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Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Detection of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

Microbiologie de la chaîne alimentaire — Réaction de polymérisation en chaîne (PCR) pour la détection de micro-organismes pathogènes dans les aliments — Détection des Yersinia enterocolitica *et* Yersinia pseudotuberculosis *pathogènes*

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Foreword

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The committee responsible for this document is 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

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are zoonotic bacterial pathogens causing food-borne infection (yersiniosis) in humans worldwide. The main reservoir for pathogenic *Y. enterocolitica* is domestic pigs[3] and for *Y. pseudotuberculosis* a wide range of domestic and wild animals such as rodents, deer, birds, and various farm animals serve as potential reservoirs.[4] Some of the biotypes of *Y. enterocolitica* are associated with human infection. In contrast, all *Y. pseudotuberculosis* are considered potentially pathogenic to humans.[9]

The chromosomally located gene *ail* (attachment invasion locus) is present in all bio(sero)types of *Y. enterocolitica* associated with disease and a variant of it is also present in *Y. pseudotuberculosis*.[8] The *ail* gene is the target gene used for detection in this Technical Specification, and the developed primer/probe sets target different sites of the *ail* gene for the two pathogens.[7][8][13][14]

Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Detection of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

1 Scope

This Technical Specification specifies two horizontal methods for detection of the pathogenic bioserotypes of *Y. enterocolitica* and one for detection of *Y. pseudotuberculosis* by using real-time PCR-based methods. The described methods allow for the detection of the two pathogens in enrichments and allow the isolation of colonies. *Y. pestis*, the causative agent of bubonic and pneumonic plague harbours a variant of the *ail* gene as well and will be detected by the same primer/probe set as *Y. pseudotuberculosis*. However, *Y. pestis* is normally not associated with food. This Technical Specification is applicable to products for human consumption, animal feeding stuffs, and environmental samples.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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 10273, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of presumptive pathogenic* Yersinia enterocolitica

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, *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 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*

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 following terms and definitions given in ISO 22174 and ISO 22119 apply.

4 Principles

4.1 General

The method comprises the following consecutive steps:

a) Microbial enrichment (4.2);

b) Nucleic acid extraction (4.3);

c) Amplification and detection (4.4);

d) Isolation (4.5).

4.2 Microbial enrichment

The number of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* bacterial cells is increased by growth in a non-selective or semi-selective liquid nutrient medium.

4.3 Nucleic acid extraction

Bacteria cells are separated from the nutrient broth, lysed, and the nucleic acid extracted for use in the PCR reaction.

4.4 Amplification and detection

The extracted nucleic acid is amplified using a probe-based real-time PCR. Detection of the target sequence is achieved by monitoring a clear increase in the fluorescence signal above the cycle threshold, *Ct*.

NOTE Probe-based real-time PCR combines amplification, detection, and confirmation of the target DNA.

4.5 Isolation

After a PCR-positive result is obtained, the target organism can be isolated by using culture methods as described in this Technical Specification.

5 Reagents

5.1 General

For the stages in 4.1 b)-c), molecular grade reagents and consumables suitable for molecular biology shall be used as given in ISO 20837 and ISO 20838.

Requirements are specified in ISO 20838.

The following media and reagents should be used.

5.2 Culture media

5.2.1 General

See ISO 7218 and ISO 11133 for the preparation, production, and performance testing of culture media.

5.2.2 Diluent

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

5.2.3 Enrichment media

5.2.3.1 Tryptone-soya broth supplemented with yeast, TSBY

5.2.3.1.1 Composition

Pancreatic digest of casein	17,0 g

Papaic digest of soyabean meal	3,0 g
Sodium chloride, (NaCl)	5,0 g
Dibasic potassium phosphate, (K_2 HPO_4)	2,0 g
Glucose	2,5 g
Yeast extract	6,0 g
Water	1 000 ml

5.2.3.1.2 Preparation

Dissolve the above ingredients in 1 000 ml distilled water. Adjust the pH, if necessary, so that after sterilization it is pH 7,3 ± 0,2. Dispense the medium into tubes or flasks of suitable capacity to obtain portions appropriate for the test samples. Sterilize for 15 min at 121 °C ± 1 °C.

Store the medium in the dark at room temperature and not longer than 4 weeks.

Alternatively, use dehydrated Tryptone soya broth (TSB) 30 g/l supplemented with 0,6 % yeast extract, pH 7,3 ± 0,2.

5.2.3.2 Peptone-sorbitol-bile-salt broth, PSB[15]

5.2.3.2.1 Composition

Peptone	5,0 g
Sorbitol	10,0 g
Sodium chloride, (NaCl)	5,0 g
Disodium hydrogen phosphate (Na_2HPO_4)	8,23 g
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$)	1,2 g
Bile salts	1,5 g
Water	1 000 ml

5.2.3.2.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, if necessary by heating.

Adjust the pH, if necessary, so that after sterilization it is pH 7,6 ± 0,2.

Dispense the medium into tubes or flasks of suitable capacity to obtain portions appropriate for the test samples. Sterilize for 15 min at 121 °C ± 1 °C.

5.2.3.3 Cold enrichment broth, PMB [5]

5.2.3.3.1 Composition

Disodium hydrogen phosphate (Na_2HPO_4)	7,6 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,0 g
Sodium chloride (NaCl)	8,5 g

Mannitol	10,0 g
Bile salts N°. 3	1,5 g
Water	1 000 ml

5.2.3.3.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, if necessary by heating.

Adjust the pH, if necessary, so that after sterilization it is pH 7,6 ± 0,2.

Dispense the medium into tubes or flasks of suitable capacity to obtain portions appropriate for the test samples. Sterilize for 15 min at 121 °C ± 1 °C.

5.2.4 Selective solid medium

5.2.4.1 Cefsulodin Irgasan Novobiocin agar, CIN[10]

5.2.4.1.1 Basic medium, composition

Enzymatic digest of gelatin	17,0 g
Enzymatic digest of casein and animal tissues	3,0 g
Yeast extract	2,0 g
Mannitol	20,0 g
Sodium pyruvate	2,0 g
Sodium chloride, (NaCl)	1,0 g
Magnesium sulfate, ($MgSO_4.7\ H_2O$)	0,01 g
Sodium desoxycholate	0,5 g
Neutral red	0,03 g
Crystal violet	0,001 g
Agar	12,5 g
Water	1 000 ml

5.2.4.1.2 Preparation

Dissolve the components or dehydrated basic medium in the water by boiling. Adjust the pH, if necessary, so that after sterilization it is pH 7,4 ± 0,2 at 25 °C. Dispense the medium into flasks of suitable capacity. Sterilize for 15 min at 121 °C ± 1 °C.

