applicants throughout the world. In this respect, the statement of the holder of this patent right is registered with ISO. Information may be obtained from:

Applied Biosystems, LLC
Scott Miller, Legal Department
5781 Van Allen Way
CARLSBAD
CA 92008
USA

Tel: +1 760 476 4387
Fax: +1 760 476 6048
e-mail: Scott.miller@lifetechnologies.com

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights other than those identified above. ISO shall not be held responsible for identifying any or all such patent rights.

ISO (www.iso.org/patents) maintains an on-line database of patents relevant to its documents. Users are encouraged to consult the databases for the most up-to-date information concerning patents.

Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Detection of botulinum type A, B, E and F neurotoxin-producing clostridia

1 Scope

This Technical Specification specifies a horizontal method for the molecular detection of clostridia carrying botulinum neurotoxin A, B, E, and F genes by a PCR method. This method detects the genes and not the toxins, therefore a positive result does not necessarily mean the presence of these toxins in the sample investigated. This Technical Specification is applicable to products for human consumption, animal feed, and environmental samples.

The PCR assays for detection of genetic sequences encoding specific toxin types are described in Annexes B and C.

2 Normative references

The following referenced documents are indispensable for the application 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 6887-1, *Microbiology of food and animal feeding stuffs — 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 7218, *Microbiology of food and animal feeding stuffs — 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 20837, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for sample preparation for qualitative detection*

ISO 20838:2006, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for amplification and detection for qualitative methods*

ISO 22174, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

3 Terms and definitions

For the purpose of this document, the terms and definitions given in ISO 22174 apply.

4 Symbols and abbreviated terms

4.1 Symbols

c substance concentration

ρ mass concentration

φ volume fraction

w mass fraction

4.2 Abbreviated terms

BoNT botulinum neurotoxin

5 Principle

5.1 General

The method comprises the following consecutive steps:

a) microbial enrichment (see 5.2);

b) nucleic acid extraction (see 5.3);

c) amplification (see 5.4);

d) detection of PCR products (see 5.5);

e) confirmation (see 5.6).

NOTE Real-time-PCR combines steps c) to e).

5.2 Microbial enrichment

The number of BoNT-producing clostridia (spores or vegetative cells) to be detected is increased by encouraging their germination and growth in non-selective liquid nutrient medium tryptone–peptose–glucose–yeast extract broth under anaerobic conditions.

5.3 Nucleic acid extraction

Bacterial cells are separated from the nutrient medium, lysed and the nucleic acids are extracted for use in the PCR reaction.

5.4 Amplification by PCR

The extracted nucleic acid is transferred to the PCR mix and the amplification is carried out in a thermal cycler.

5.5 Detection of PCR products

PCR products are detected by gel electrophoresis or an appropriate alternative.

5.6 Confirmation

The identity of the PCR products shall be confirmed by any appropriate method, e.g. sequencing, hybridization or restriction analysis.

6 Reagents

6.1 General

For all stages 5.1 b) to e), use only reagents of recognized analytical grade and consumables suitable for molecular biology applications as specified in ISO 20837 and ISO 20838.

Reagent requirements specified in ISO 20838:2006, Clause 5, apply.

6.2 Culture media

6.2.1 General

Follow ISO 11133 for the preparation, production and performance testing of culture media.

6.2.2 Diluent

Follow ISO 6887-1 and the relevant part of ISO 6887[9]–[13] dealing with the product to be examined.

6.2.3 Non–selective enrichment culture medium, tryptone–peptone–glucose–yeast extract broth (TPGY broth) (Reference [7])

6.2.3.1 General

Other approved non-selective enrichment culture media can be used provided equivalent performance is shown.

6.2.3.2 Composition and pH

Tryptone	50 g
Peptone	5 g
Yeast extract	20 g
D-Glucose	4 g
Sodium thioglycolate, $HSCH_2COONa$	1 g
Water	to 1 000 ml

pH 7,0 ± 0,2

6.2.3.3 Preparation

Dissolve the components in the water by boiling. After sterilization, adjust to pH 7,0 ± 0,2 at 25 °C. Dispense the base into flasks or bottles of appropriate capacity. Sterilize for 15 min at 121 °C. Store in a refrigerator at 5 °C ± 3 °C. Discard unused medium 4 weeks after preparation.

6.2.4 TPGY broth buffered — for acidic and acidifying foodstuffs only

6.2.4.1 Stock solution (phosphate buffer)

6.2.4.1.1 Solution 1

Sodium dihydrogenphosphate monohydrate [$NaH_2PO_4 \cdot H_2O$]	138 g
Water	to 1 000 ml

6.2.4.1.2 Solution 2

Disodium hydrogenphosphate [Na_2HPO_4]	142 g
Water	to 1 000 ml

6.2.4.1.3 Preparation

Dissolve the components in the water by boiling. To 250 ml solution 1, add solution 2 until the pH reaches 7,2. Store in a refrigerator at 5 °C ± 3 °C.

6.2.4.2 Preparation of the complete medium

Dissolve the components given for the base (6.2.3.2) in 500 ml water by boiling. Add 100 ml phosphate buffer (6.2.4.1) The final phosphate concentration of the complete medium is 0,1 mol/l. Add water up to 1 000 ml. Dispense the complete medium into flasks or bottles of appropriate capacity. Sterilize for 15 min at 121 °C. Store in a refrigerator at 5 °C ± 3 °C. Discard unused medium 4 weeks after preparation.

6.3 Nucleic acid extraction

6.3.1 Chloroform, $CHCl_3$.

6.3.2 Ethanol, $\varphi(C_2H_5OH) = 96$ %.

6.3.3 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), $C_{10}H_{14}N_2O_8Na_2$.

6.3.4 Hexadecyl(trimethyl)ammonium bromide [(cetyl(trimethyl)ammonium bromide, **CTAB]**, $C_{19}H_{42}BrN$.

6.3.5 Hydrochloric acid, $\varphi(HCl) = 37$ %.

6.3.6 Isopropanol, $CH_3CH(OH)CH_3$.

6.3.7 Proteinase-K, approximately 20 units/mg of lyophilizate.

6.3.8 Sodium chloride, NaCl.

6.3.9 Sodium hydroxide, NaOH.

6.3.10 Tris(hydroxymethyl)aminomethane (tris), $C_4H_{11}NO_3$.

6.3.11 CTAB extraction buffer, ρ(CTAB) = 20 g/l, c(NaCl) = 1,4 mol/l, c(tris) = 0,1 mol/l, $c(Na_2EDTA)$ = 0,02 mol/l.

Adjust to pH 8,0 with HCl or NaOH.

6.3.12 CTAB-precipitation buffer, ρ(CTAB) = 5 g/l, c(NaCl) = 0,04 mol/l.

6.3.13 Sodium chloride solution, c(NaCl) = 1,2 mol/l.

6.3.14 Ethanol solution, $\varphi(C_2H_5OH) = 70\ \%$.

6.3.15 Proteinase-K solution, $\rho = 20$ mg/ml, dissolved in sterile water.

Do not autoclave. Store at −20 °C, but avoid repeated freezing and thawing.

6.3.16 Tris–EDTA (TE) buffer, c(tris) = 0,01 mol/l, $c(Na_2EDTA) = 0{,}001$ mol/l.

Adjust to pH 8,0 with HCl or NaOH.

6.4 Reagents for PCR

6.4.1 Thermostable DNA polymerase, as specified in ISO 20838 and ISO 22174.

6.4.2 Deoxyribonucleoside triphosphates (dNTPs) containing dATP, dCTP, dGTP and dTTP or dUTP, as specified in ISO 20838 and ISO 22174.

6.4.3 PCR buffer solution, as specified in ISO 20838 and ISO 22174.

The PCR buffer solution is usually delivered with the DNA polymerase, which may or may not include $MgCl_2$ in a concentration specified by the manufacturer. The final $MgCl_2$ concentrations are method specific and are therefore listed in the annexes. It is possible that ready-to-use reagents are commercially available. If so, follow the manufacturer's instructions for use.

6.4.4 Primers and probes

Primers and probes for specific detection of the neurotoxin gene sequences are listed in Annexes B and C.

7 Apparatus and equipment

7.1 General

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

7.2 Equipment for sample preparation prior to enrichment

7.2.1 Water bath, capable of being maintained at 50 °C ± 1 °C.

7.2.2 Centrifuge, for 50 ml and 100 ml tubes and with an adjustable acceleration of up to 12 000 × g.

7.2.3 Membrane filter, nitrocellulose-filter, pore size 0,45 µm.

7.2.4 Centrifuge tubes, of capacities of 50 ml and 100 ml.

7.3 Equipment for microbial enrichment

7.3.1 Water baths, capable of being maintained at 30 °C ± 1 °C, 65 °C ± 1 °C and 100 °C ± 1 °C.

7.3.2 Anaerobic jar or anaerobic cabinet, capable of being maintained at 30 °C ± 1 °C, according to ISO 7218.

7.3.3 Incubator, capable of operating at 30 °C ± 1 °C.

7.3.4 Flasks or bottles, of appropriate capacity.

7.4 Equipment used for nucleic acid extraction

Appropriate equipment according to ISO 20837 and, in particular, the following.

7.4.1 Microcentrifuge tubes, of capacities of 1,5 ml and 2,0 ml.

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

7.4.3 Graduated pipettes and pipette filter tips, for volumes between 1 µl and 1 000 µl.

7.4.4 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*.

7.4.5 Mixer, e.g. vortex type.

7.5 Equipment used for PCR

Appropriate equipment according to the method and, in particular, the following.

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

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

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

7.5.4 Thermal cycler.

7.6 Equipment used for the detection of the PCR product

Appropriate equipment according to the method and, in particular, the following.

7.6.1 Gel-based PCR

7.6.1.1 Horizontal gel system.

7.6.1.2 Power supply.

7.6.1.3 Ultraviolet (UV) transilluminator or UV light box.

7.6.1.4 Gel documentation system.

7.6.2 Real-time PCR

7.6.2.1 Real-time PCR thermal cycler.

7.6.2.2 Appropriate detection and analysis software.

8 Sampling

Sampling is not part of the method specified in this Technical Specification. 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 the subject.

It is important the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

9 Procedure

9.1 Sample preparation prior to enrichment

9.1.1 General

See Figure A.1.

It is recommended that at least 25 g be analysed, particularly for honey samples; however, if supply is limited, smaller sample sizes may be used.

9.1.2 Preparation of the sample

Prepare and homogenize the sample according to ISO 6887-1 and the relevant parts of ISO 6887[9]–[13] concerning the relevant matrix.

9.1.3 Preparation of honey samples

Place the vessel of honey in a water bath (7.2.1) at 50 °C ± 1 °C for 30 min to melt the honey. Invert the vessel several times to mix the sample.

Weigh 25 g ± 2 g of honey into a sterile centrifuge tube with a capacity of 100 ml (7.2.4) and add at least 50 ml of sterile distilled or deionized water containing 1 % volume fraction polysorbate 80, preheated to 50 °C ± 1 °C. Mix until the solution is homogeneous. Centrifuge the mixture at 12 000 × *g* (7.2.2) for 30 min. Remove the supernatant carefully and pass it through a 0,45 µm membrane filter (7.2.3). In case of blockage, pass any remaining supernatant through a fresh filter. Use all filters in the subsequent steps. Store the sediment temporarily at 5 ± 3 °C.

9.2 Microbial enrichment

9.2.1 Inoculation

9.2.1.1 General

Remove dissolved oxygen from the enrichment medium (6.2) by boiling for 10 min to 15 min in a water bath (7.3.1).

If the sample is acidic or acidifying, the enrichment broth shall be prepared according to 6.2.4.

