Real-time PCR Detection of *E. coli* O157:H7 in Food Samples

Using spin-column-based DNA isolation methods

for use with:
PrepSEQ™ Rapid Spin Sample Preparation Kits
MicroSEQ™ E. coli O157:H7 Detection Kit
Applied Biosystems™ 7500 Fast Real-Time PCR Instrument
RapidFinder™ Express Software

Catalog Numbers 4407760, 4413269, 4426714, 4426715, 4427409, 4445657
Publication Number MAN0013440
Revision B

ABI 29/03 – 03/11
ALTERNATIVE ANALYTICAL METHODS
FOR AGRIBUSINESS
http://nf-validation.afnor.org/en/

For testing of Food and Environmental samples only.

ThermoFisher SCIENTIFIC

Revision history: MAN0013440 B (English)

Revision	Date	Description
B	9 August 2024	• Troubleshooting was added for possible instance of varying morphology of PCR pellets. • The software version was updated for RapidFinder™ Express Software. • Characteristics were added for the 7500 Fast Real-Time PCR Instrument.
A.0	7 November 2018	New document for the *Real-time PCR Detection of E. coli O157:H7 in Food Samples User Guide (Spin-Column DNA Isolation, AFNOR)*. • Includes the complete workflow that covers enrichment, spin-column DNA isolation, and real-time PCR detection methods certified by NF VALIDATION™ by AFNOR™ Certification. • Supersedes: – *PrepSEQ™ Rapid Spin Sample Preparation Kit User Guide* (Pub. No. 4426519) – *MicroSEQ™ E. coli O157:H7 Detection Kit User Guide* (Pub. No. 4426511)

The information in this guide is subject to change without notice.

Contents

1 Overview

IMPORTANT! Before using these products, read and understand the information in the "Safety" appendix in this document.

General overview

In the context of NF VALIDATION™ by AFNOR™ Certification, this guide describes the following workflows for detection of *E. coli* O157:H7 in raw beef and vegetable samples:

1. Enrichment of food samples by one of the following methods (see “Overview of the enrichment and DNA isolation options” on page 6):
   - 25 g samples in prewarmed Brain Heart Infusion (BHI) Broth (workflow A)
   - 25 g samples in Buffered Peptone Water (BPW) (workflow B)
2. Spin-column-based preparation of PCR-ready DNA using a PrepSEQ™ Rapid Spin Sample Preparation Kit.
3. Real-time PCR detection of *E. coli* O157:H7 DNA using the MicroSEQ™ E. coli O157:H7 Detection Kit and RapidFinder™ Express Software on the Applied Biosystems™ 7500 Fast Real-Time PCR Instrument.
4. Confirmation testing of positive samples.

   See Chapter 5, “Recommended confirmation methods” for detailed information.

Go to **thermofisher.com/foodsafety** for a list of workflows for detection of *E. coli* (Pub. No. MAN0009419).

Overview of the enrichment and DNA isolation options

Select an enrichment and DNA isolation workflow based on the sample amount and your preferred media and enrichment time. See “NF VALIDATION™ by AFNOR™ Certification” on page 30 for validated sample matrices.

Table 1 Enrichment and DNA isolation options

Workflow	Sample amount (food)	Enrichment media	Enrichment time[1]	Volume of enriched sample for DNA isolation	Food type/PK[2] requirement
A	25 g	Prewarmed (42±1°C) Brain Heart Infusion (BHI) Broth	6–8 hr	750 µL	Animal products[3]: with PK
					Non-animal products: without PK
B	25 g	Buffered Peptone Water (BPW)	16–20 hr	750 µL	Animal products: with PK [3]
					Non-animal products: without PK

[1] All enrichments are incubated at 42±1°C.
[2] Proteinase K
[3] Animal products include ground beef and beef trim.

Required materials

Unless otherwise indicated, all materials are available through the Thermo Fisher Microbiology ordering process or **thermofisher.com**. They may also be available through Fisher Scientific (**fisherscientific.com**), MLS, or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Note: Parts may ship separately depending on configuration and storage conditions.