5.2.4.2 Supplements

5.2.4.2.1 **Cefsulodin solution** (15 mg/ml)

Dissolve 1,5 g Cefsulodin in 100 ml water. Sterilize by filtration.

5.2.4.2.2 **Irgasan™**[5-chloro-2-(2,4-dichlorophenoxy)phenol], ethanolic solution (4 mg/ml).

Dissolve Irgasan in ethanol, store the solution at about –20 °C for not more than 4 weeks.

5.2.4.2.3 **Novobiocin solution** (2,5 mg/ml)

Dissolve novobiocin in water. Sterilize by filtration.

5.2.4.3 Composition of the complete medium

Basic medium (5.2.4.1.1)	997 ml
Cefsulodin solution (5.2.4.2.1)	1 ml
Irgasan solution (5.2.4.2.2)	1 ml
Novobiocin solution (5.2.4.2.3)	1 ml

5.2.4.4 Preparation

Add each antibiotic solution aseptically to the basic medium, cooled to about 45 °C, and mix. Pour approximately 15 ml of the complete medium into sterile petri dishes.

5.2.5 Potassium hydroxide in saline solution, KOH

5.2.5.1 Composition

Potassium hydroxide (KOH)	0,25 g/0,50 g
Saline solution	100 ml

NOTE It is recommended to use freshly prepared 0,5 % KOH for pathogenic *Y. enterocolitica* and 0,25 % for *Y. pseudotuberculosis.*

5.2.5.2 Preparation

Dissolve the potassium hydroxide in the saline solution. Dispense the solution into flasks of a suitable capacity. Sterilize for 15 min at 121 °C ± 1 °C. Prepare the solution the day before use.

5.3 Nucleic acid extraction

Nucleic acid extraction procedure and reagents appropriate for Gram-negative bacteria shall be used.

NOTE Commercial kits can also be used.

5.4 Reagents for PCR

See ISO 22119 and ISO 20838.

5.5 Primers and probes

The primers and probes for detection of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* are listed in Annex B and Annex C.

6 Apparatus and equipment

6.1 General

Microbiology equipment (see ISO 7218, ISO 20837, and ISO 22119), in particular, the following

6.2 Equipment for sample preparation prior to enrichment

Peristaltic blender and sterile bags with filter.

NOTE Filter of small pore size suitable for PCR is recommended.

6.3 Equipment for microbial enrichment

Incubators, capable of operating at 25 °C ± 1 °C and 30 °C ± 1 °C.

6.4 Equipment for nucleic acid extraction

6.4.1 Micro-centrifuge tubes, with capacities of 1,5 ml and 2,0 ml.

6.4.2 Centrifuge, for reaction tubes with a capacity of 1,5 ml and 2,0 ml and capable of achieving an acceleration up to approximately 14 000 × *g*.

6.4.3 Thermoblock, with heating capacity of up to 100 °C.

6.4.4 Graduated pipettes and **pipette filter tips**, for volumes between 1 µl to 1 000 µl.

6.4.5 Mixer.

6.5 Equipment for real-time PCR

6.5.1 Real-time PCR thermal cycler.

6.5.2 96-well plates and/or 8-well strips.

7 Sampling

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

8 Procedure

See diagram in Annex A.

8.1 Sample preparation prior to enrichment

8.1.1 General

It is recommended to analyse at least 25 g or 25 ml of sample. However, if sample amount is limited other sample sizes can be used.

8.1.2 Preparation of the sample

Prepare and homogenize the sample according to ISO 6887-1 and the relevant part of ISO 6887 dealing with the specific product type intended for analysis (see ISO 6887-1 to ISO 6887-5).

A test portion of the sample is added to the enrichment medium to obtain a ratio of the test portion to medium of 1/10.

8.2 Microbial enrichment

Depending on the target, most suitable enrichment medium should be used.

8.2.1 Pathogenic *Y. enterocolitica*

PSB enrichment medium should be used prior to detection by PCR.

PSB enrichment is performed at 25 °C ± 1 °C for 24 h ± 3 h.

If isolation of colonies is required, enrichment time should be increased up to 48 h. It is recommended to refer to ISO 10273.

If thermal lysis is used to extract DNA (method Annex B), prolonged enrichment for 48 h in PSB may be needed.

8.2.2 *Y. pseudotuberculosis*

TSBY enrichment medium should be used prior to detection by PCR.

TSBY enrichment is performed at 25 °C ± 1 °C for 24 h ± 3 h.

NOTE The procedure does not allow isolation of the bacterium. Isolation involves re-enrichment of the sample by selective culture as described in 8.3.2 and C.2.

If there is a limited amount of sample or swab sample that cannot be divided for PCR screening (TSBY) and isolation of colonies, PMB should be used instead of TSBY, see C.2, 5.2.3.3, and 8.3.2.

8.2.3 Pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*

For the simultaneous detection of *Y. pseudotuberculosis* and pathogenic *Y. enterocolitica* by PCR, TSBY medium may be used.

The risk of false-negative results for *Y. enterocolitica* may increase when TSBY is used.

8.3 Isolation of colonies, optional

8.3.1 Pathogenic *Y. enterocolitica*

After enrichment for 24 h ± 3 h, transfer 0,5 ml of the PSB enrichment into 4,5 ml 0,5 % potassium hydroxide solution, KOH (5.2.5) and mix gently for 25 s ± 5 s. Inoculate 1 µl, 10 µl, and 100 µl of the mixture after streaking on the surface of three CIN-agar plates. Continue incubation of the enrichment for further 24 h. Incubate CIN agar plates at 30 °C ± 1 °C for 24 h ± 3 h. Plates are examined (preferably by stereo microscope) and typical colonies with bull's eye appearance are picked for confirmation. Second plating on CIN agar is made after 48 h ± 4 h enrichment, performed in the same way as for the plating after 24 h.