9.2.1.2 Inoculation of test portion

9.2.1.2.1 Recovery of vegetative cells and spores

Adjust the temperature of the enrichment medium to 30 °C ± 1 °C in a water bath (7.3.1). Transfer the test portion into the degassed enrichment medium to give a final dilution of 10^{-1}.

9.2.1.2.2 Recovery of spores

Adjust the temperature of the enrichment medium to 65 °C ± 1 °C in a water bath (7.3.1). Transfer the test portion into the preheated enrichment medium to give a final dilution of 10^{-1} at 65 °C ± 1 °C. After inoculation, maintain the flask or bottle at 65 °C ± 1 °C for further 10 min, and then quickly cool to 30 °C ± 1 °C in a water bath (7.3.1).

9.2.1.3 Inoculation of honey test portions

Adjust the temperature of the enrichment broth to 65 °C ± 1 °C (7.3.1). Transfer the sediment (9.1.3) to one flask or bottle (7.3.4) and the filter or filters (9.1.3) into a second flask or bottle (7.3.4) each containing at least 10 ml of the heated enrichment broth, but in all cases ensure sufficient liquid covers the filters. Incubate both flasks or bottles at 65 °C ± 1 °C for 10 min in a water bath (7.3.1).

NOTE Honey samples are only analysed for spores.

9.2.2 Incubation

Incubate under anaerobic conditions (7.3.2) at 30 °C ± 1 °C. After 24 h ± 2 h of incubation, remove 1 ml of the enrichment for the PCR analysis and return immediately to anaerobic conditions.

If the result of the first PCR is negative, continue incubation under the same conditions for a further 48 h ± 2 h, then transfer 1 ml of the enrichment into a flask or bottle (7.3.4) containing 9 ml of fresh enrichment broth (6.2.3). Incubate anaerobically (7.3.2) at 30 °C ± 1 °C for 18 h ± 2 h and perform a second PCR run.

9.2.3 Process controls

Positive and negative process controls shall be included according to ISO 22174.

An example of a method for the preparation of spores is given in Annex D.

9.3 Nucleic acid preparation

9.3.1 General

An appropriate nucleic acid extraction procedure for Gram-positive bacteria shall be used.

An example of a procedure is given in 9.3.2 to 9.3.4. This procedure consists of a lysis step — thermal lysis in the presence of CTAB — followed by several extraction steps in order to remove inhibitors, such as polysaccharides and proteins.

Once the matrix test portion has been prepared, apply the DNA extraction and purification protocol given in 9.3.2 to 9.3.4.

Scale adaptation of masses and buffer volumes is required as a function of the selected size of the test portion.

9.3.2 Sample extraction

Transfer 1 000 µl of the enrichment culture (9.2.2) into a microcentrifuge tube (7.4.1). Centrifuge (7.4.4) for 5 min at approximately 12 000 × *g*. Discard the supernatant (aqueous).

Add 500 µl prewarmed (65 °C) CTAB extraction-buffer (6.3.11) to the pellet and mix gently until the pellet is lysed. Incubate for 30 min at 65 °C, under agitation (7.4.2). Add 20 µl of proteinase-K solution (6.3.7), gently mix the tube and incubate for 30 min at 65 °C, under agitation (7.4.2). Centrifuge for 10 min at approximately 12 000 × *g*. Transfer the supernatant to a new tube, add 0,7 to 1 volume of chloroform (6.3.1) and mix thoroughly.

Centrifuge for 15 min at approximately 12 000 × *g*. Transfer the supernatant (aqueous) to a new tube.

9.3.3 CTAB precipitation

Add 2 volumes of the CTAB precipitation buffer (6.3.12). Incubate for 60 min at room temperature without agitation. Centrifuge for 15 min at 12 000 × *g*. Discard the supernatant. Dissolve the precipitated DNA by adding 350 µl of NaCl solution (6.3.13). Add 350 µl of chloroform (6.3.1) and mix thoroughly. Centrifuge for 10 min at 12 000 × *g*. Transfer the aqueous phase into a new tube.

NOTE CTAB-precipitation is not necessary for all matrices, only for protein- and polysaccharide-rich matrices. Alternatively, a solid-phase purification of the DNA (e.g. by the use of spin columns) is possible assuming the results are equivalent.

9.3.4 DNA precipitation

Add 0,6 volume of isopropanol (6.3.6), mix smoothly by inverting the tube and keep the tube at room temperature for 20 min. Centrifuge for 15 min at 12 000 × *g*. Discard the supernatant. Add 500 µl of ethanol solution (6.3.14) to the tube and invert several times. This is the critical step ensuring the complete removal of CTAB. Centrifuge for 10 min at 12 000 × *g*. Discard the supernatant. Dry the DNA pellet and redissolve it into 100 µl of an appropriate buffer, e.g. TE buffer (6.3.16). This is the DNA master stock. The DNA can be stored at –20 °C until use.

9.4 PCR amplification

Different procedures for PCR amplification can be used. The detection of the PCR product can be either gel based or by detection of the fluorescence signal.

All requirements for the PCR amplification are specified in ISO 20838.

Examples of gel-based PCR methods are described in Annex B and of real-time PCR methods in Annex C.

9.4.1 PCR controls

PCR controls shall be in accordance with ISO 22174.

9.4.2 Detection of PCR products

Different procedures for detection of PCR products can be used. Examples of gel-based PCR methods are described in Annex B and of real-time PCR methods in Annex C.

9.5 Confirmation of a positive PCR result

Follow the procedure specified in ISO 20838.

9.5.1 Interpretation of the results

The results obtained, including the controls specified in ISO 22174, should be unambiguous and the controls should yield expected results, otherwise the procedure shall be repeated.

The PCR result is:

a) positive, if a specific PCR product has been detected and confirmed and all the controls give expected results;

b) negative within the limits of detection, if a specific PCR product has not been detected, and all controls give expected results.

A positive PCR result does not automatically indicate the presence of botulinum neurotoxins. To confirm the presence of the neurotoxins suitable methods targeting the toxins directly shall be applied.

Annex A
(normative)

Flow-diagram of the procedure

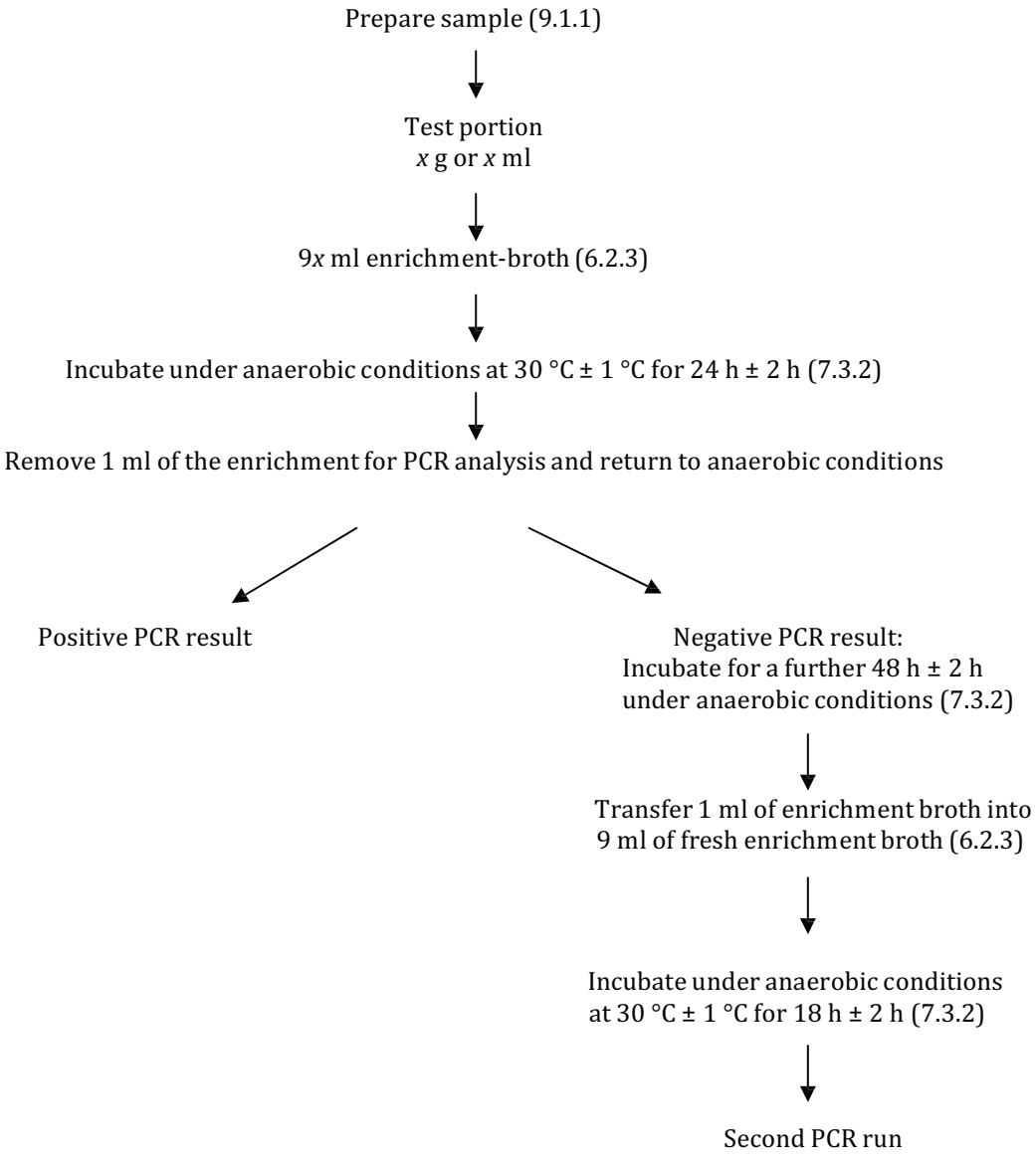

Figure A.1

Annex B
(informative)

Multiplex PCR assays for detection of genes encoding botulinum neurotoxin types A, B, E, and F using agarose gel electrophoresis

B.1 Method 1

B.1.1 Introduction

This clause describes a method for the specific amplification and detection of the genes encoding botulinum neurotoxin types A, B, E, and F using agarose gel electrophoresis.

For limitations see B.1.7.6.

B.1.2 Performance characteristics

B.1.2.1 General

The method has been validated for DNA extracted from various *C. botulinum* type A, B, E, and F reference strains and from naturally contaminated samples.

The method appears in References [1][2][5].

B.1.2.2 **Theoretical** evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database, where EMBL is the European Molecular Biology Laboratory and DDBJ is the DNA Database of Japan (Reference [16], 2009-09-20). The result of the search confirmed a complete identity only with the expected target sequences.

B.1.2.3 Selectivity

B.1.2.3.1 Inclusivity test

The inclusivity of the method was tested with 86 *C. botulinum* type A strains, 70 *C. botulinum* type B, 15 *C. botulinum/butyricum* type E and six *C. botulinum* type F strains, see Table B.1.