Materials for enrichment of food samples

Table 2 Equipment, accessories, and consumables

Item	Source
Incubator, 42±1°C	**thermofisher.com**

Table 2 Equipment, accessories, and consumables *(continued)*

Item	Source
Homogenizer laboratory blender or diluter, one of the following or equivalent:	
• Homogenizer Laboratory Blender • Diluflux™ Pro Automated Gravimetric Dilutor with simple (non-robotic) dispensing arm • Diluflux™ Pro Automated Gravimetric Dilutor with robotic dispensing arm	DB5000A DB4100A DB4150A
Homogenizer bag appropriate for the sample type	
Homogenizer bag, with mesh, 6” × 9”, 24 oz (Whirl-Pak™ Filter Bag for Homogenizer Blenders, or equivalent)	Nasco #B01348WA or equivalent
Homogenizer bag, 6” × 9”, 24 oz (Whirl-Pak™ Sample Bag, or equivalent)	Nasco #B01297WA or equivalent
Enrichment media appropriate for the sample and workflow chosen	
Brain Heart Infusion (BHI) Broth (workflow A)	One of the following or equivalent: • Oxoid™ CM1135B • Remel™ R060270
Buffered Peptone Water (BPW) (workflow B)	Oxoid™ CM1049B or equivalent

Materials for DNA isolation

Choose the PrepSEQ™ Rapid Spin Sample Preparation Kit appropriate for your sample type and enrichment method.

Table 3 PrepSEQ™ Rapid Spin Sample Preparation Kit (100 reactions, Cat. No. 4407760)

Contents	Amount	Storage[1]
Spin columns	100	Room temperature (23±5°C)
Microcentrifuge tubes, 1.5 mL	100	
Lysis Buffer, 1 bottle	5 mL	5±3°C

[1] See the expiration date on the box.

Table 4 PrepSEQ™ Rapid Spin Sample Preparation Kit – Extra Clean (100 reactions, Cat. No. 4413269)

Contents	Amount	Storage[1]
Spin columns	100	Room temperature (23±5°C)
Microcentrifuge tubes, 1.5 mL	2 × 100	
Lysis Buffer, 1 bottle	5 mL	5±3°C

[1] See the expiration date on the box.

Table 5 PrepSEQ™ Rapid Spin Sample Preparation Kit with Proteinase K (100 reactions, Cat. No. 4426714)

Contents	Amount	Storage[1]
Spin columns	100	Room temperature (23±5°C)
Microcentrifuge tubes, 1.5 mL	100	
Lysis Buffer, 1 bottle	5 mL	5±3°C
Proteinase K (20 mg/mL), 1 tube	1.25 mL	Below –18°C

[1] See the expiration date on the box.

Table 6 PrepSEQ™ Rapid Spin Sample Preparation Kit – Extra Clean with Proteinase K (100 reactions, Cat. No. 4426715)

Contents	Amount	Storage[1]
Spin columns	100	Room temperature (23±5°C)
Microcentrifuge tubes, 1.5 mL	2 × 100	
Lysis Buffer, 1 bottle	5 mL	5±3°C
Proteinase K (20 mg/mL), 1 tube	1.25 mL	Below –18°C

[1] See the expiration date on the box.

Table 7 Required materials not included in the PrepSEQ™ Rapid Spin Sample Preparation Kit

Item	Source
Equipment	
Block heater, 95°C	MLS
Block heater, 56°C; for workflows that use Proteinase K	MLS
Rack for 1.5-mL tubes	MLS
Benchtop microcentrifuge	Eppendorf 5415 D or equivalent
Laboratory mixer, Vortex or equivalent	MLS

Table 7 Required materials not included in the PrepSEQ Rapid Spin Sample Preparation Kit *(continued)*

Item	Source
Pipettors: • Positive-displacement • Air-displacement	MLS
Additional consumables	
Disposable gloves	MLS
Micropipette tips, aerosol-resistant	MLS
Reagents	
Nuclease-Free Water (not DEPC-Treated)	AM9938