Presumptive pathogenic *Y. enterocolitica* colonies should be confirmed according to ISO 10273.

NOTE Only for samples with high expected *Y. enterocolitica* contamination levels, enrichment in PSB can be used to isolate colonies as described in this Technical Specification. For samples with low suspected *Y. enterocolitica* contamination levels, it is recommended to follow ISO 10273 for isolation of colonies.

8.3.2 *Y. pseudotuberculosis*

Test sample is inoculated (dilution 1/10) into the cold enrichment medium PMB (5.2.3.3), homogenized and incubated at 4 °C ± 1 °C for 7 days and 14 days. If PCR detection of Y. *pseudotuberculosis* has to be performed after enrichment in PMB (see 8.2.2), nucleid acid extraction and further PCR amplification should be carried out after 72 h ± 8 h of incubation (see 8.4 and 8.5).

Transfer 0,5 ml of the incubated enrichment (PMB) into 4,5 ml 0,25 % potassium hydroxide solution, KOH, (5.2.5) and mix gently for 25 s ± 5 s. Transfer the mixture by using a 10 µl loop onto the surface of a CIN-agar plate(performed in duplicate). Incubate CIN-agar plates at 30 °C ± 1 °C for a total of 48 h ± 4 h but examine the plates for typical growth after 24 h. Plates are examined by stereo microscope and typical colonies are picked for confirmation. Typical colonies are small (usually <0,5 mm in diameter after 24 h incubation) and irregular-edged. Colonies usually have deep-red diffuse centre with no clear borderline. The transparent or translucent zone surrounding the centre has ground-glass appearance and may be thin and hardly visible after 24 h. For confirmation, biochemical or PCR tests can be used.

8.3.3 Process controls

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

8.4 Nucleic acid extraction

Nucleic acid is extracted from a portion, usually 1 ml, of the enriched culture. Any nucleic acid extraction procedure appropriate for Gram-negative bacteria tested suitable for this purpose can be used, for example the CTAB extraction method.[2] Also commercial kits can be used.

NOTE It is known that for certain matrices a nucleic acid extraction method producing high yield and high purity DNA is needed (see Annexes B, C, and D for the tested matrices).

8.5 PCR amplification

8.5.1 General

Different protocols for probe-based real-time PCR amplification can be used.

Examples of real-time PCR assays for detection of pathogenic *Y. enterocolitica* are given in Annex B.

Example of a real-time PCR assay for *Y. pseudotuberculosis* is given in Annex C.

Example of a real-time PCR assay for simultaneous detection of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* is given in Annex D.

8.5.2 PCR controls

PCR controls shall be in accordance with ISO 22174.

8.6 Confirmation of the PCR product

8.6.1 General

According to ISO 20838 a probe-based PCR-positive result does not require additional confirmation of the PCR product.

8.6.2 Interpretation of the PCR result

The result obtained, including the controls specified in ISO 22174, should be unambiguous otherwise the PCR shall be repeated.

The PCR result will be either

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

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

9 Test report

The test report shall conform to the requirements of ISO 22174.

Additionally, it shall specify which method within this Technical Specification has been used, in particular when the simultaneous detection of pathogenic *Y. enterolitica* and *Y. pseudotuberculosis* is performed.

Annex A
(normative)

PCR detection and isolation of pathogenic *Y. enterocolitica* (see Figure A.1)

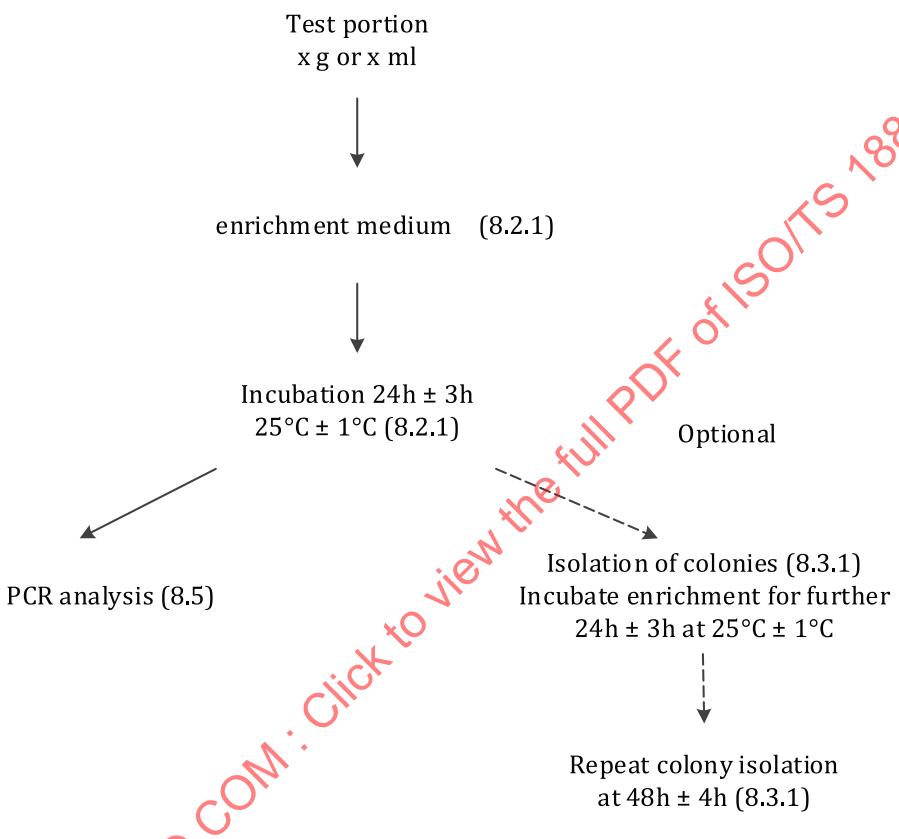

Figure A.1 — Flow diagram for PCR detection and isolation of pathogenic *Y. enterocolitica*

Annex B
(informative)

Real-time PCR for detection of *Y. enterocolitica*

B.1 Real-time PCR for detection of *Y. enterocolitica* — Method 1

B.1.1 General

This is a method for the detection of pathogenic *Yersinia enterocolitica* by amplification of a sequence specific for the *ail* locus of *Y. enterocolitica*. This gene is part of the pathogenicity mechanism of *Y. enterocolitica*.