Table B.1 — Inclusivity of the multiplex PCR using target strains

Strain, type and subtype	Number of strains	Type of botulinum neurotoxin gene detected			
		Type A	Type B	Type E	Type F
C. botulinum type A subtype A1[a]	3	3	0	0	0
C. botulinum type A subtype A2[b]	9	9	0	0	0
C. botulinum type A subtype A3[c]	1	1	0	0	0
C. botulinum type A subtype A5[d]	4	4	0	0	0
C. botulinum type A undetermined subtype[e]	69	69	0	0	0
C. botulinum type Ba subtype A4[f]	1	1	1	0	0
C. botulinum type Ab subtype A2[g]	4	4	4	0	0
C. botulinum type B[h]	70	0	70	0	0
C. botulinum type E[i]	4	0	0	4	0
C. butyricum type E[j]	11	0	0	11	0
C. botulinum type F[k]	6	0	0	0	6

a *C. botulinum* National Collection of Type Cultures (NCTC 4587), *C. botulinum* strain 62A, *C. botulinum* NCTC 7272.

b Strains isolated from Italian National Reference Centre for Botulism (NRCB).

c *C. botulinum* NCTC 2012 Loch Maree.

d *C. botulinum* strains from Institute of Food Research (IFR), UK.

e A total of 64 strains from NRCB collection and five strains from the Consultant Laboratory for Anaerobic Bacteria (CLAB) of the University of Leipzig, Germany.

f *C. botulinum* strain 657Ba from IFR, UK.

g Strains isolated by NRCB, Italy.

h *C. botulinum* NCTC 7273 and 69 strains from NRCB collection.

i Three strains from the CLAB and one strain from NRCB.

j Seven strains isolated in Italy by NRCB, four strains provided by Reference [17].

k *C. botulinum* NCTC 10281: three strains isolated in Italy by NRCB; two strains isolated in Germany by CLAB.

B.1.2.3.2 Exclusivity test

The exclusivity of the method was tested with 34 non-target organisms, see Table B.2. No cross-reactivity was observed with the non-target bacteria.

Table B.2 — Exclusivity of the multiplex PCR using non-target strains

Strains[a]	Number of strains	Type of botulinum neurotoxin gene detected			
		Type A	Type B	Type E	Type F
C. sporogenes WDCM 00008	1	0	0	0	0
C. perfringens (WDCM 00007 and field strains)	3	0	0	0	0
C. carnis NCTC 13036	1	0	0	0	0
C. histolyticum NCTC 503	1	0	0	0	0
C. butyricum NCTC 7423	1	0	0	0	0
C. barati NCTC 10986	1	0	0	0	0
Bacillus subtilis WDCM 00003	1	0	0	0	0
B. cereus (NCTC 11143 and field strains)	3	0	0	0	0
Thermophilic *Campylobacter* spp. (field strains)	2	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 00017 and 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
Lactobacillus fermentum (field strain)	1	0	0	0	0
Aspergillus spp. (field strains)	2	0	0	0	0
Saccharomyces cervisiae (field strain)	1	0	0	0	0
a WDCM ≡ World Data Centre for Microorganisms.					

B.1.2.4 Sensitivity

B.1.2.4.1 Sensitivity tests using artificially contaminated samples (Reference [2])

The limit of detection was assessed by measuring artificially contaminated samples with different inoculation levels (0,1 cfu to 10 cfu and 10 cfu to 100 cfu before enrichment) in 10 g of various food matrices (canned fish, canned sausages, and honey) under investigation using the culture method (Reference [1]). According to the results, the false-positive rate for the method using artificially contaminated samples is 0 % and the false-negative rate is 0 %. The limit of detection for the method described is 1 cfu to 10 cfu or spores per 10 g before enrichment.

B.1.2.4.2 Sensitivity tests using naturally contaminated samples (Reference [2])

The limit of detection was assessed by measuring 382 naturally contaminated samples (e.g. honey, vegetable matter, and canned meats) using the mouse bioassay as reference method. According to the results the false-positive rate for the method using naturally contaminated samples is 0 % and the false-negative rate is 0 %.

NOTE The experiments were performed with test portions of 10 g.

B.1.2.5 Analytical controls

All tests were performed using positive and negative process controls and additionally for PCR an internal amplification control (IAC) described in B.1.6.

B.1.2.6 Instruments and reagents

Validation was carried out with the MyCycler®[1)] and with the Mastercycler® Gradient[2)] using the 2× Multiplex qPCR MasterMix®[3)] (Reference [1]) and the MasterMix according to Table B.7 (References [2][5]).

B.1.3 Principle

Specific DNA fragments of the BoNT/A, B, E, and F genes are amplified by multiplex PCR in combination with a homologous IAC using eight primers. The detection of the PCR products is done using agarose gel electrophoresis.

B.1.4 Reagents

B.1.4.1 General

For the quality of reagents to be used, see ISO 22174.

B.1.4.2 Reagents for PCR

B.1.4.2.1 Nuclease-free water.

B.1.4.2.2 PCR buffer solution, 10×.[4)]

The PCR buffer solution is usually delivered with the DNA polymerase, which may or may not include $MgCl_2$ in a concentration specified by the manufacturer. The final $MgCl_2$ concentrations are method specific and are therefore listed in Table B.7. It is possible that ready-to-use reagents are commercially available. If so, follow the manufacturer's instructions for use.

B.1.4.2.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

B.1.4.2.4 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

B.1.4.2.5 dNTP solution, c(dNTP) = 10 mmol/l.

B.1.4.2.6 Oligonucleotides.

Sequences of the oligonucleotides are listed in Table B.3.

---

1) MyCycler® is the trade name of a product supplied by BioRad. 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.

2) Mastercycler® Gradient is the trade name of a product supplied by Eppendorf. 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.

3) 2× Multiplex qPCR MasterMix® is the trade name of a product supplied by Qiagen. 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.

4) 10× means 10-fold; i.e. the concentration of the PCR buffer.

Table B.3 — Sequences of oligonucleotides

Type	Primer	Sequence (5´ — 3´)	Amplicon size bp
A	IA_O3_fw	ggg CCT AgA ggT AgC gTA R[a]Tg	101
	IA_O4_rev	TCT TY[a] A TTT CCA gAA gCA TAT TTT	
B	CBMLB1	CAg gAg AAg Tgg AgC gAA AA	205
	CBMLB2	CTT gCg CCT TTg TTT TCT Tg	
E	CBMLE1	CCA AgA TTT TCA TCC gCC TA	389
	CBMLE2	gCT ATT gAT CCA AAA Cgg TgA	
F	CBMLF1	Cgg CTT CAT TAg AgA ACg gA	543
	CBMLF2	TAA CTC CCC TAg CCC CgT AT	

[a] In the sequence of oligonucleotides R = A,G and Y = C,T.

B.1.4.3 Reagents for gel electrophoresis

B.1.4.3.1 General. 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.

B.1.4.3.2 Agarose, suitable for DNA electrophoresis and for the intended size separation of the DNA molecules.

B.1.4.3.3 Boric acid , H_3BO_3, for the TBE buffer system only.

B.1.4.3.4 Bromophenol blue, $C_{19}H_9Br_4O_5SNa$ and/or xylene cyanol FF, $C_{25}H_{27}N_2O_6S_2Na$.

B.1.4.3.5 DNA molecular mass standard, e.g. a commercial preparation containing DNA fragments from very high to very low molecular mass.

B.1.4.3.6 Glacial acetic acid, CH_3COOH, for the TAE buffer system only.

B.1.4.3.7 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), $C_{10}H_{14}N_2O_8Na_2$.

B.1.4.3.8 Ethidium bromide (EthBr), $C_{21}H_{20}N_3Br$.

B.1.4.3.9 Glycerol, $C_3H_8O_3$.

B.1.4.3.10 Sodium acetate, $C_2H_3O_2Na$, for the TAE buffer system only.

B.1.4.3.11 Hydrochloric acid, $\varphi(HCl) = 37\ \%$.

B.1.4.3.12 Sodium hydroxide, NaOH.

B.1.4.3.13 Tris(hydroxymethyl)aminomethane (tris), $C_4H_{11}NO_3$.

B.1.4.3.14 Tris–acetate–EDTA (TAE) buffer solution (1×), $c(\text{tris}) = 0{,}050$ mol/l, $c(C_2H_3O_2Na) = 20$ mmol/l, $c(Na_2EDTA) = 0{,}001$ mol/l.

Adjust to pH 8,0 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.

B.1.4.3.15 Tris–borate–EDTA (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 to pH 8,0 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.

B.1.4.3.16 Sample loading buffer solution (5×), φ(glycerol) = 50 %, ρ(bromophenol blue) = 2,5 g/l and/or ρ(xylene cyanol) = 2,5 g/l, dissolved in electrophoresis buffer solution (B.1.4.3.14 or B.1.4.3.15).

B.1.4.3.17 Ethidium bromide solution, c(EthBr) = 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 (EthBr is light-sensitive). It is also advisable to avoid weighing EthBr. The stock solution should be prepared by dissolving an appropriate amount of water in the vessel already containing the EthBr powder, or alternatively, by employing preweighed EthBr tablets. Solubilization of EthBr should be carried out protected from light, under agitation at room temperature. This usually takes approximately 1 h.

WARNING — Ethidium bromide is a known mutagen. Follow safe handling procedures.

B.1.5 Apparatus

B.1.5.1 General

Appropriate equipment according to the method and, in particular, the following.

B.1.5.2 Equipment used for PCR

B.1.5.2.1 Pipettes and **pipette filter tips**, having a capacity between 1 µl and 1 000 µl.

B.1.5.2.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

B.1.5.2.3 Thin-walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

B.1.5.2.4 Thermal cycler.

B.1.5.2.5 Equipment used for the detection of the PCR product.

B.1.5.2.6 Microwave oven or **boiling water bath**.

B.1.5.2.7 Horizontal gel system.

B.1.5.2.8 Power supply.

B.1.5.2.9 UV transilluminator or **UV light box**.

B.1.5.2.10 Gel documentation system.

B.1.6 Internal amplification control (Reference [2])

B.1.6.1 Principle

A DNA fragment of the pUC 19 plasmid is amplified by PCR using two specific primers. The primers possess over-hanging 5'-ends, which are identical to the primers used for the detection of the BoNT/F gene, whereas their 3'-ends are complementary to the pUC 19 predetermined DNA sequence of defined length and sequence. The DNA fragment is used as a template for the competitive IAC.

B.1.6.2 Reagents

B.1.6.2.1 General

For the quality of reagents to be used, see ISO 22174.

B.1.6.2.2 Nuclease-free water.

B.1.6.2.3 Magnesium chloride solution, $c(MgCl_2)$ = 25 mmol/l.

B.1.6.2.4 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

B.1.6.2.5 dNTP solution, c(dNTP) = 10 mmol/l.

B.1.6.2.6 Potassium chloride solution, c(KCl) = 0,5 mol/l.

B.1.6.2.7 Tris(hydroxymethyl)aminomethane hydrochloride, $c(C_4H_{11}NO_3\ HCl)$ = 0,1 mol/l.

Adjust to pH 8,3 with HCl or NaOH.

B.1.6.2.8 pUC 19 plasmid (accession number L09137).

B.1.6.2.9 Oligonucleotides

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

Table B.4 — Sequences of oligonucleotides

Primer	Sequence (5′ — 3′)	Amplicon size of IAC bp
IAC_f	Cgg CTT CAT TAg AgA ACg gAC gTT Tgg TAT ggC TTC ATT C	698
IAC_r	TAA CTC CCC TAg CCC CgT ATT AgA CgT CAg gTg gCA CTT T	

B.1.6.3 PCR-Setup for the construction of the IAC

The method is described for a total PCR volume of 50 µl per reaction with the reagents as listed in Table B.5. The PCR can also be carried out in a smaller volume if the solutions are adjusted appropriately. The final values of reagents as outlined in Table B.5 have proven to be suitable.