Materials for PCR detection

Table 8 MicroSEQ™ E. coli O157:H7 Detection Kit (96 reactions; Cat. No. 4427409, 4445657)

Contents [1]	Amount	Cap color	Storage
E. coli O157:H7 Assay Beads, 8-tube strips	12 strips (96 tubes) 1 rack	Orange (rack)	5±3°C Protect from light and moisture.[2]
MicroAmp™ Optical 8-Cap Strips	12 strips (96 caps)	N/A	
Pathogen Detection Negative Control[3]	1.5 mL	Red	5±3°C

[1] Cat. No. 4445657 includes the PrepSEQ™ Rapid Spin Sample Preparation Kit – Extra Clean with Proteinase K.

[2] Excessive exposure to light may affect the fluorescent probes. To protect the beads from moisture, do not remove the desiccant from the pouch, and seal the pouch tightly each time you remove assay bead strips.

[3] The Pathogen Detection Negative Control is included in a separate box and may be shipped separately.

Item	Source
Instruments and equipment	
Applied Biosystems™ 7500 Fast Real-Time PCR Instrument	A30304 (desktop) A30299 (laptop) Contact your local microbiology sales representative.
RapidFinder™ Express Software Version 2.0 or higher	Download the latest version at **thermofisher.com/rapidfinder-express-software**
7500 Fast Precision Plate Holder for MicroAmp™ Tube Strips	A29252

Item	Source
MicroAmp™ 96-Well Base	N8010531
MicroAmp™ Cap Installing Tool	4330015
MicroAmp™ Multi-removal Tool	4313950
Benchtop microcentrifuge with 8-tube strip adapter *or* Plate centrifuge	MLS
Laboratory mixer (vortex mixer or equivalent)	MLS
Pipettors: • Positive-displacement • Air-displacement • Multichannel	MLS
Consumables	
Aerosol-resistant pipette tips	MLS
Disposable gloves	MLS
MicroAmp™ Fast 8-Tube Strip, 0.1-mL[1]	4358293
MicroAmp™ Optical 8-Cap Strip, 300 strips[1]	4323032
Reagents	
Nuclease-Free Water (not DEPC-Treated)	AM9938

[1] Required to evenly distribute the clamping load applied to the tube strips during PCR processing. Do not use other tube strips, which could result in crushed tubes.

2 Enrich 25 g of food sample in BHI and isolate DNA (workflow A)

Workflow

Enrich food samples in BHI Broth

(25 g or 25 mL sample, 225 mL prewarmed BHI, 6–10 hours, 42±1°C)

▼

Isolate DNA using Proteinase K (raw beef samples)

▼

Prepare Proteinase K-Lysis Buffer and preheat block heaters

▼

Filter 750 µL of enriched culture through the spin column

▼

Lyse the sample

(55 µL Proteinase K-Lysis Buffer)

(Optional transfer to clean tube, for high-fat samples)

Isolate DNA without Proteinase K (raw vegetable samples)

▼

Preheat the block heater to 97°C

▼

Filter 750 µL of enriched culture through the spin column

▼

Lyse the sample

(50 µL Lysis Buffer)

(Optional transfer to clean tube, for high-fat samples)

Important procedural guidelines for enrichment and DNA isolation

Guidelines for sample enrichment

- Use proper aseptic technique while handling samples to avoid cross-contamination.
- Use a forced air incubator and ensure sufficient space between enrichment bags to allow for air flow.

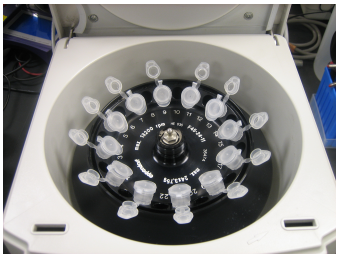
Guidelines for DNA isolation

PCR-clean water

Use nuclease-free water for all procedures described in this protocol that require water. Nuclease-free water is considered “PCR-clean” water. In contrast, autoclaved water should not be considered "PCR-clean" water.