The detection system was developed as duplex real-time PCR in combination with a heterologous internal amplification control based on the plasmid pUC 19. By real-time PCR, a fragment spanning from the lac operon from M13mp18 to sequences of pBR322 is amplified. This sequence does not occur naturally.

B.1.2 Performance characteristics

B.1.2.1 General

The method is applicable for the detection of *Y. enterocolitica* strains representing the bioserotypes associated with pathogenicity in humans.

The method has been published.[7]

B.1.2.2 Selectivity

Selectivity was performed using the primers ye-ail-F2 and ye-ail-R2, in combination with the hydrolysis probe ye-ail-tmp in combination with the internal amplification control using the primer-probe system pUC 18-F, pUC 18-R, and Tm-pUC 18.

B.1.2.2.1 Inclusivity test

Inclusivity of the PCR assay was tested on 50 strains, 100 % inclusivity was obtained (see Table B.1).

The strains tested were isolated from samples of human, animal, and food origin.

Table B.1 — Inclusivity using 50 target strains

Species and bio/serotype (Number of strains)		Number of strains resulting in amplification of the target fragment
Y. enterocolitica 4/O:3	(33)	33
Y. enterocolitica 2/O:9	(3)	3
Y. enterocolitica 2/O:9	(11)	11
Y. enterocolitica 1B/O:8	(1)	1
Y. enterocolitica 2/O:5,27	(2)	2
Total	(50)	50

B.1.2.2.2 Exclusivity test

Exclusivity of the PCR assay was tested on 51 non-target strains, 100 % exclusivity was obtained (see Table B.2) The strains tested were isolated from samples of human, animal, and food origin.

Table B.2 — Exclusivity using 51 non-target strains

Species and bio/serotype (Number of strains)		Number of strains resulting in amplification of the target fragment
Y. enterocolitica 1A/O:5	(2)	0
Y. enterocolitica 1A/O:6, 30	(1)	0
Y. enterocolitica 1A/O:10	(1)	0
Y. frederiksenii	(2)	0
Y. kristensenii	(2)	0
Y. intermedia	(2)	0
Y. aldovae	(1)	0
Y. mollaretii	(1)	0
Y. pseudotuberculosis	(3)	0
Diverse food-related bacterial species	(34)	0
Saccharomyces cerevisiae	(1)	0
Aspergillus niger	(1)	0
Total	(51)	0

B.1.2.2.3 Molecular selectivity

According to a BLAST search (16.5.2011) the primers and probe have been validated *in silico.*

B.1.2.3 Sensitivity

B.1.2.3.1 Reaction sensitivity

The limit of detection is 5 genome equivalents [25 femtograms (fg)] per single reaction. All analyses were performed in the presence of approximately 25 copies of the internal amplification control (IAC).

B.1.2.3.2 Method sensitivity

The detection level LOD_{95}[17] of the PCR method was 10,7 cfu per 25 g of food. The limit of detection of the method was assessed measuring four different food matrices, i.e. Mortadella sausage, cabbage, pasteurized milk, and uncooked fish, inoculated with 5 cfu and 50 cfu in 25 g with six replicates for each contamination level. After 48 h of enrichment in PSB, the limit of detection in Mortadella sausage, uncooked fish, and pasteurized milk was 5 cfu in 25 g and in cabbage it was 50 cfu in 25 g.

B.1.2.3.3 Performance parameters relative accuracy, relative sensitivity, relative specificity

Relative accuracy	AC	89,5 %
Relative sensitivity	SE	100 %
Relative specificity	SP	76,7 %

The performance parameters were assessed by comparison with the culture method according to ISO 10273:2003, Clause 5.

NOTE These data were obtained by the analysis of artificially contaminated samples.

B.1.2.4 Robustness

The robustness of the PCR was tested by applying the following modifications in the reaction setup.

— Increasing and decreasing the primer and probe concentrations by ±20 % in the presence of 25 copies of the internal amplification control.

— Use of two different formulations of the Taq-polymerase mastermixes from two suppliers.

The modifications did not influence the reaction performance.

B.1.2.5 Instruments

Evaluation was carried out using the Roche LightCycler® 480[1)] and the ABI 7900HT real-time PCR instruments[1)].

Different real-time PCR instruments did not have a significant influence in the method's performance.

B.1.3 Procedure

B.1.3.1 Principle

A specific DNA fragment of the *ail* locus of *Yersinia enterocolitica* is amplified and detected by real-time PCR. The real-time PCR system is based on a specific hydrolysis probe which is labelled with Carboxyfluorescein (FAM) as reporter molecule and Dabcyl as non-fluorescent quencher molecule.

B.1.3.2 Reagents

B.1.3.2.1 General

For quality of reagents used, see ISO 22174.

B.1.3.2.2 Reagents for PCR

See ISO 22119 and ISO 20838.

B.1.3.2.3 Oligonucleotides

B.1.3.2.3.1 Oligonucleotides, pathogenic *Y. enterocolitica*

Table B.3 — Sequences of the oligonucleotides

Primer and probe	DNA sequence of the oligonucleotide (5' – 3')	Size of the PCR product
ye-ail-F2	5'-GGT TAT GCA CAA AGC CAT GTA AA -3'	93 bp
ye-ail-R2	5'-AAA CGA ACC TAT TAC TCC CCA GTT-3'	
[a] FAM: 6 Carboxyfluorescein, DB: Dabcyl.		

1) The Roche LightCycler® 480 and the ABI 7900HT real-time PCR instruments, are examples of suitable products available commercially from Roche Diagnostics and Life Technologies respectively. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of these products. Equivalent products can be used if they can be shown to lead to equivalent results.

Table B.3 *(continued)*

Primer and probe	DNA sequence of the oligonucleotide (5' – 3')	Size of the PCR product
ye-ail-tmp-probe	5'-FAM-AAC CTG AAG TAC CGT TAT GAA CTC GAT GA-DB-3'[a]	

[a] FAM: 6 Carboxyfluorescein, DB: Dabcyl.