Table B.5 — Addition of reagents

Reagent	Final value	Volume per sample µl
Template DNA (pUC 19), 0,5 µg/µl	0,5 µg	1
Nuclease-free water		26
KCl solution, c(KCl) = 0,5 mol/l	50 mmol/l	5
Tris·HCl (pH 8,3), c(tris·HCl) = 0,1 mol/l	10 mmol/l	5
$MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l	2,5 mmol/l	5
dNTP solution, c(dNTP) = 10 mmol/l	0,8 mmol/l	4
Primer IAC_f, 5 µmol/l	0,1 µmol/l	1
Primer IAC_r, 5 µmol/l	0,1 µmol/l	1
Thermostable DNA polymerase, 5 IU/µl	2,5 IU	1

B.1.6.4 Temperature–time programme

The temperature–time programme as outlined in Table B.6 has been used for the validation study using thermal cyclers MyCycler®[1)] or the Mastercycler® Gradient[2)] and *Taq* DNA polymerase.[5)] The use of other thermal cyclers might make an adaptation necessary. The time for activation and initial denaturation depends on the polymerase used. If using a hot-start polymerase, follow the manufacturer's instructions for use.

Table B.6 — Temperature–time programme

Amplification	60 s/95 °C
	60 s/55 °C
	2 min/72 °C
Number of cycles (amplification)	30
Final extension	10 min/72 °C

B.1.6.5 Use of the IAC

Purify the PCR product using an appropriate method. Generally 1 500 copies/well of IAC are suitable for gel-based PCR methods. The IAC described is a competitive IAC and is constructed in competition with the primers amplifying type F neurotoxin gene.

B.1.7 Procedure

B.1.7.1 PCR set-up

The method is described for a total PCR volume of 50 µl per reaction with the reagents as listed in Table B.7. The PCR can also be carried out in a smaller volume if the solutions are adjusted accordingly. The final values of reagents as outlined in Table B.7 have proven to be suitable.

5) Applied Biosystems is an example of a suitable supplier of *Taq* DNA polymerase. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to give the same results.

Table B.7 — Addition of reagents

Reagent	Final value	Volume per sample µl
Template DNA	10 ng to 50 ng	3
Nuclease-free water		2,6
PCR buffer 10× (without $MgCl_2$)[a]	1×	5
$MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l	4,8 mmol/l	9,6
dNTP solution, c(dNTP) = 10 mmol/l	0,8 mmol/l	4
PCR primers (according to Table B.3) 5 µmol/l	0,3 µmol/l each	3 each
IAC, 1 500 copies/µl	1 500 copies	1
Thermostable DNA polymerase, 5 IU/µl	2 IU	0,8

[a] If the PCR buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture is adjusted to 4,8 mmol/l.

B.1.7.2 PCR controls

B.1.7.2.1 General.

In accordance with ISO 22174, the controls in B.1.7.2.2 to B.1.7.2.4 are necessary.

B.1.7.2.2 Negative PCR control.

DNA-free water is used as a negative control.

B.1.7.2.3 Positive PCR control.

A mixture of DNA from BoNT-producing clostridia, positive for all four target sequences (type A, B, E, and F neurotoxin genes), approximately1 000 copies each.

B.1.7.2.4 Amplification control.

Use an appropriate IAC or external amplification control (EAC). An example of an IAC is shown in B.1.6.

B.1.7.3 Temperature–time programme

The temperature–time programme outlined in Table B.8 has been used for the validation studies with the My Cycler® and with the Mastercycler® Gradient[2)] using the 2× Multiplex qPCR MasterMix[3)] (Reference [1]) and the MasterMix according to Table B.7 (References [2][5]). The use of other thermal cyclers might make an adaptation necessary. The time for activation and initial denaturation depends on the polymerase used.[6)] If using a hot-start polymerase, follow the manufacturer's instructions for use.

6) Qiagen is an example of a suitable supplier of *Taq* DNA polymerase. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to give the same results.

Table B.8 — Temperature–time programme

Activation and initial denaturation	15 min/95 °C
Amplification	30 s/95 °C
	30 s/56 °C
	90 s/72 °C
Number of cycles (amplification)	35
Final extension	7 min/72 °C

B.1.7.4 Detection of PCR products (gel electrophoresis)

B.1.7.4.1 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.

B.1.7.4.2 Agarose gel preparation

The amplified PCR products should be detected using a 2,0 % agarose gel. Weigh an appropriate amount of agarose (B.1.4.3.2) and add it to the electrophoresis buffer solution (B.1.4.3.14 or B.1.4.3.15). Allow the solution to boil in a microwave oven or in a water bath (B.1.5.2.6) until the agarose is completely dissolved. Replace the volume lost by evaporation with an equivalent amount of water, mix by swirling (avoid air bubbles trapping), cool the solution down to about 60 °C and keep it at this temperature until usage. Prepare a gel support (gel tray) with a suitable comb in place. Pour the agarose solution on to the gel tray and allow the gel to solidify at room temperature (1 h is usually recommended).

B.1.7.4.3 Agarose gel eletrophoresis

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 (B.1.4.3.14 or B.1.4.3.15). Overlay the gel with approximately 2 mm of the same buffer.

Mix the loading buffer with the PCR products (10 µl) (B.1.4.3.16) in the ratio 1→5 (e.g. add 2,5 µl of loading buffer to 10 µl of PCR products), mix and apply the mixture to the sample slots (wells) with a micropipette. If the unknown samples are suspected to be too concentrated, also provide some dilutions of them to be loaded on to the gel.

To determine the size of the PCR products, add the loading buffer (B.1.4.3.16) and DNA molecular mass standard (B.1.4.3.5) 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.

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 conditions described, 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.

B.1.7.4.4 Staining

After completing the electrophoresis, incubate the gel for 15 min to 50 min in the ethidium bromide solution (B.1.4.3.17) 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, EthBr can be added to the gel before pouring it. In this case, EthBr 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 60 °C. To minimize the problems of EthBr movement in the gel, some EthBr

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 stains.

B.1.7.4.5 Gel recording

Transfer the gel to the transilluminator surface, switch on the UV light and record the DNA fluorescence by photography or video-documentation.

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. See Table B.9. For the interpretation of the results, see ISO 22174.

Table B.9 — Size of amplification products

Type	Primer	Product size bp
A	IA_O3_fw/IA_O4_rev	101
B	CBMLB1/CBMLB2	205
E	CBMLE1/CBMLE2	389
F	CBMLF1/CBMLF2	543
IAC	IAC_f/IAC_r	698

B.1.7.5 Confirmation of a positive PCR result

A positive PCR result shall be confirmed, e.g. by sequencing of the PCR products. Other appropriate methods for confirmation may also be used.

B.1.7.6 Limitation of the method

The method has been tested for the detection of the recent types and subtypes of neurotoxin-producing clostridia type A, B, E, and F. At the time of publication, there is no confirmation whether the primers listed in Table B.3 can detect new subtypes of BoNT/A, B, E, and F.

B.2 Method 2

B.2.1 Introduction

This clause describes a method for the specific amplification and detection of the genes encoding botulinum neurotoxin types A, B, E, and F using agarose gel electrophoresis.

For limitations see B.2.7.6.

B.2.2 Performance characteristics

B.2.2.1 General

The method has been adapted for DNA extracted from different *C. botulinum* types A, B, E, and F reference strains and from naturally contaminated samples.

Parts of the method have been published in Reference [5].

B.2.2.2 Theoretical evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database (Reference [16], 2009-09-20). The result of the search confirmed a complete identity only with the expected target sequences.

B.2.2.3 Selectivity

B.2.2.3.1 Inclusivity test

The inclusivity of the method was tested with 19 *C. botulinum* type A strains, 19 *C. botulinum* type B, 17 *C. botulinum* type E and nine *C. botulinum* type F strains, see Table B.10.

Table B.10 — Inclusivity of the multiplex PCR using target strains

Strain, type and subtype	Number of strains	Type of botulinum neurotoxin gene detected			
		Type A	Type B	Type E	Type F
C. botulinum type A	19	19	0	0	0
C. botulinum type B	19	0	19	0	0
C. botulinum type E	17	0	0	17	0
C. botulinum type F	9	0	0	0	9
NOTE Strains isolated from: Italian National Reference Centre for Botulism (NRCB); IFR; Consultant Laboratory for Anaerobic Bacteria (CLAB) of the University of Leipzig, Germany.					

B.2.2.3.2 Exclusivity test

The exclusivity of the method was tested with 34 non-target organisms, see Table B.11. No cross-reactivity was observed with the non-target bacteria.

Table B.11 — Exclusivity of the multiplex PCR using non-target strains

Strains	Number of strains	Type of botulinum neurotoxin gene detected			
		Type A	Type B	Type E	Type F
C. sporogenes WDCM 00008	1	0	0	0	0
C. perfringens (WDCM 00007 and field strains)	3	0	0	0	0
C. carnis NCTC 13036	1	0	0	0	0
C. histolyticum NCTC 503	1	0	0	0	0
C. butyricum NCTC 7423	1	0	0	0	0
C. barati NCTC 10986	1	0	0	0	0
B. subtilis WDCM 00003	1	0	0	0	0
B. cereus (NCTC 11143 and field strains)	3	0	0	0	0
Thermophilic *Campylobacter* spp. (field strains)	2	0	0	0	0
E. 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
B. thermospacta WDCM 00071	1	0	0	0	0
E. faecalis WDCM 00087	1	0	0	0	0
C. freundii WDCM 00078	1	0	0	0	0
Pseudomonas spp. (field strains)	3	0	0	0	0
Y. enterocolitica (field strains)	3	0	0	0	0
L. fermentum (field strain)	1	0	0	0	0
Aspergillus spp. (field strains)	2	0	0	0	0
S. cerevisiae (field strain)	1	0	0	0	0

B.2.2.4 Sensitivity tests using artificially contaminated samples (Reference [5])

The limit of detection was assessed by measuring artificially contaminated samples with different inoculation levels (0,1 cfu to 10 cfu and 10 cfu to 100 cfu before enrichment) in 10 g of various food matrices (canned fish, canned sausages, and honey) under investigation using the culture method (Reference [5]). According to the results, the false-positive rate for the method using artificially contaminated samples is 0 % and the false-negative rate is 0 %. The limit of detection for the method described is 1 cfu to 10 cfu or spores per test portion before enrichment.

NOTE The experiments were performed with test portions of 10 g.

B.2.2.5 Analytical controls

All tests were performed using positive and negative process controls and additionally for PCR an IAC described in B.2.6.5.

B.2.2.6 Instruments and reagents

Validation was carried out for the Mastercycler® Gradient[2)] using the 2× Multiplex qPCR MasterMix®[3)] and the MasterMix according to Table B.16.

B.2.3 Principle

Specific DNA fragments of the BoNT/A, B, E, and F genes are amplified by multiplex PCR in combination with a homologous IAC using eight primers. The detection of the PCR products is done using agarose gel electrophoresis.

B.2.4 Reagents

B.2.4.1 General

For the quality of reagents to be used, see ISO 22174.

B.2.4.2 Reagents for PCR

B.2.4.2.1 Nuclease-free water.

B.2.4.2.2 PCR buffer solution (without $MgCl_2$), 10×.

The PCR buffer solution is usually delivered with the DNA polymerase, which may or may not include $MgCl_2$ in a concentration specified by the manufacturer. The final $MgCl_2$ concentrations are method specific and are therefore listed in Table B.14. It is possible that ready-to-use reagents are commercially available. If so, follow the manufacturer's instructions for use.

B.2.4.2.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

B.2.4.2.4 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

B.2.4.2.5 dNTP solution, c(dNTP) = 10 mmol/l.

B.2.4.2.6 Oligonucleotide.

Sequences of the oligonucleotides are listed in Table B.12.