Position of the spin column/tube assembly in the microcentrifuge

Place the tube cap hinge toward the inside of the rotor, and position the cap in the opposite direction of rotation.



Incorrect position of tube caps

Correct position of tube caps

For high-fat samples: remove fat layer before lysis

For samples that contain a distinct, top, fat layer following centrifugation, remove the fat layer and supernatant as follows:

Type of fat layer	Fat layer and supernatant removal
Liquid	1. Use a P1000 pipettor to remove fat from the top surface by aspirating in a circular motion without disturbing the pellet. 2. Continue to collect supernatant from the top surface until all the supernatant is removed. 3. Discard the supernatant into a waste container.
Solid	1. Use a pipette tip to gently dislodge the fat layer without disturbing the pellet. 2. Aspirate the supernatant from the top surface using a pipettor until all the supernatant is removed. 3. Discard the supernatant into a waste container.

Enrich food samples in BHI Broth

1. Prepare 225 mL of Brain Heart Infusion (BHI) Broth for each 25 g food of sample, according to the instructions of the manufacturer, and prewarm to 42±1°C.

2. Combine the food sample with 225 mL of prewarmed BHI Broth in a homogenizer bag, and homogenize.

   A filtered bag may be used for enrichment of samples with particulates.

Sample type	Method
Coarse food types	Homogenize the sample thoroughly using a laboratory blender. Hand massage foods that cannot be processed in a homogenizer: squeeze the bag 5–10 times.

3. Incubate the sample for 6–8 hours at 42±1°C under static conditions.

Isolate DNA using Proteinase K (raw beef samples)

Before each use of the kit

- Prepare Proteinase K-Lysis Buffer: combine the following components for the number of samples required; store on ice until use.

Component	Volume per sample	Volume for *n* samples[1]
Proteinase K, 20 mg/mL	5 µL	5.5 µL × *n*
Lysis Buffer	50 µL	55 µL × *n*

[1] Includes 10% overage.

- Preheat block heaters to 97±2°C and 56±1°C.

Filter 750 µL of enriched culture through the spin column

Gently mix the enriched culture before transferring the sample to the spin column.

1. Insert a spin column into a labeled tube, transfer 750 µL of the enriched sample from the filtered side of the enrichment bag to the spin column, and cap the column.
2. Microcentrifuge the spin column assembly at 12,000–16,000 × *g* for about 3 minutes.
   Follow "Position of the spin column/tube assembly in the microcentrifuge" on page 12.
3. Remove the assembly from the microcentrifuge and discard the used spin column.
4. Gently aspirate the supernatant without disturbing the pellet, then discard the supernatant.
   To remove liquid on the sides of the tube, push droplets into the supernatant by circling the inside of the tube with the pipettor before aspiration.
   (Optional) If necessary, follow "For high-fat samples: remove fat layer before lysis" on page 12.

Lyse the sample

1. Add 55 µL of Proteinase K-Lysis Buffer to the pellet, and pipet up and down or vortex until the pellet is well dispersed.
2. (*Optional*) **Rapid Spin Extra Clean protocol (for samples with high lipid content)**: transfer the mixture to a clean 1.5-mL tube, avoiding residual fat.
   The pellet must be well dispersed in the Lysis Buffer prior to transfer.

Avoid contact with residual fat on the sides of the original tube, and transfer only the Lysis Buffer containing the resuspended pellet.

3. Cap the tube, then incubate at 56±1°C for at least 30 minutes.
4. Incubate at 97±2°C for 12±2 minutes, then allow the sample to cool for about 2 minutes at room temperature (23±5°C).
5. Microcentrifuge the tube at 12,000–16,000 × *g* for about 1 minute to collect the contents at the bottom of the tube.
6. Add 250 µL of nuclease-free water, and mix thoroughly.
7. Microcentrifuge the tube at 12,000–16,000 × *g* for 1–2 minutes to pellet any remaining particulate material.

   The microbial DNA is in the supernatant.

Proceed directly to real-time PCR. Alternatively, store the DNA in one of the following ways:

- At 5±3°C for up to 24 hours.
- Below –18°C for up to 1 year.