B.1.3.2.3.2 Oligonucleotides, internal amplification control, IAC

Table B.4 — Sequences of the oligonucleotides

Name	DNA sequence of the oligonucleotide (5' – 3')	Size of the PCR product
pUC 18-F	5'-TGT CGT GCC AGC TGC ATT A-3'	83 bp
pUC 18-R	5'-GAG CGA GGA AGC GGA AGA G-3'	
Tm-pUC18-probe	5'-TexasRed - AAT CGG CCA ACG CGC GG -BHQ2–3'[a]	

[a] TexasRed:Sulforhodamin-101-Sulfonylchlorid, BHQ2:N-Nitrophenylazo-dimethoxy-phenylazo-phenyl-N-dimethoxytrityloxy-ethyl-2-aminoethyl-1-oxyglycolate (Black-Hole Quencher 2™).

NOTE Equivalent reporter molecule and/or quencher molecule can be used.

B.1.4 Molecular procedure

B.1.4.1 DNA extraction

1 ml of each aliquot of the above listed samples is transferred into a 1,5 ml reaction tube and centrifuged for 10 min at 13 000 × *g*. The bacterial pellet is washed twice in 1 ml 0,9 % NaCl and finally resuspended in 200 µl molecular grade water. After 10 min incubation at 95 °C, 2,5 µl is used as template for the real-time PCR.

B.1.4.2 PCR setup

The total PCR volume is 25 µl per PCR reaction. The reagents are listed in Table B.5.

Table B.5 — Addition of reagents

Reagent (Stock conc.)	Final concentration	Volume per reaction (µl)
LightCycler® 480 Probes Master[a] (containing Fast Start Taq DNA polymerase, reaction buffer, dNTP-mix with dUTP instead of dTTP and 6,4 mmol/l MgCl2)	1 x	12,5
ye-ail-F2 (10 µmol/l)	300 nmol/l	0,75
ye-ail-R2 (10 µmol/l)	300 nmol/l	0,75
ye-ail-tmp (10 µmol/l)	125 nmol/l	0,312
pUC 18-F (10 µmol/l)	250 nmol/l	0,625
pUC 18-R (10 µmol/l)	250 nmol/l	0,625
Tm-pUC18 (10 µmol/l)	100 nmol/l	0,25

[a] Roche LC 480 Probes Master is an example of a suitable product available commercially from Roche Diagnostics. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of this product. Equivalent products can be used if they can be shown to give to the same results.

Table B.5 *(continued)*

Reagent (Stock conc.)	Final concentration	Volume per reaction (µl)
IAC plasmid pUC19	~25 copies (0,1 pg)	1
Test sample		2,5
Sterile distilled water	Adjust the volume to 25 µl	5,687
[a] Roche LC 480 Probes Master is an example of a suitable product available commercially from Roche Diagnostics. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of this product. Equivalent products can be used if they can be shown to give to the same results.		

B.1.4.3 PCR controls

PCR controls shall be in accordance with ISO 22174.

B.1.4.3.1 Negative PCR control

DNA-free water is used as a negative control.

B.1.4.3.2 Positive PCR control

A solution containing a defined amount and/or copy number of target DNA is used as a positive control.

B.1.4.3.3 Amplification control

An example of an IAC is given in Table B.4. This IAC is based on *E. coli* plasmid pUC19 DNA as target molecule. Also commercially available amplification controls can be used.

NOTE The plasmid vectors pUC18 and pUC19 (Accession number L08752; 2686 Bp) are identical except that they contain polycloning sites arranged in opposite directions.

B.1.4.4 Temperature - time programme

The temperature-time programme stated in Table B.6 has proven suitable.

Table B.6 — Temperature/time programme

Activation/initial denaturation	95 °C 10 min
Denaturation	95 °C 10 s
Amplification	60 °C 30 s
Number of cycles	45

B.1.4.5 Limitations of the assay

A high correlation has been found between the presence of the *ail* gene and virulence of *yersiniae*,[8] however, in rare occasions *Yersinia enterocolitica* biotype 1A strains, which are generally considered as non-pathogenic, may carry a variant of the *ail* gene.[6][11] However, there are indications that a small proportion of the 1A strains may be pathogenic to humans.[12]

B.2 Real-time PCR for detection of pathogenic *Y. enterocolitica* — Method 2

B.2.1 General

This is a method for the detection of pathogenic *Yersinia enterocolitica* by amplification of a sequence specific for the ail locus of *Yersinia enterocolitica*. This gene is part of the pathogenicity mechanism of *Yersinia enterocolitica*.

The detection system was developed as duplex real-time PCR in combination with a heterologous internal amplification control based on the plasmid pUC 19.

B.2.2 Performance characteristics

B.2.2.1 General

The method is applicable for the detection of *Y. enterocolitica* strains representing the bioserotypes associated with patogenicity in humans.

The method has been published.[13][14]

B.2.2.2 Selectivity

Selectivity was performed using the primers R-real 9A and F-real 10A, in combination with a MGB™-probe[2)] or a TAMRA-quenched probe (*ail* probe) in combination with the internal amplification control using the primer-probe system IAC-fw, IAC-re, and probe pUC 19.

B.2.2.2.1 Inclusivity test

Inclusivity of the PCR assay was tested on 102 strains, 100 % inclusivity was obtained, see Table B.7.

The strains tested were isolated from samples of human (n = 70), animal (n = 15), food (n = 8), and unknown (n = 9) origin.

Table B.7 — Inclusivity using 102 target strains

Species and bio/serotype (Number of strains)		Number of strains resulting in amplification of the target fragment
Y. enterocolitica 4/O:3	(81)	81
Y. enterocolitica NT/O:9	(3)	3
Y. enterocolitica NT/O:8	(8)	8
Y. enterocolitica NT/O:5,27	(4)	4
Y. enterocolitica 1B/O:18	(1)	1
Y. enterocolitica NT/O:20	(2)	2
Y. enterocolitica 1B/O:21	(1)	1
Y. enterocolitica 3/O:1,2,3	(1)	1
Y. enterocolitica 5/O:2,3	(1)	1
Total	102	102
NT = not typed.		

B.2.2.2.2 Exclusivity test

Exclusivity of the PCR assay was tested on 71 non-target strains, 100 % exclusivity was obtained (see Table B.8) The strains tested were isolated from samples of human (n = 20), animal (n = 1), food (n = 21), and unknown (n = 29) origin.