Table B.12 — Sequences of oligonucleotides

Type	Primer	Sequence (5′ — 3′)	Amplicon size bp
A	CBMLA3-F	ATT CAg gAT ggA AAg TAT CAC TTA AT	698
	CBMLA3-R	TTC TAC gCC TgC CTg TgA Tg	
B	CBMLB1	CAg gAg AAg Tgg AgC GAA AA	205
	CBMLB2	CTT gCg CCT TTg TTT TCT Tg	
E	CBMLE1	CCA AgA TTT TCA TCC gCC TA	389
	CBMLE2	gCT ATT gAT CCA AAA Cgg TgA	
F	CBMLF1	Cgg CTT CAT TAg AgA ACg gA	543
	CBMLF2	TAA CTC CCC TAg CCC CgT AT	

B.2.4.3 Reagents for gel electrophoresis

As specified in B.1.4.3.

B.2.5 Apparatus

As specified in B.1.5.

B.2.6 Internal amplification control

B.2.6.1 Principle

A DNA fragment of the pUC 19 plasmid is amplified by PCR using two specific primers. The primers possess over-hanging 5'-ends, which are identical to the primers used for the detection of the BoNT/F gene, whereas their 3'-ends are complementary to the pUC 19 predetermined DNA sequence of defined length and sequence. The DNA fragment is used as a template for the competitive IAC.

B.2.6.2 Reagents

B.2.6.2.1 General.

For the quality of reagents to be used, see ISO 22174.

B.2.6.2.2 Nuclease-free water.

B.2.6.2.3 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

B.2.6.2.4 Thermostable DNA polymerase (for hot-start PCR), 5 IU/µl.

B.2.6.2.5 dNTP solution, c(dNTP) = 10 mmol/l.

B.2.6.2.6 KCl solution, c(KCl) = 0,05 mol/l.

B.2.6.2.7 Tris(hydroxymethyl)aminomethane hydrochloride, $c(C_4H_{11}NO_3 \cdot HCl)$ = 0,01 mol/l.

Adjust to pH 8,3 with HCl or NaOH.

B.2.6.2.8 pUC 19 plasmid (accession number L09137).

B.2.6.2.9 Oligonucleotides.

Sequences of the oligonucleotides are listed in Table B.13.

Table B.13 — Sequences of oligonucleotides

Primer	Sequence (5´ — 3´)	Amplicon size of IAC bp
IOF-new- F	Cgg CTT CAT TAg AgA ACg GAT gTC gTg CCA gCT gCA TTA A	800
IOF-new- R	TAA CTC CCC TAg CCC CgT ATgCC ggA TCA AgA gCT ACC AAC	

B.2.6.3 PCR setup for the construction of the IAC

The method is described for a total PCR volume of 50 µl per reaction with the reagents as listed in Table B.14. The PCR can also be carried out in a smaller volume if the solutions are adjusted appropriately. The final values of reagents as outlined in Table B.14 have proven to be suitable.

Table B.14 — Addition of reagents

Reagent	Final value	Volume per sample µl
Template DNA (pUC 19), 0,5 µg/µl	0,5 µg	1
Nuclease-free water		27
KCl solution, c(KCl) = 0,05 mol/l	50 mmol/l	5
Tris·HCl (pH 8,3), c(tris·HCl) = 0,01 mol/l	10 mmol/l	5
$MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l	2,5 mmol/l	5
dNTP solution, c(dNTP) = 10 mmol/l	0,8 mmol/l	4
Primer IAC_f, 5 µmol/l	0,1 µmol/l	1
Primer IAC_r, 5 µmol/l	0,1 µmol/l	1
Thermostable DNA polymerase, 5 IU	2,5 IU	1

B.2.6.4 Temperature–time programme

The temperature–time programme outlined in Table B.15 has been used for the validation study using thermal cyclers MyCycler®[1)] or the Mastercycler® Gradient[2)] and *Taq* DNA polymerase.[5)]

The use of other thermal cyclers might make an adaptation necessary. The time for activation and initial denaturation depends on the polymerase used. If using a hot-start polymerase, follow the manufacturer's instructions for use.

Table B.15 — Temperature–time programme

Activation and initial denaturation	15 min/95 °C
Amplification	60 s/95 °C
	60 s/55 °C
	2 min/72 °C
Number of cycles (amplification)	30
Final extension	10 min/72 °C

B.2.6.5 Use of the IAC

Purify the PCR product using an appropriate commercial kit. Generally 1 500 copies/well of IAC are suitable for gel-based PCR methods. The IAC described is a homologous IAC and is constructed in competition with the primers amplifying type F neurotoxin gene.

B.2.7 Procedure

B.2.7.1 PCR set-up

The method is described for a total PCR volume of 50 µl per reaction with the reagents listed in Table B.16. The PCR can also be carried out in a smaller volume if the solutions are adjusted appropriately. The final values of reagents outlined in Table B.16 have proven to be suitable.

Table B.16 — Addition of reagents

Reagent	Final value	Volume per sample µl
Template DNA	10 ng to 50 ng	3
Nuclease-free water		2,6
PCR buffer 10× (without $MgCl_2$)[a]	1×	5
$MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l	4,8 mmol/l	9,6
dNTP solution, c(dNTP) = 10 mmol/l	0,8 mmol/l	4
PCR primers (according to Table B.3) 5 µmol/l	0,3 µmol/l each	3 each
IAC, 1 500 copies/µl	1 500 copies	1
Thermostable DNA polymerase, 5 IU/µl	2 IU	0,8

[a] If the PCR buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture is adjusted to 4,8 mmol/l.

B.2.7.2 PCR controls

B.2.7.2.1 General.

In accordance with ISO 22174, the controls in B.2.7.2.2 to B.2.7.2.4 are necessary.

B.2.7.2.2 Negative PCR control.

DNA-free water without PCR inhibitors is used as a negative control.

B.2.7.2.3 Positive PCR control.

A mixture of DNA from BoNT-producing clostridia, positive for all four target sequences (type A, B, E, and F neurotoxin genes), approximately 1 000 copies each.

B.2.7.2.4 Amplification control.

Use an appropriate IAC or EAC. An example of an IAC is shown in B.2.6.

B.2.7.3 Temperature–time programme

The temperature–time programme outlined in Table B.17 has been used for the validation study using thermal cycler Mastercycler® Gradient[2)] and *Taq* DNA polymerase.[6)] The use of other thermal cyclers might make an adaptation necessary. The time for activation and initial denaturation depends on the polymerase used. If using a hot-start polymerase, follow the manufacturer's instructions for use.

Table B.17 — Temperature–time programme

Activation and initial denaturation	15 min/95 °C
Amplification	30 s/95 °C
	30 s/60 °C
	85 s/72 °C
Number of cycles (amplification)	27
Final extension	3 min/72 °C

B.2.7.4 Detection of PCR products (gel electrophoresis)

B.2.7.4.1 General

The agarose gel electrophoresis may be carried out as TAE buffer electrophoresis or as TBE buffer electrophoresis. Use the same buffer to dissolve the agarose and to fill the electrophoresis tank. The agarose gel preparation, the DNA sample preparation, the submarine electrophoresis, and the staining are described in B.1.7.4.

B.2.7.4.2 Gel recording

Transfer the gel to the transilluminator surface, switch on the UV light and record the DNA fluorescence by photography or video-documentation.

The target sequences are presumed to have been detected if the size of the PCR product corresponds to the expected length of the target DNA sequences. See Table B.18. The detection of fragments with a size of 205 bp, 389 bp, 543 bp and/or 698 bp indicate that the sample DNA solution contains amplifiable DNA of BoNT-producing clostridia respectively.

Table B.18 — Size of amplification products

Type	Primer	Product size bp
A	CBMLA3-F/CBMLA3-R	698
B	CBMLB1/CBMLB2	205
E	CBMLE1/CBMLE2	389
F	CBMLF1/CBMLF2	543
IAC	IOF-new-F/IOF-new-R	800

B.2.7.5 Confirmation of a positive PCR result

A positive PCR result shall be confirmed, e.g. by sequencing of the PCR products. Other appropriate methods for confirmation may also be used.

B.2.7.6 Limitation and interpretation of the results

The method is tested for the detection of the recent types and subtypes of neurotoxin-producing clostridia type A, B, E, and F. At the time of publication, there is no confirmation whether the primers shown in Table B.12 can detect new subtypes of BoNT/A, B, E, and F.

Annex C
(informative)

Assays for detection of genes encoding botulinum neurotoxin types A, B, E, and F using real-time PCR

C.1 Method 1 — Multiplex PCR assay

C.1.1 Introduction

This annex describes a probe-based multiplex real-time PCR method based on TaqMan®[7] technology for the detection of genes, encoding botulinum neurotoxin types A, B, E, and F.

For limitations see C.1.6.5.

C.1.2 Performance characteristics

C.1.2.1 General

The method has been validated for DNA extracted from various *C. botulinum* type A, B, E, and F reference strains and from naturally contaminated samples.

The method has been published in Reference [6].

C.1.2.2 Theoretical evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database (Reference [16], 2009-09-20). The result of the search confirmed a complete identity only with the expected target sequences.

C.1.2.3 Selectivity

C.1.2.3.1 Inclusivity test

The inclusivity of the method was tested with 14 *C. botulinum* type A strains, 20 *C. botulinum* type B, two *C. botulinum* type E and one *C. botulinum* type F strains, see Table C.1.

7) TaqMan® is a trademark of Roche Molecular Systems. 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.

Table C.1 — Inclusivity of the multiplex PCR using target strains

Strain, type and subtype	Number of strains	Type of botulinum neurotoxin gene detected			
		Type A	Type B	Type E	Type F
C. botulinum type A	14	14	0	0	0
C. botulinum type B	20	0	20	0	0
C. botulinum type E	2	0	0	2	0
C. botulinum type F	1	0	0	0	1
NOTE Strains isolated from: the Consultant Laboratory for Anaerobic Bacteria (CLAB) of the University of Leipzig, Germany; the University of Giessen, Germany; the Federal Institute for Animal Health, Germany; and the Bavarian Health and Food Safety Authority, Germany.					

C.1.2.3.2 Exclusivity test

The exclusivity of the method was tested with 34 non-target organisms. No cross-reactivity was observed with the non-target bacteria, see Table C.2.

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

Strains	Number of strains	Type of botulinum neurotoxin gene detected			
		Type A	Type B	Type E	Type F
C. sporogenes WDCM 00008	1	0	0	0	0
C. perfringens (WDCM 00007 and field strains)	3	0	0	0	0
C. carnis NCTC 13036	1	0	0	0	0
C. histolyticum NCTC 503	1	0	0	0	0
C. butyricum NCTC 7423	1	0	0	0	0
C. barati NCTC 10986	1	0	0	0	0
B. subtilis WDCM 00003	1	0	0	0	0
B. cereus (NCTC 11143 and field strains)	3	0	0	0	0
Thermophilic *Campylobacter* spp. (field strains)	2	0	0	0	0
E. 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
B. thermospacta WDCM 00071	1	0	0	0	0
E. faecalis WDCM 00087	1	0	0	0	0
C. freundii WDCM 00078	1	0	0	0	0
Pseudomonas spp. (field strains)	3	0	0	0	0
Y. enterocolitica (field strains)	3	0	0	0	0
L. fermentum (field strain)	1	0	0	0	0
Aspergillus spp. (field strains)	2	0	0	0	0
S. cerevisiae (field strain)	1	0	0	0	0

C.1.2.4 Sensitivity

C.1.2.4.1 Sensitivity tests using artificially contaminated samples (Reference [5])

The limit of detection was assessed by measuring artificially contaminated samples with different inoculation levels (0, 1 cfu to 10 cfu and 10 cfu to 100 cfu before enrichment) in 10 g of various food

matrices (canned fish, canned sausages, and honey) under investigation using the culture method (Reference [4]). According to the results, the false-positive rate for the method using artificially contaminated samples is 0 % and the false-negative rate is 0 %. The limit of detection for the method described is 1 cfu to 10 cfu or spores per test portion before enrichment.