If required, validate storage of the DNA according to EN ISO 20837:2006.

Isolate DNA without Proteinase K (raw vegetable samples)

Preheat the block heater to 97°C

Before each use of the kit, preheat the block heater to 97°C.

Filter 750 µL of enriched culture through the spin column

Gently mix the enriched culture before transferring the sample to the spin column.

1. Insert a spin column into a labeled tube, transfer 750 µL of the enriched sample from the filtered side of the enrichment bag to the spin column, and cap the column.
2. Microcentrifuge the spin column assembly at 12,000–16,000 × *g* for about 3 minutes.

   Follow “Position of the spin column/tube assembly in the microcentrifuge” on page 12.
3. Remove the assembly from the microcentrifuge and discard the used spin column.
4. Gently aspirate the supernatant without disturbing the pellet, then discard the supernatant.

   To remove liquid on the sides of the tube, push droplets into the supernatant by circling the inside of the tube with the pipettor before aspiration.

   (Optional) If necessary, follow “For high-fat samples: remove fat layer before lysis” on page 12.

Lyse the sample

1. Add 50 µL of Lysis Buffer to the pellet, and pipet up and down or vortex until the pellet is well dispersed in the Lysis Buffer mix.
2. (*Optional*) **Rapid Spin Extra Clean protocol (for samples with high lipid content)**: transfer the mixture to a clean 1.5-mL tube, avoiding residual fat.

   The pellet must be well dispersed in the Lysis Buffer prior to transfer.

   Avoid contact with residual fat on the sides of the original tube, and transfer only the Lysis Buffer containing the resuspended pellet.
3. Cap the tube, then incubate at 97±2°C for 12±2 minutes.
4. Allow the sample to cool for about 2 minutes at room temperature (23±5°C).
5. Microcentrifuge the tube at 12,000–16,000 × *g* for about 1 minute to collect the contents at the bottom of the tube.
6. Add 250 µL of nuclease-free water, and mix thoroughly.
7. Microcentrifuge the tube at 12,000–16,000 × *g* for 1–2 minutes to pellet any remaining particulate material.

   The microbial DNA is in the supernatant.

Proceed directly to real-time PCR. Alternatively, store the DNA in one of the following ways:

- At 5±3°C for up to 24 hours.
- Below –18°C for up to 1 year.

3 Enrich 25 g of food sample in BPW and isolate DNA (workflow B)

Workflow

Enrich food samples in BPW

(25 g or 25 mL sample, 225 mL BPW, 42±1°C, 16–20 hours)

▼

Isolate DNA using Proteinase K (raw beef samples)

▼

Before each use of the kit

▼

Filter 750 µL of enriched culture through the spin column

(750 µL enriched culture)

▼

Lyse the sample

(55 µL Proteinase K-Lysis Buffer)

(Optional transfer to clean tube, for high-fat samples)

Isolate DNA without Proteinase K (raw vegetable samples)

▼

"Preheat the block heater to 97°C" on page 14

▼

Filter 750 µL of enriched culture through the spin column

(750 µL enriched culture)

▼

Lyse the sample

(50 µL Lysis Buffer)

(Optional transfer to clean tube, for high-fat samples)

Important procedural guidelines for enrichment and DNA isolation

Guidelines for sample enrichment

- Use proper aseptic technique while handling samples to avoid cross-contamination.
- Use a forced air incubator and ensure sufficient space between enrichment bags to allow for air flow.

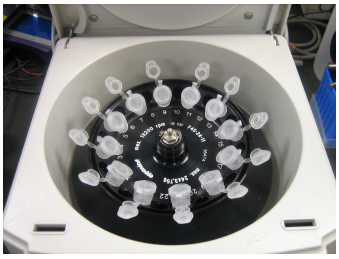
Guidelines for DNA isolation

PCR-clean water

Use nuclease-free water for all procedures described in this protocol that require water. Nuclease-free water is considered “PCR-clean” water. In contrast, autoclaved water should not be considered "PCR-clean" water.