2) Minor groove binder (MGB™) is example of a suitable product available commercially from Applied Biosystems. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of this product. Equivalent products can be used if they can be shown to give to the same results.

Table B.8 — Exclusivity using 71 non-target strains

Species and bio/serotype (Number of strains)		Number of strains resulting in amplification of the target fragment
Y. enterocolitica 1A	(5)	0
Y. enterocolitica NT	(9)	0
Y. frederiksenii	(7)	0
Y. kristensenii	(2)	0
Y. intermedia	(2)	0
Y. pseudotuberculosis	(25)	0
Diverse food-related species	(21)	0
Total	(71)	0
NT = not typed.		

B.2.2.3 Reaction Sensitivity

The limit of detection is 15-20 genome equivalents (85 fg) per single reaction. All analyses were performed in the presence of 100 copies of the internal amplification control (IAC).

B.2.2.4 Method sensitivity

The detection level LOD_{95}[17] of the PCR method was 23 cfu/10 g of food. The limit of detection of the method was assessed measuring five different food matrices, i.e. milk, cold-smoked sausage, fish, carrots, and minced meat inoculated with 1 cfu to 55 cfu in 10 g with six replicates for each contamination level. After 18 h to 20 h of enrichment the limit of detection in milk was 1 cfu in 10 g, in cold-smoked sausage it was 5,5 cfu and in carrots, fish, and minced meat it was 55 cfu in 10 g.

B.2.2.5 Performance parameters relative accuracy, relative sensitivity, relative specificity

The relative accuracy of the real-time PCR method (expected/obtained results) was 99 %, relative sensitivity was 98 % and relative specificity was 100 % in a study of 90 samples of ham, carrot, and minced meat when levels of 10 cfu to 300 cfu *Y. enterocolitica* in 25 g of food sample were used. In this study setting, dilution of the extracted DNA 1/10 before run was always used. The same study revealed relative accuracy of 60 % for the culture method EN/ISO 10273:2003 and a relative sensitivity of 40 % and relative specificity of 100 %.

NOTE These data were obtained by the analysis of artificially contaminated samples. A comparison between expected and obtained results was calculated using NMKL procedure n° 20, 2007.[1]

B.2.2.6 Robustness

Robustness of PCR was tested by varying the annealing-extension temperatures ±2 °C and the PCR-reagent concentrations ±20 % by using 50 copies of genomic *Y. enterocolitica* DNA (SLV-408) in the presence of 100 copies of the internal amplification control (IAC). The *Ct* deviations were ±1 *Ct* value from the given method conditions.

B.2.2.7 Instruments

Evaluation was carried out using the ABI 7300, 7500[3)] and the BioRad CFX96™ real-time PCR instruments[3)]. Different real-time PCR instruments did not have a significant influence in the method's performance.

3) ABI 7300, 7500 and the BioRad CFX96TM real-time PCR instruments, are examples of suitable products available commercially from Applied Biosystems and BioRad, respectively. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of this product. Eqvivalent products can be used if they can be shown to give to equivalent results.

B.2.3 Procedure

B.2.3.1 Principle

A specific DNA fragment of the chromosomally located virulence-associated gene attachment invasion locus (*ail*) is amplified and detected using a probe-based real-time PCR.[13] The real-time PCR system is based on a hydrolysis probe which is labelled with 6-carboxyfluorescein (FAM) as reporter molecule and at the 3′end either a special chemical compound called a minor groove binder, MGB (*ail* probe), or alternatively tetramethyl-6-carboxyrhodamine, TAMRA (*Ye* probe).

B.2.3.2 Reagents

B.2.3.2.1 General

For quality of reagents used, see ISO 22174.

B.2.3.2.2 Reagents for PCR

See ISO 22119 and ISO 20838.

B.2.3.2.2.1 Oligonucleotides, pathogenic *Y. enterocolitica*

Table B.9 — Sequences of oligonucleotides

Primer and probe	DNA sequence of the oligonucleotide (5' – 3')	Size of the PCR product
R-real 9A[a]	CCCAGTAATCCATAAAGGCTAACATAT	163 bp
F- real 10A[a]	ATGATAACTGGGGAGTAATAGGTTCG	
Ye probe[b]	FAM-TCTATGGCAGTAATAAGTTTGGTCACGGTGATCT-TAMRA	
ail probe[b]	FAM-TGACCAAACTTATTACTGCCATA-MGB™[c]	

[a] Reverse; Forward

[b] Ye probe and ail probe are used alternatively.

[c] Minor groove binder (MGB™) is an example of a suitable product available commercially from Applied Biosystems, Forster City, CA, USA. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of this product. Eqvivalent products can be used if they can be shown to give to equivalent results.

B.2.3.2.2.2 Oligonucleotides, internal amplification control (IAC)

Table B.10 — Sequences of oligonucleotides

Name	DNA sequence of the oligonucleotide	Size of the PCR product
IAC-fw	TGTGAAATACCGCACAGATG	119
IAC-re	AGCTGGCGTAATAGCGAAG	
Probe-pUC19	Z_1-TAAGGAGAAAATACCGCATCAGGCGCC-Z_2 .	

Z_1 = a reporter dye different from the dye used for the target detection probe; Z_2 = any suitable quencher. For example, the Cyanine colour dye CY5 as reporter molecule and at the 3′end the Blackberry quencher, BBQ.

NOTE Equivalent reporter molecule dyes and/or quencher molecule dyes can be used.

B.2.3.3 DNA extraction

DNA was extracted from enriched homogenates, broth cultures, or cell suspensions using the DNeasy blood and tissue kit or MasterPure complete DNA purification kit.[4)] The procedure was conducted according to the manufacturer's instruction. Also, other commercial ready-to-use purification kits can be used.

B.2.3.4 PCR setup

The total PCR volume is 25 µl per reaction. The reagents are listed in Table B.11. The final concentrations of reagents as outlined in the table have proven to be suitable.