NOTE The experiments were performed with test portions of 10 g.

C.1.2.4.2 Sensitivity tests using naturally contaminated samples

The limit of detection was assessed by measuring 20 naturally contaminated samples (e.g. honey, vegetable matter, and canned meats) using the culture method as reference method. According to the results, the false-positive rate for the method using naturally contaminated samples is 0 % and the false-negative rate is 0 %.

NOTE The experiments were performed with test portions of 10 g.

C.1.2.5 Analytical controls

All tests were performed using positive and negative process controls and additionally for PCR a heterologous IAC.

C.1.2.6 Instruments and reagents

Validation was carried out with the Stratagene MX 3000P[8] and with the Stratagene MX 3005P[9] using the 2× Brilliant Multiplex qPCR MasterMix[10] (Reference [6]).

C.1.3 Principle

Specific DNA fragments of the BoNT/A, B, E, and F genes are amplified by multiplex real-time PCR. The PCR products are detected by measuring the fluorescence of the probes.

C.1.4 Reagents

C.1.4.1 General.

For the quality of reagents to be used, see ISO 22174.

C.1.4.2 Nuclease-free water.

C.1.4.3 Ready to use MasterMix for real-time-PCR.

A ready-to-use MasterMix contains PCR buffer solution, $MgCl_2$ solution, dNTP solution, an optional decontamination system (dUTP including uracil *N*-glycosylase), and *Taq* polymerase and is mostly adapted to the thermal cycler used. Follow the manufacturer's instructions for use.

C.1.4.4 pUC 19 plasmid (accession number L09137).

---

8) Stratagene MX 3000P is the trade name of a product supplied by Agilent Technologies. 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.

9) Stratagene MX 3005P is the trade name of a product supplied by Agilent Technologies. 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.

10) 2× Brilliant Multiplex qPCR MasterMix is the trade name of a product supplied by Agilent Technologies. 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.

C.1.4.5 Oligonucleotides.

Sequences of the oligonucleotides are listed in Table C.3.

Table C.3 — Sequences of oligonucleotides

Type	Primer	Sequence (5´ — 3´)
A	CBOT A fw	TCT TAC gCg AAA Tgg TTA Tgg
	CBOT A re	TgC CTg CAC CTA AAA gAg gA
	CBOT A S	[c]HEX-Tgg TTT TgA ggA gTC ACT TgA A-TAMRA[b]
B	CBOT B fw	AggA gAA gTg gAg CGR[e]AAA
	CBOT B re	TTC CCT TgA TgC AAA ATg AT
	CBOT B S	[a]FAM-CCT ggg CCA gTT TTA AAT gA-TAMRA[b]
E	CBOT E fw	TCA gCA CCT ggA CTT TCA gA
	CBOT E re	CAT gTT gTT CTA TAT CAC TTg TTC CA
	CBOT E S	[a]FAM-TCC AAA ATg ATg CTT ATA TAC CAA AA-TAMRA[b]
F	CBOT F fw	ATA Cgg ggC TAg ggg AgT TA
	CBOT F re	AAA TCC TgA CCT CCA AAg gTT
	CBOT F S	[c]HEX-CCg AAA AAC CCA TAA ggC TA-TAMRA[b]
Internal amplification control	IAC_pUC_fw	TgT gAA ATA CCg CAC AgA Tg
	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'-(hexachlorofluorescein).

[d] ROX = carboxy-X-rhodamine.

[e] In the sequence of oligonucleotides R is (A,G).

NOTE The use of other fluorescence labels for the probes has not been tested or validated.

C.1.5 Apparatus

C.1.5.1 General

Appropriate equipment according to the method and, in particular, the following.

C.1.5.2 Equipment used for PCR

C.1.5.2.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

C.1.5.2.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

C.1.5.2.3 Thin walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

C.1.5.2.4 Real-time PCR instrument.

C.1.6 Procedure

C.1.6.1 PCR-Setup

The method is described for a total PCR volume of 25 µl per reaction with the reagents as listed in Tables C.4 and C.5. The PCR can also be carried out in a larger volume if the solutions are adjusted appropriately. The final values of reagents as outlined in Tables C.4 and C.5 have proven to be suitable.

Two multiplex systems are used, one system for the detection of genes, encoding type A and B and the IAC and one system for the detection of genes, encoding type E and F.

Table C.4 — Addition of reagents (triplex real-time-PCR-system, BoNT/A, B and IAC)

Reagent		Final value	Volume per sample µl
Template DNA		maximum 250 ng	5
TaqMan®[7] DNA polymerase	2× Brilliant Multiplex qPCR MasterMix[10]	1 x	12,5
PCR buffer			
$MgCl_2$ solution			
dNTP solution			
PCR primers for BoNT/A, B and the IAC (according to Table C.3), 10 µmol/l		0,3 µmol/l each	0,75 each
PCR probes for BoNT/A, B and the IAC (according to Table C.3), 10 µmol/l		0,2 µmol/l each	0,5 each
Nuclease-free water			0,5
pUC 19-plasmid, 1 fg		1 fg	1

Table C.5 — Addition of reagents (duplex real-time-PCR-system, BoNT/E and F)

Reagent		Final value	Volume per sample µl
Template DNA		maximum 250 ng	5
TaqMan®[7] DNA polymerase	2× Brilliant Multiplex qPCR MasterMix[10]	1 x	12,5
PCR buffer			
$MgCl_2$ solution			
dNTP solution			
PCR primers for BoNT/E and F (according to Table C.3), 10 µmol/l		0,5 µmol/l each	1,25 each
PCR probes for BoNT/E and F (according to Table C.3), 10 µmol/l		0,2 µmol/l each	0,5 each
Nuclease-free water			1,5

C.1.6.2 PCR controls

C.1.6.2.1 General.

In accordance with ISO 22174, the controls in C.1.6.2.2 to C.1.6.2.4 are necessary.

C.1.6.2.2 Negative PCR control.

DNA-free water is used as a negative control.

C.1.6.2.3 Positive PCR control.

A mixture of DNA from BoNT-producing clostridia, positive for all four target sequences (type A, B, E, and F neurotoxin genes), approximately 100 copies each.

C.1.6.2.4 Amplification control.

Use an appropriate IAC. An example for an heterologous IAC is given in C.1.6.

C.1.6.3 Temperature–time programme

The temperature–time programme as outlined in Table C.6 has been used for the validation study using the Stratagene MX 3000P[8)] and the Stratagene MX 3005P[9)] system in combination with the 2× Brilliant Multiplex qPCR MasterMix®.[10)] The use of other thermal cyclers might make an adaptation necessary. The time for activation and initial denaturation depends on the polymerase used. If using a hot-start polymerase, follow the manufacturer's instructions for use unless the protocol states otherwise.

Table C.6 — Temperature–time programme

Activation and initial denaturation	10 min/95 °C
Number of cycles (amplification)	45
Amplification	15 s/95 °C
	60 s/55 °C

C.1.6.4 Interpretation of the results

The threshold value to determine the cycle of threshold, C_t, shall be defined 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.[15] 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.

C.1.6.5 Limitation of the method

The method is tested for the detection of the recent types and subtypes of neurotoxin-producing clostridia type A, B, E, and F. At the time of publication, there is no confirmation whether the primers shown in Table C.3 can detect new subtypes of BoNT/A, B, E, and F.

C.2 Method 2 — PCR assay

C.2.1 Introduction

This clause describes a probe-based real-time PCR method based on TaqMan®[7)] technology for the detection of genes, encoding botulinum neurotoxin types A, B, E, and F.

For limitations see C.2.6.5.

C.2.2 Performance characteristics

C.2.2.1 General

The method has been validated for DNA extracted from various *C. botulinum* type A, B, E, and F reference strains and from naturally contaminated samples (Reference [2]).

C.2.2.2 Theoretical evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database (Reference [16], 2009-11-20). The result of the search confirmed a complete identity only with the expected target sequences.

C.2.2.3 Selectivity

C.2.2.3.1 Inclusivity test

The inclusivity of the method was tested with 60 *C. botulinum* type A strains, 68 *C. botulinum* type B, four *C. botulinum* type AB/Ab strains, 21 *C. botulinum/butyricum* type E and seven *C. botulinum/baratii* type F strains,[2] see Table C.7.

Table C.7 — Inclusivity of the multiplex PCR using target strains

Strain, type and subtype	Number of strains	Type of botulinum neurotoxin gene detected			
		Type A	Type B	Type E	Type F
C. botulinum type A	60	60	0	0	0
C. botulinum type B	68	0	68	0	0
C. botulinum type AB	3	3	3	0	0
C. botulinum type Ab	1	1	1	0	0
C. botulinum/butyricum type E	21	0	0	21	0
C. botulinum/baratii type F	7	0	0	0	7

C.2.2.3.2 Exclusivity test

The exclusivity of the method was tested with 27 non-target organisms. No cross-reactivity was observed with the non-target bacteria (Reference [2]). See Table C.8.

Table C.8 — Exclusivity of the multiplex PCR using non-target strains

Strains	Number of strains	Type of botulinum neurotoxin gene detected			
		Type A	Type B	Type E	Type F
Clostridia spp. other than *C. botulinum*	24	0	0	0	0
Non-*Clostridia* spp.	3	0	0	0	0

C.2.2.4 Sensitivity

C.2.2.4.1 Sensitivity tests using 10-fold dilutions of extracted DNA (Reference [2])

The limit of detection was assessed by measuring 10-fold dilutions of purified DNA. The limit of detection for BoNT type A is 25 genome copies, for BoNT type B and F is between 25 genome copies and 250 genome copies, for BoNT type E is 250 genome copies.

C.2.2.4.2 Sensitivity tests using naturally contaminated samples

The limit of detection was assessed by measuring 11 naturally contaminated food samples using the mouse bioassay as reference method. According to the results, the false-positive rate for the method using naturally contaminated samples is 0 % and the false-negative rate is 0 %.

NOTE The experiments were performed with test portions of 10 g.

C.2.2.4.3 Collaborative trial

Primers and probes have been also evaluated in an international collaborative study performed in four European laboratories using different real–time-PCR platforms. Results of the study have been reported in Reference [2].

C.2.2.5 Analytical controls

All tests were performed using positive and negative process controls and additionally for PCR an EAC.

C.2.2.6 Instruments and reagents

Validation was carried out for the ABI 7700[11)] and the Stratagene MX 3005P[9)] for the tests (Reference [2]) using the TaqMan® Universal PCR MasterMix[12)] or qPCR MasterMix buffer.[13)]

C.2.3 Principle

Specific DNA fragments of the BoNT/A, B, E, and F genes are amplified by real-time PCR. The PCR products are detected by measuring the fluorescence of the probes.

C.2.4 Reagents

C.2.4.1 General

For the quality of reagents to be used, see ISO 22174.

C.2.4.2 Nuclease-free water.

C.2.4.3 Ready to use MasterMix for real-time-PCR

A ready-to-use MasterMix contains PCR buffer solution, $MgCl_2$-solution, dNTP-solution, an optional decontamination system (dUTP including uracil *N*-glycosylase) and *Taq* polymerase and is mostly adapted to the thermal cycler used. Follow the manufacturer's instructions for use.

C.2.4.4 Oligonucleotides

Sequences of the oligonucleotides are listed in Table C.9.

11) ABI 7700 is the trade name of a product supplied by Applied Biosystems. 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.

12) TaqMan® Universal PCR MasterMix is the trade name of a product supplied by Applied Biosystems. 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.

13) qPCR MasterMix buffer is the trade name of a product supplied by Eurogentec. 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.