Position of the spin column/tube assembly in the microcentrifuge

Place the tube cap hinge toward the inside of the rotor, and position the cap in the opposite direction of rotation.



Incorrect position of tube caps

Correct position of tube caps

For high-fat samples: remove fat layer before lysis

For samples that contain a distinct, top, fat layer following centrifugation, remove the fat layer and supernatant as follows:

Type of fat layer	Fat layer and supernatant removal
Liquid	1. Use a P1000 pipettor to remove fat from the top surface by aspirating in a circular motion without disturbing the pellet. 2. Continue to collect supernatant from the top surface until all the supernatant is removed. 3. Discard the supernatant into a waste container.
Solid	1. Use a pipette tip to gently dislodge the fat layer without disturbing the pellet. 2. Aspirate the supernatant from the top surface using a pipettor until all the supernatant is removed. 3. Discard the supernatant into a waste container.

Enrich food samples in BPW

1. Prepare 225 mL of Buffered Peptone Water (BPW) for each 25 g of food sample, according to the manufacturer's instructions.
2. Combine the food sample with 225 mL of BPW in a homogenizer bag, and homogenize.

   A filtered bag may be used for enrichment of samples with particulates.

Sample type	Method
Coarse food types	Homogenize the sample thoroughly using a laboratory blender. Hand massage foods that cannot be processed in a homogenizer: squeeze the bag 5–10 times.

3. Incubate the sample at 42±1°C under static conditions for 16–20 hours.

Isolate DNA using Proteinase K (raw beef samples)

Before each use of the kit

- Prepare Proteinase K-Lysis Buffer: combine the following components for the number of samples required; store on ice until use.

Component	Volume per sample	Volume for *n* samples[1]
Proteinase K, 20 mg/mL	5 µL	5.5 µL × *n*
Lysis Buffer	50 µL	55 µL × *n*

[1] Includes 10% overage.

- Preheat block heaters to 97±2°C and 56±1°C.

Filter 750 µL of enriched culture through the spin column

Gently mix the enriched culture before transferring the sample to the spin column.

1. Insert a spin column into a labeled tube, transfer 750 µL of the enriched sample from the filtered side of the enrichment bag to the spin column, and cap the column.
2. Microcentrifuge the spin column assembly at 12,000–16,000 × *g* for about 3 minutes.
   Follow "Position of the spin column/tube assembly in the microcentrifuge" on page 12.
3. Remove the assembly from the microcentrifuge and discard the used spin column.
4. Gently aspirate the supernatant without disturbing the pellet, then discard the supernatant.
   To remove liquid on the sides of the tube, push droplets into the supernatant by circling the inside of the tube with the pipettor before aspiration.
   (Optional) If necessary, follow "For high-fat samples: remove fat layer before lysis" on page 12.

Lyse the sample

1. Add 55 µL of Proteinase K-Lysis Buffer to the pellet, and pipet up and down or vortex until the pellet is well dispersed.
2. (*Optional*) **Rapid Spin Extra Clean protocol (for samples with high lipid content)**: transfer the mixture to a clean 1.5-mL tube, avoiding residual fat.
   The pellet must be well dispersed in the Lysis Buffer prior to transfer.

Avoid contact with residual fat on the sides of the original tube, and transfer only the Lysis Buffer containing the resuspended pellet.

3. Cap the tube, then incubate at 56±1°C for at least 30 minutes.
4. Incubate at 97±2°C for 12±2 minutes, then allow the sample to cool for about 2 minutes at room temperature (23±5°C).
5. Microcentrifuge the tube at 12,000–16,000 × *g* for about 1 minute to collect the contents at the bottom of the tube.
6. Add 250 µL of nuclease-free water, and mix thoroughly.
7. Microcentrifuge the tube at 12,000–16,000 × *g* for 1–2 minutes to pellet any remaining particulate material.

   The microbial DNA is in the supernatant.

Proceed directly to real-time PCR. Alternatively, store the DNA in one of the following ways:

- At 5±3°C for up to 24 hours.
- Below –18°C for up to 1 year.