Table B.11 — Addition of reagents

Reagent (Stock conc.)	Final concentration	Volume per reaction (µl)
10xTaqMan® buffer A[a,b]	1x	2,5
$MgCl_2$ (25 mmol/l)[a]	3,5 mmol/l	3,5
dNTPs (50 mmol/l)[a]	200 µmol/l	0,1
AmpliTaqGold® (5 U/µl)[a]	0,02 U/µl	0,25
R-real 9A (10 µmol/l)	300 nmol/l	0,75
F-real 10A (10 µmol/l)	300 nmol/l	0,75
Ye probe (20 µmol/l)	200 nmol/l	0,25
IAC pUC19 DNA	~100 copies (0,3 pg)	1,0
IAC-fw (20 µmol/l)	500 nmol/l	0,625
IAC-re (20 µmol/l)	500 nmol/l	0,625
Probe-pUC19 (20 µmol/l)	200 nmol/l	0,25
Test sample		5,0
Adjust the volume to 25 µl using sterile distilled water		

[a] These components can be replaced by similar buffers or ready-made mastermixes from other suppliers. These buffers include all reagents in the same solution. The commercially available TaqMan® 2x Universal PCR MasterMix was used in performance testing.

[b] 10xTaqMan buffer A, AmpliTaqGold® is an example of a suitable product available commercially from Applied Biosystems. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of this product. Equivalent products can be used if they can be shown to give to the same results.

B.2.3.5 PCR controls

PCR controls shall be in accordance with ISO 22174.

B.2.3.6 Amplification control

An example of an IAC is given in Table B.10. This IAC is based on *E. coli* plasmid pUC19 DNA as target molecule. Also commercially available amplification controls can be used.

4) DNeasy blood and tissue kit and MasterPure complete DNA purification kit are examples of suitable products available commercially from Qiagen Gmbh, (Hilden, Germany) and Epicentre Biotechnology (Madison, WI, USA), respectively. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of this product. Equivalent products can be used if they can be shown to give to equivalent results.

B.2.3.7 Temperature-time programme

The temperature-time programme as outlined in Table B.12 has been used in the evaluation study.

NOTE Use of other real-time PCR thermal cycler than those mentioned above (B.2.2.7) may require further adjustment. The time for activation/initial denaturation depends on the polymerase used.

Table B.12 — Temperature-time programme

Activation/initial denaturation	95 °C 10 min
Denaturation	95 °C 15 s
Amplification	60 °C 1 min
Number of cycles	45

B.2.3.8 Limitations of the real-time PCR assay

A high correlation has been found between the presence of the *ail* gene and virulence of *Yersinia* spp,[8] however, in rare occasions *Yersinia enterocolitica* biotype 1A strains, which are generally considered as non-pathogenic, may carry a variant of the *ail* gene.[6][11] However, there are indications that a small proportion of the 1A strains may be pathogenic to humans.[12]

Annex C
(informative)

Detection and isolation of *Y. pseudotuberculosis*

C.1 Real-time PCR for detection of *Y. pseudotuberculosis*

C.1.1 General

This Annex describes a probe-based real-time PCR method for the detection of *Y. pseudotuberculosis.*

C.1.2 Performance characteristics

C.1.2.1 General

The method is applicable for detection of *Y. pseudotuberculosis* strains considered potentially pathogenic to humans.

The method has been published.[14]

C.1.2.2 Selectivity

Selectivity was performed using the primers F-Yps 1 and R-Yps 2, in combination with a MGB™-probe or a TAMRA-quenched probe in combination with the internal amplification control using the primer-probe system IAC-fw, IAC-re and probe pUC 19.

C.1.2.2.1 Inclusivity test

Inclusivity of the PCR assay was tested on 44 target strains, 95 % inclusivity was obtained. The primer/probe sets did not detect the serotypes O:11 and O:12.

Table C.1 — Inclusivity using 44 target strains

Species and bio/serotype (Number of strains)		Number of strains resulting in amplification of the target fragment
Y. pseudotuberculosis O:1-O:15	(33)	31
Y. pseudotuberculosis NT	(11)	11
Total	(44)	42
NT = not typed.		

C.1.2.2.2 Exclusivity test

Exclusivity of the assay was tested on 23 non-target strains, 100 % exclusivity was obtained. The strains tested were isolated from samples of human (n = 24), animal (n = 2), food (n = 10), environment (n = 4), and unknown (n = 27) origin.

Table C.2 — Exclusivity using 23 non-target strains

Species and bio/serotype (Number of strains)		Number of strains resulting in amplification of the target fragment
Y. enterocolitica, pathogenic	(9)	0
Y. enterocolitica, nonpathogenic	(2)	0
Diverse food-related species	(12)	0
Total	(23)	0
NT = not typed.		

C.1.2.3 Sensitivity

C.1.2.3.1 Reaction sensitivity

The limit of detection LOD_{95}[17] is 15 genome equivalents (75 fg) per single reaction.

C.1.2.3.2 Method sensitivity

The level of detection of the method was assessed by measuring food samples inoculated at two levels (13 and 200 cfu) in 25 g of three different food matrices, i.e. non-pasteurized milk, minced beef meat, and a mixed vegetable salad (grated carrots and iceberg lettuce). Ten samples per food type were used. After 24 h of enrichment, the combined level of detection, LOD_{95},[17] for all three food matrices was 13 cfu in 25 g. For limit of detection data on more matrices, see B.1.2.

C.1.2.4 Performance parameters relative accuracy, relative sensitivity, relative specificity

The relative accuracy, sensitivity, and specificity of the real-time PCR method (expected/obtained results) were 100 % in a study of 90 food samples (non-pasteurized milk, minced beef meat, and a mixed vegetable salad) when low levels of *Y. pseudotuberculosis*, i.e. 10 cfu to 200 cfu per 25 g of food sample were used. In this study setting, dilution of the extracted DNA 1/10 before run was always used.

NOTE These data were obtained by the analysis of artificially contaminated samples. A comparison between expected and obtained results was calculated using NMKL procedure n° 20, 2007.[1]

C.1.2.5 Instrument

Evaluation was carried out using the ABI 7300, 7500[5] and the BioRad CFX96™ real-time PCR instruments[5].

C.1.3 Procedure

C.1.3.1 Principle

A specific DNA fragment of the chromosomally located virulence-associated gene attachment invasion locus (*ail*) is amplified using a probe-based real-time PCR to detect *Y. pseudotuberculosis*.[14]

C.1.3.2 Reagents

C.1.3.2.1 General

For quality of reagents used, see ISO 22174.