Table C.9 — Sequences of oligonucleotides

Type	Primer	Sequence (5` — 3`)
A	A1	ggA gTC ACT TGA AGT TGA TAC AAA TC
	A2	gCT AAT gTT ACT gCT ggA TCT gTA g
	A3	FAM[b]-TCT TTT Agg TgC Agg CAA ATT T-BHQ1[c]
B	B1	gAT gAA CAg CCA ACA TAT AgT TgT CA
	B2	gTT TCC TTT TTA CCT CTT TTA AgT ACC ATT
	B3	FAM[b]-TgA TgA K[a]AT Agg ATT gAT Tgg TAT TCA-BHQ1[c]
E	E1	CTA TCC AAA ATg ATg CTT ATA TAC CAA A
	E2	ggC ACT TTC TgT gCA TCT AAA TA
	E3	FAM[b]-ATg ATT CTA ATg gAA CAA gTg ATA TAg AAC AAC ATg ATg T -BHQ1[c]
F	F1	gCA ATA TAg gAT TAC TAg gTT TTC ATT C
	F2	gAA ATA AAA CTC CAA AAg CAT CCA TT
	F3	FAM[b]-TTg gTT gCT AgT AgT Tgg TAT TAT AAC AA-BHQ1[c]
External amplification control	EAC1	TAC CAT Agg Agg AAg CCA C
	EAC2	CTT CTT CCT AAT CTC TAC gCA T
	EAC3	HEX[d]-gTg CCA gCA gCC gCg gTA ATA Cg-BHQ1[c]

[a] In the sequence of oligonucleotides K is (G,T).

[b] FAM: 6-carboxyfluorescein.

[c] BlackHole™ Dark Quencher 1. BlackHole is the trademark of a product supplied by Biosearch Technologies. 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.

[d] HEX: 5'-(Hexachlorofluorescein).

NOTE The use of other fluorescence labels for the probes has not been tested or validated.

C.2.4.5 External amplification control

C.2.4.5.1 Principle

DNA extracted from *C. butyricum* ATCC® 19398™[14)] is amplified by PCR using the specific primers described in Table C.9 in a separate reaction tube together with the template DNA (Reference [8]).

C.2.4.5.2 *C. butyricum* strain ATCC® 19398™

Grow *C. butyricum* strain ATCC® 19398™ in TPGY broth at 30 °C ± 1 °C for 24 h ± 2 h under anaerobic conditions. Extract DNA from enriched culture (9.3). Use approximately 2 500 copies of DNA as EAC.

C.2.4.5.3 Use of the EAC

The size of the amplification product of *C. butyricum* strain ATCC® 19398™ is 232 bp. A total of 2 500 copies of DNA gives a C_t value of approximately 30.

C.2.5 Apparatus

C.2.5.1 General

Appropriate equipment according to the method and, in particular, the following.

14) ATCC® 19398™ is the trademark of a product supplied by ATCC. 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.

C.2.5.2 Equipment used for PCR

C.2.5.2.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

C.2.5.2.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

C.2.5.2.3 Thin walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

C.2.5.2.4 Real-time PCR instrument.

C.2.6 Procedure

C.2.6.1 PCR-Setup

The method is described for a total PCR volume of 25 µl per reaction with the reagents as listed in Tables C.10 and C.11. The PCR can also be carried out in a larger volume if the solutions are adjusted accordingly. The final values of reagents as outlined in Tables C.10 and C.11 have proven to be suitable.

The primer systems are divided into five different reactions: four separate toxin type-specific PCR tests and one for the EAC.

Table C.10 — Addition of reagents (real-time-PCR-systems BoNT/A, B, E or F)

Reagent		Final value	Volume per sample µl
Template DNA		maximum 250 ng	5
TaqMan®[7] DNA polymerase	2× TaqMan® Universal PCR MasterMix[12]	1×	12,5
PCR buffer			
$MgCl_2$ solution			
dNTP solution			
PCR primers for BoNT/A, B, E or F (according to Table C.9) 10 µmol/l		0,3 µmol/l each	0,75 each
PCR probes for BoNT/A, B, E or F (according to Table C.9), 10 µmol/l		0,1 µmol/l each	0,25 each
Nuclease-free water			0,75

Table C.11 — Addition of reagents (EAC)

Reagent		Final value	Volume per sample µl
Template DNA		maximum 250 ng	5
TaqMan®[7] DNA polymerase	2× TaqMan® Universal PCR MasterMix[12]	1×	12,5
PCR buffer			
$MgCl_2$ solution			
dNTP solution			
PCR primers for the EAC (according to Table C.9) 10 µmol/l		0,9 µmol/l each	2,25 each
PCR probes for BoNT/E and F (according to Table C.9), 10 µmol/l		0,3 µmol/l each	0,75 each
C. butyricum DNA (2 500 copies/µl)		2 500 copies	1
Nuclease-free water			1,25

C.2.6.2 PCR controls

C.2.6.2.1 General.

In accordance with ISO 22174, the controls in C.2.6.2.2 to C.2.6.2.4 are necessary.

C.2.6.2.2 Negative PCR control.

DNAse-free water is used as a negative control.

C.2.6.2.3 Positive PCR control.

A mixture of DNA from BoNT-producing clostridia, positive for all four target sequences (type A, B, E, and F neurotoxin genes), approximately 1 000 copies each.

C.2.6.2.4 Amplification control.

Use an appropriate IAC or EAC. An example for an EAC is given in C.2.4.5.

C.2.6.3 Temperature–time programme

The temperature–time programme as outlined in Table C.12 has been used for the validation study using the ABI 7700[11)] and the Stratagene MX 3005P[9)] system in combination with TaqMan® Universal PCR MasterMix[12)] or qPCR MasterMix buffer.[13)] The use of other thermal cyclers might make an adaptation necessary. The time for activation and initial denaturation depends on the polymerase used. If using a hot-start polymerase, follow the manufacturer's instructions for use unless the protocol states otherwise.

Table C.12 — Temperature–time programme

Activation and initial denaturation	10 min/95 °C
Number of cycles (amplification)	35
Amplification	15 s/95 °C
	60 s/60 °C

C.2.6.4 Interpretation of the results

The threshold value to determine the cycle of threshold, C_t, shall be defined 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.[15] 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.

C.2.6.5 Limitation of the method

The method is tested for the detection of the recent types and subtypes of neurotoxin-producing clostridia type A, B, E, and F. At the time of publication, there is no confirmation whether the primers shown in Table C.9 can detect new subtypes of BoNT/A, B, E, and F.

Annex D
(informative)

Preparation of *C. botulinum* spores

D.1 Introduction

This annex describes the preparation of *C. botulinum* spores (proteolytic and non-proteolytic strains) for the process control (9.2.3). See Figure D.1 for an overview. Background details appear in Reference [4].

D.2 Culture media

D.2.1 General

Follow ISO 11133 for the preparation, production, and performance testing of culture media.

D.2.2 Modified Anellis broth

Other approved enrichment culture media can be used provided equivalent performance is shown.

D.2.2.1 Agar phase-medium

D.2.2.1.1 Composition and pH

Tryptone	25 g
Peptone	2,5 g
Yeast extract	0,5 g
Agar	0,5 g
Potassium hydrogenphosphate [K_2HPO_4]	0,625 g
Water	to 500 ml

pH 7,5 ± 0,1

D.2.2.1.2 Preparation

Dissolve the components in the water by boiling. Adjust the pH so that after sterilization it will be pH 7,5 ± 0.1 at 25 °C. Dispense the base into flasks or bottles of appropriate capacity. Sterilize for 15 min at 121 °C. The medium will become solid (solid part of the Modified Anellis Broth). It is highly recommended that the medium be used on the day of preparation.

D.2.2.2 Fluid medium

D.2.2.2.1 Composition and pH

Yeast extract	0,25 g
Ammonium sulfate [$(NH_4)_2SO_4$] (w ± 2 %)	5 g

Thiamine hydrochloride [$C_{12}H_{17}ClN_4OS{\cdot}HCl$] ($w$ ± 0.0001 %)	0,25 µg
Water	to 250 ml

pH 7,5 ± 0,1

D.2.2.2.2 Preparation

Dissolve the components in the water by boiling. Adjust the pH so that after sterilization it is pH 7,5 ± 0,1 at 25 °C. Dispense the base into flasks or bottles of appropriate capacity. Sterilize for 15 min at 121° C. Store in a refrigerator at 5 °C ± 3 °C until use. It is highly recommended that the medium be used on the day of preparation.

D.2.2.3 Preparation of the complete medium

For the final medium use a solid to liquid volume of 4 + 1. After cooling the solid agar phase to 20 °C to 30 °C, transfer a suitable amount of a proteolytic *C. botulinum* strain culture (see D.5) to the solid medium and overlay it with the fluid medium.

D.2.3 Biphase medium

Other approved enrichment culture media can be used provided equivalent performance is shown.

D.2.3.1 Agar phase (liver agar)

D.2.3.1.1 Composition and pH

Infusion from fresh liver	30 g
Peptone	0,6 g
Sodium chloride, NaCl	0,3 g
Agar	1,2 g
Water	to 500 ml

pH 6,9 ± 0,1

D.2.3.1.2 Preparation

Dissolve the components in the water by boiling. Adjust the pH so that after sterilization it is pH 6,9 ± 0,1 at 25 °C. Dispense the base into flasks or bottles of appropriate capacity. Sterilize for 15 min at 121 °C. Store in a refrigerator at 5 °C ± 3 °C until use. It is highly recommended that the medium be used on the day of preparation.

D.2.3.2 Fluid medium

Distilled water	500 ml

D.2.3.3 Preparation of the complete medium

For the final medium use a solid to liquid volume of 2 + 1. After cooling the solid agar-phase to 20 °C to 30 °C, transfer a suitable amount of a non-proteolytic *C. botulinum* strain culture (see D.5) to the solid medium and overlay it with the fluid medium.

D.3 Reagents

D.3.1 Reagents for the cleaning of spores

D.3.1.1 Sodium chloride, NaCl.

D.3.1.2 Sodium chloride solution, ρ(NaCl) = 0,85 g/l, dissolved in sterile water.

D.3.2 Reagents for Gram-staining

D.3.2.1 Crystal violet, $[(CH_3)_2NC_6H_4]_2C{:}C_6H_4{:}N^+(CH_3)_2Cl^-$.

D.3.2.2 Ethanol, $\varphi(C_2H_5OH)$ = 96 %.

D.3.2.3 Ammonium oxalate, $NH_4OCOCOONH_4$.

D.3.2.4 Water.

D.3.2.5 Iodine crystals.

D.3.2.6 Potassium iodide.

D.3.2.7 Safranin, $C_{20}H_{19}ClN_4$.

D.3.2.8 Safranin solution, $\rho(C_{20}H_{19}ClN_4)$ = 0,1 g/l.

D.3.2.9 Crystal violet stain.

Dissolve 2,0 g crystal violet (D.3.2.1) in 20 ml of ethanol (D.3.2.2) and 0,8 g of ammonium oxalate in 80 ml of water. Gently mix the two solutions together. The mixture is stable for 2 to 3 years.

D.3.2.10 Gram's iodine

Dissolve 2 g of potassium iodide (D.3.2.6) in 2 ml of the distilled water. The crystals dissolve and the solution becomes very cold. Dissolve the iodine crystals (D.3.2.5) in the potassium iodide solution. Dilute the mixture with 298 ml of water. The mixture is stable for 2 to 3 years.

D.3.2.11 Immersion oil.

D.4 Apparatus

D.4.1 General

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

D.4.2 Equipment for microbial enrichment

D.4.2.1 Water baths, capable of being maintained at 30 °C ± 1 °C.

D.4.2.2 Anaerobic jar or anaerobic cabinet, capable of being maintained at 30 °C ± 1 °C, according to ISO 7218.