If required, validate storage of the DNA according to EN ISO 20837:2006.

Isolate DNA without Proteinase K (raw vegetable samples)

Preheat the block heater to 97°C

Before each use of the kit, preheat the block heater to 97°C.

Filter 750 µL of enriched culture through the spin column

Gently mix the enriched culture before transferring the sample to the spin column.

1. Insert a spin column into a labeled tube, transfer 750 µL of the enriched sample from the filtered side of the enrichment bag to the spin column, and cap the column.
2. Microcentrifuge the spin column assembly at 12,000–16,000 × *g* for about 3 minutes.

   Follow “Position of the spin column/tube assembly in the microcentrifuge” on page 12.
3. Remove the assembly from the microcentrifuge and discard the used spin column.
4. Gently aspirate the supernatant without disturbing the pellet, then discard the supernatant.

   To remove liquid on the sides of the tube, push droplets into the supernatant by circling the inside of the tube with the pipettor before aspiration.

   (Optional) If necessary, follow “For high-fat samples: remove fat layer before lysis” on page 12.

Lyse the sample

1. Add 50 µL of Lysis Buffer to the pellet, and pipet up and down or vortex until the pellet is well dispersed in the Lysis Buffer mix.
2. (*Optional*) **Rapid Spin Extra Clean protocol (for samples with high lipid content)**: transfer the mixture to a clean 1.5-mL tube, avoiding residual fat.

   The pellet must be well dispersed in the Lysis Buffer prior to transfer.

   Avoid contact with residual fat on the sides of the original tube, and transfer only the Lysis Buffer containing the resuspended pellet.
3. Cap the tube, then incubate at 97±2°C for 12±2 minutes.
4. Allow the sample to cool for about 2 minutes at room temperature (23±5°C).
5. Microcentrifuge the tube at 12,000–16,000 × *g* for about 1 minute to collect the contents at the bottom of the tube.
6. Add 250 µL of nuclease-free water, and mix thoroughly.
7. Microcentrifuge the tube at 12,000–16,000 × *g* for 1–2 minutes to pellet any remaining particulate material.

   The microbial DNA is in the supernatant.

Proceed directly to real-time PCR. Alternatively, store the DNA in one of the following ways:

- At 5±3°C for up to 24 hours.
- Below –18°C for up to 1 year.

4 Perform PCR with the MicroSEQ™ E. coli O157:H7 Detection Kit and RapidFinder™ Express Software

Important procedural guidelines for PCR

Software

RapidFinder™ Express Software determines the Run Layout (plate layout) during creation of the run file, therefore it must be set up before distributing DNA samples to the assay beads.

For additional information, refer to the *Applied Biosystems™ RapidFinder™ Express Software Quick Reference* (Pub. No. 4480999) or the online help within the software.

Sample handling

- If DNA samples have been stored or the pellet has dispersed, thaw the samples (if necessary), vortex, then centrifuge at 12,000–16,000 × *g* for 1–2 minutes. This step will avoid cross-contamination and exclude particulate matter from the PCR.
- Use a new pipette tip for each sample.
- If you mix the assay beads with the DNA samples by pipetting up and down, keep the pipette tip at the bottom of the tube to minimize aerosol formation and cross-contamination.
- Follow the recommendations in "Good laboratory practices for PCR" on page 31.

For high-fat samples after lysis: collection of DNA sample for PCR

After lysis, food samples with high fat or oil content can form a top layer containing fat and debris over the aqueous phase containing the DNA. Collect the DNA sample for PCR from the clear middle phase, avoiding the top layer and bottom pellet.

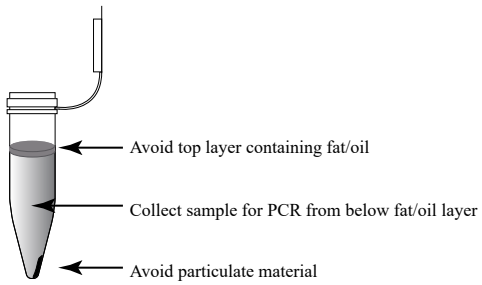


Figure 1 High-fat samples: collect sample from middle phase after lysis

MicroAmp™ tube strips

- Follow these instructions to ensure proper storage of the tube strips:
  - Cut the storage pouch at the notch above the resealable strip.
  - Always reseal the storage pouch with desiccant, and replace at 5±3°C.
- 8-tube strips can be cut apart with scissors.