5) ABI 7300, 7500 and the BioRad CFX96™ real-time PCR instruments, are examples of suitable products available commercially from Life Technologies and Bio-Rad respectively. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of these products. Equivalent products can be used if they can be shown to lead to equivalent results.

C.1.3.2.2 Reagents for PCR

C.1.3.2.2.1 General

See ISO 22119 and ISO 20838.

C.1.3.2.2.2 Oligonucleotides, *Y. pseudotuberculosis*

Table C.3 — Sequences of oligonucleotides

Primer and probe	DNA sequence of the oligonucleotide (5' – 3')	Size of the PCR product
F-Yps 1[a]	CGTCTGTTAATGTGTATGCCGAAG	157 bp
R-Yps 2[a]	GAACCTATCACTCCCCAGTCATTATT	
Probe Yps[b, c]	VIC-CGTGTCAAGGACGATGGGTACAAGTTGG-TAMRA	
Probe Yps_2[b, c]	NED-ATGCTCAAAGTCGTGTCAA-MGB™[d]	

[a] Forward; Reverse.

[b] TAMRA™; VIC® MGB™, minor groove binder; NED™.

[c] Probe Yps and Probe Yps_2 are used alternatively.

[d] MGB™, minor groove binder; NED™are examples of suitable products available commercially from Applied Biosystems. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of these products. Equivalent products can be used if they can be shown to lead to equivalent results.

C.1.3.3 DNA extration

See B.2.3.3.

C.1.3.4 PCR setup

The total PCR volume is 25 µl per reaction. The reagents are listed in Table C.4. The final concentrations of reagents as outlined in the table have proven to be suitable.

Table C.4 — PCR reaction reagents

Reagent (Stock conc.)	Final concentration	Volume per sample (µl)
TaqMan®2x Universal PCR[a]	1x	12,5
F-Yps 1 (10 µmol/l)	300 nmol/l	0,75
R-Yps 2 (10 µmol/l)	300 nmol/l	0,75
Probe Yps (20 µmol/l)	200 nmol/l	0,25
IAC pUC19 DNA	approximately 100 copies (0,3 pg)	1,0
IAC-fw (20 µmol/l)	500 nmol/l	0,625
IAC-re (20 µmol/l)	500 nmol/l	0,625
Probe-pUC19 (20 µmol/l)	200 nmol/l	0,25
Test sample		5,0
Adjust the volume to 25 µl using sterile distilled water		

[a] TaqMan®2x Universal PCR is an example of a suitable product available commercially from Applied Biosystems. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement of these products. Equivalent products can be used if they can be shown to lead to equivalent results.

C.1.3.5 PCR controls

PCR controls shall be in accordance with ISO 22174.

C.1.3.6 Amplification control

An example of an IAC is given in Table B.10. This IAC is based on *E. coli* plasmid pUC19 DNA as target molecule. Also commercially available amplification controls can be used.

C.1.3.7 Temperature-time programme

See B.2.3.7.

C.1.4 Limitations of the real-time PCR assay

All *Y. pseudotuberculosis* strains are considered potentially pathogenic to humans.[9] The real-time PCR assay presented in this Technical Specification does not amplify the target genes of the serotypes O:11 and O:12, the pathogenicity of which is unknown. This primer/probe set will detect also *Y. pestis*, which, however is normally not associated with food.

C.2 Isolation of *Y. pseudotuberculosis*

C.2.1 Flow diagram for isolation (and PCR detection) of *Y. pseudotuberculosis*

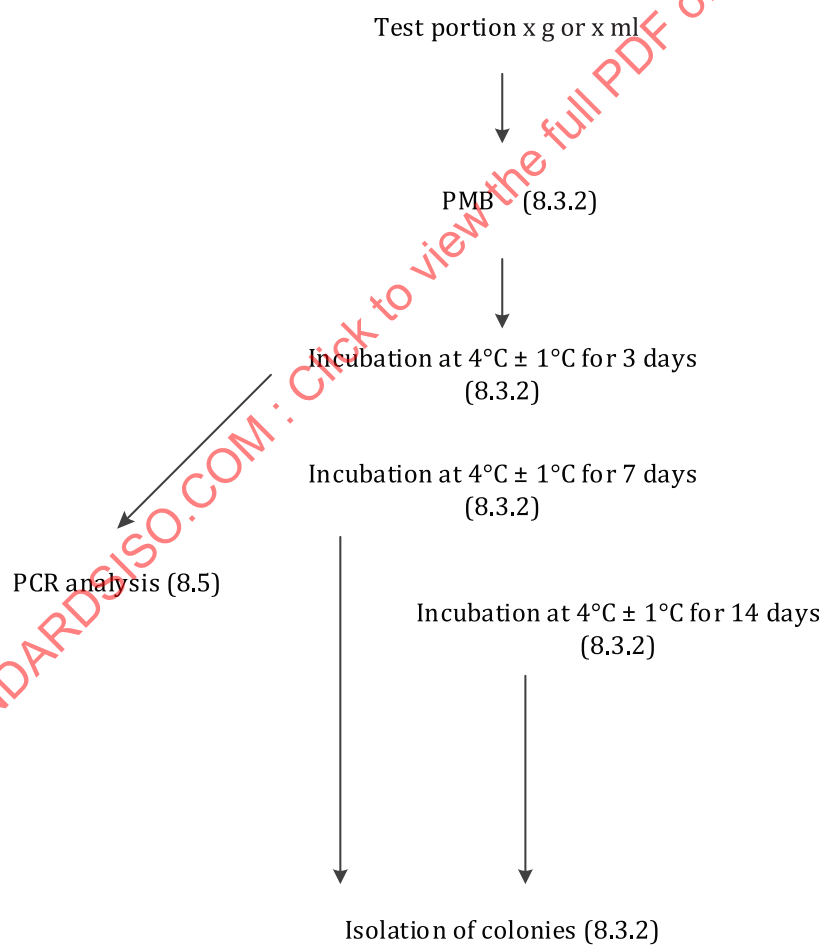

Figure C.1 — Flow diagram for isolation (and PCR detection) of *Y. pseudotuberculosis*