D.4.2.3 Incubator, capable of operating at 30 °C ± 1 °C.

D.4.2.4 Flasks or bottles, of appropriate capacity.

D.4.3 Equipment for the cleaning of the spores

D.4.3.1 Ultrasonic cleaning bath with a operating frequency up to 38 Hz.

D.4.3.2 Centrifuge, for 50 ml and 100 ml tubes and with adjustable acceleration of up to 6 000 × *g*.

D.4.3.3 Light microscope with a magnification value of the objective lens up to 100×.

D.4.3.4 Microscope slides, 75 mm by 25 mm.

D.5 Procedure

D.5.1 Inoculation

D.5.1.1 Proteolytic *C. botulinum* strains (Modified Anellis broth)

For the final medium use a solid to liquid volume of 4 + 1. After cooling the solid agar-phase to 20 °C to 30 °C, inoculate the solid phase (D.2.2.1) of the medium with 1 ml of a TPGY culture (9.2) of a proteolytic *C. botulinum* strain and overlay it with the fluid medium (D.2.2.2).

D.5.1.2 Non-proteolytic *C. botulinum* strains (Biphase medium)

For the final medium, use a solid to liquid volume of 2 + 1. After cooling the solid agar-phase to 20 °C to 30 °C inoculate the solid phase (D.2.3.1) of the medium with 1 ml of a TPGY culture (9.2) of a non-proteolytic *C. botulinum* strain and overlay it with the fluid medium (D.2.3.2).

D.5.2 Enrichment

Incubate proteolytic *C. botulinum* strains in modified Anellis broth, non-proteolytic strains in a two phase medium for 7 days at 30 °C ± 1 °C under anaerobic conditions (D.4.2.3).

D.5.3 Isolation and cleaning of spores from the enrichment culture

Centrifuge 20 ml of the fluid part of the enrichment culture for 15 min at 6 000 × *g* (D.4.3.2) and discard the supernatant.

Resuspend the pellet in 20 ml of sodium chloride solution (D.3.1.2.), centrifuge the sample at 6 000 × *g* for 15 min and sonicate the sample for 5 min in an ultrasonic cleaning bath (D.4.3.1). Repeat the centrifugation, resuspension, and sonication steps five times.

Suspend the pellet in 1 ml of sodium chloride solution (D.3.1.2) centrifuge the sample for 1 h at 6 000 × *g* and discard the supernatant.

Resuspend the pellet in 20 ml of sodium chloride solution (D.3.1.2), centrifuge the sample at 6 000 × *g* for 15 min (D.4.3.2), discard the supernatant and sonicate the sample for 5 min in an ultrasonic cleaning bath (D.4.3.1). Repeat the centrifugation, resuspension, and sonication steps four times.

Resuspend the pellet in 1 ml of sodium chloride solution (D.3.1.2) and store the spore suspension at 1 °C until use.

D.5.4 Confirmation of the result

D.5.4.1 General

Different procedures for staining of bacteria and spores and microscopy can be used for the confirmation of the isolation and cleaning results. An example is given in D.5.4.2.

D.5.4.2 Gram staining

D.5.4.2.1 Smear preparation

Transfer 1 drop or 2 drops of the spore suspension to a microscope slide (D.4.3.4) and spread into an even thin film. Dry on a flat surface. After air drying, pass the slide through a flame two or three times. Cool the slide before staining.

D.5.4.2.2 Staining

Flood the fixed smear with the crystal violet stain (D.3.2.9); allow the stain to remain for 30 s. Wash briefly in water to remove excess crystal violet. Flood with Gram's iodine (D.3.2.10) for 3 s. Wash briefly in water, do not let the section dry out. Rinse gently with flowing tap water. Decolourize by letting the ethanol solution (D.3.2.2) flow over the smear and stop when the runoff becomes clear. Wash in tap water and counterstain with safranin solution (D.3.2.8) for 15 s. Remove excess counterstain with a gentle flow of tap water. Drain the slide, and dry it in air in an upright position.

D.5.4.2.3 Microscopical examination

Examine the smear under a light microscope with a magnification value of the objective lens of 100× (D.4.3.3) with immersion oil (D.3.2.11). *C. botulinum* vegetative cells are Gram-positive rod-shaped bacteria and generate subterminal endospores. The vegetative cells are blue. The spores can be located within the cell or exist as free spores. There should only be a few vegetative cells in the visual field of the microscope.

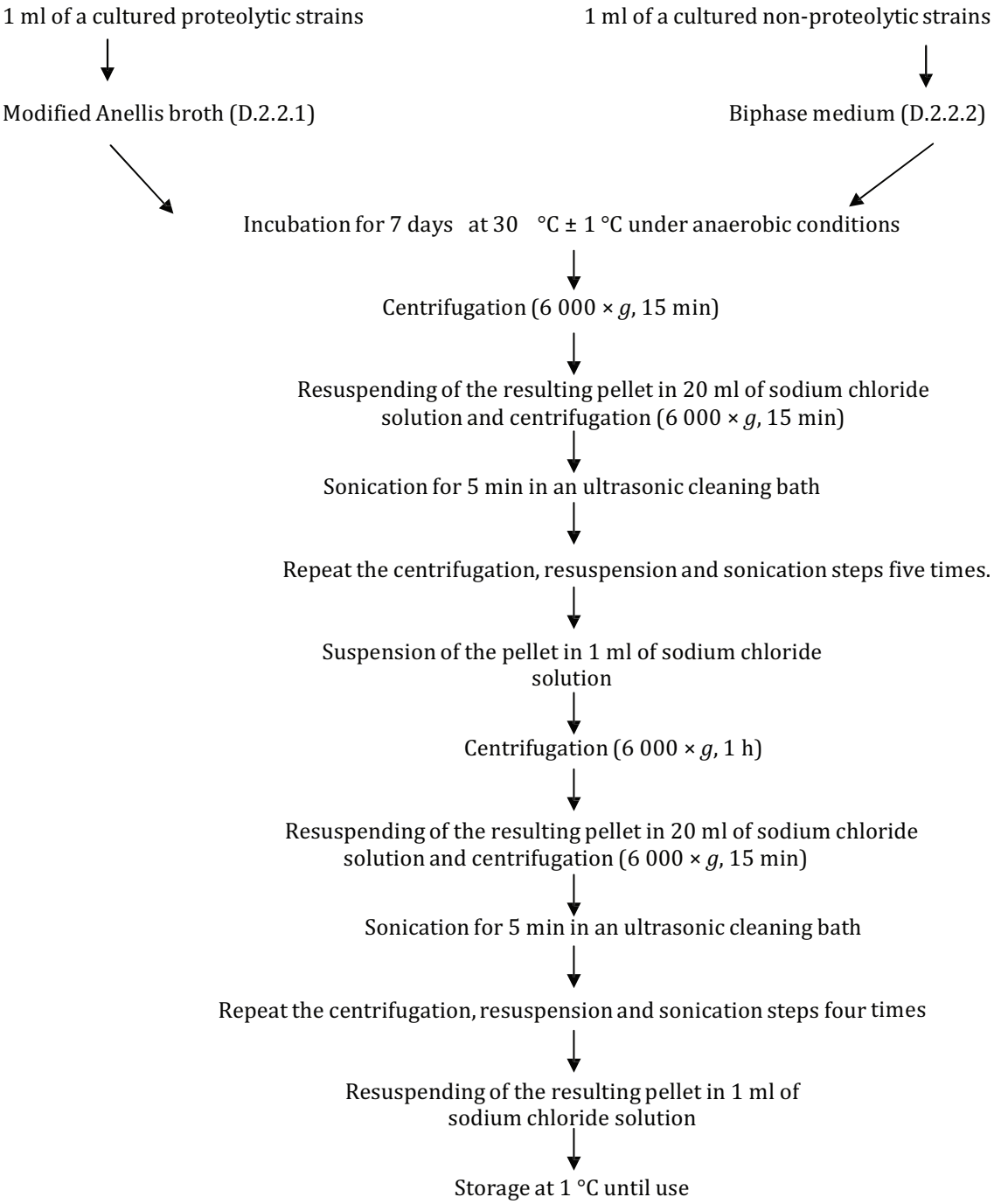

Figure D.1 — Flow diagram of the preparation of spores

Bibliography

[1] De Medici D., Anniballi F., Wyatt G.M., Lindström M., Messelhäusser U., Aldus C.F., Delibato E., Korkeala H., Peck M.W., Fenicia L Multiplex PCR to detect botulinum neurotoxin-producing clostridia in clinical, food and environmental samples. *Appl. Environ. Microbiol.* 2009, **75** pp. 6457–6461

[2] Fach P., Fenicia L., Knutsson R., Wielinga P.R., Anniballi F., Delibato E., Auricchio B., Woudstra C., Agren J., Segerman B., De Medici D., Van Rotterdam BJ An innovative molecular detection tool for tracking and tracing *Clostridium botulinum* types A, B, E, F and other botulinum neurotoxin producing clostridia based on the GeneDisc cycler. *Int. J. Food Microbiol.* 2011, **145** (Suppl 1) pp. S145–S151

[3] Fenicia L., De Medici D., Anniballi F., Delibato E., Aureli P. Multiplex PCR method to detect Botulinum neurotoxins producing clostridia in food and clinical samples. Abstract Book of MED-VET-NET 2nd Annual General meeting 2006-05-03/06, Malta

[4] Fenicia L., Fach P., Van Rotterdam B., Author S.V.A., Anniballi F., Wielinga P., Auricchio B., Woudstra C., Delibato E., De Medici D., Knutsson R. Towards an international standard for detection and typing botulinum neurotoxin-producing clostridia types A, B, E and F in food, feed and environmental samples: A European ring trial study to evaluate a real-time PCR assay. *Int. J. Food Microbiol.* 2011, **145** (Suppl 1) pp. S152–S157

[5] Grecz N., Anellis A., Schneider M.D. Procedure for cleaning of *Clostridium botulinum* spores. *J. Bacteriol.* 1962, **84** pp. 552–558

[6] Lindström M., Keto R., Markkula A., Nevas M., Hielm S., Korkeala H. Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. *Appl. Environ. Microbiol.* 2001, **67** pp. 5694–5699

[7] Messelhäusser U., Zucker R., Ziegler H., Elmer-Englhard D., Kleih W., Höller C., Busch U. Nachweis von *Clostridium botulinum* Typ A, B, E und F mittels real-time-PCR [Detection of *Clostridium botulinum* type A, B,E and F with real-time-PCR]. *J. Verbrauch. Lebensm*. 2007, **2** pp. 198–201

[8] Center for Food Safety and Applied Nutrition. M151: Trypticase–peptone–glucose–yeast extract broth. In: *Bacteriological analytical manual online*. Silver Spring, MD: U.S. Food & Drug Administration, 2001. Available (viewed 2013-04-22) at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm063710.htm

[9] ISO 6887-2, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products*

[10] ISO 6887-3, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*

[11] ISO 6887-4, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products*

[12] ISO 6887-5, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products*

[13] ISO 6887-6, *Microbiology of food and animal feed — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 6: Specific rules for the preparation of samples taken at the primary production stage*

[14] ISO 22118, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection and quantification of food-borne pathogens — Performance characteristics*

[15] ISO 22119, *Microbiology of food and animal feeding stuffs — Real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

[16] National Center for Biotechnology Information (NCBI) Blast[15] *search,* European Molecular Biology Laboratory (EMBL) database. Available (viewed 2013-04-22) at: http://blast.ncbi.nlm.nih.gov/Blast.cgi

[17] Nakamura S School of Medicine, Kanazawa University, Kanazawa, Japan

15) 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.

ICS 07.100.30

Price based on 47 pages