  If necessary, trim any remaining connector material from the cut to allow a better fit against adjacent tubes in the 7500 Fast Precision Plate Holder for MicroAmp™ Tube Strips.
- MicroAmp™ Tube Strips are labeled 1–8 on the side of the tubes to orient tube strips during handling.

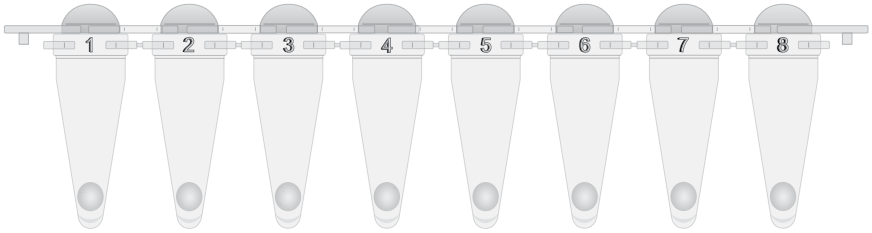


Figure 2 MicroAmp™ Tube Strip labeling The tube strip is shown with tinted dome caps, as shipped. For PCR, replace the dome caps with the optical cap strips provided in the kit.

If necessary for visual reference from above, mark the tab at one end of the cap strip. Do not mark any of the caps (this could interfere with real-time PCR detection).

- Seal the tubes with the transparent, optical cap strips provided in the kit. Do not use colored caps or tubes for real-time PCR reactions, because they may affect dye-signal readings during real-time PCR.
- Always use intact 8-cap strips, even if empty tubes have been added next to reaction tubes.
- Use the MicroAmp™ 96-Well Base and the MicroAmp™ Cap Installing Tool to seal the assay tubes with the optical cap strips. This avoids collapsing, bending, or misaligning the tubes.

  Confirm that the strips are straight and that each tube is in line with the adjacent tube.
- Use a plate adapter for vortexing the tube strips, or hold the strips in the MicroAmp™ 96-Well Base while vortexing.

Create or edit a run file in RapidFinder™ Express Software

On the main page of the RapidFinder™ Express Software, select **Create/Edit a Run File**, then select the target pathogen, number of samples, replicates, and positive and negative controls for each target at the prompts.

The software determines the sample layout based on the information entered and creates a run file.

Prepare the assay beads

Follow the plate layout determined by the RapidFinder™ Express Software.

1. Transfer the appropriate number of individual tubes or 8-tube strips from the storage pouch to a 96-well base at room temperature (23±5°C).

2. If required by the plate layout, place empty MicroAmp™ Fast 8-Tube Strips (or partial strips) to balance the tray when the assay tubes are placed in the instrument later.

Set up the PCR reactions

For step-by-step instructions, select **Pipette Samples** on the main page in RapidFinder™ Express Software.

1. If necessary, thaw samples and controls completely, then mix each sample or control thoroughly.

   If the DNA samples have been stored or the pellet has dispersed, see “Sample handling” on page 21.

   If the sample contains oil droplets or food particulate residue, see “For high-fat samples after lysis: collection of DNA sample for PCR” on page 21.

2. Following the layout determined by RapidFinder™ Express Software, add 30 µL of sample or control to each assay bead at room temperature (23±5°C), then mix by gently pipetting up and down a few times.

   Beads dissolve in 1–5 seconds.

   Alternatively, vortex the assay tubes after they are capped in the final step.

3. Seal the tubes with the transparent, optical cap strips provided in the kit.

4. Ensure that the reactions are thoroughly mixed: if reactions were not previously mixed during the pipetting step, vortex at high speed for 5–10 seconds.

5. Ensure that the reagents are at the bottom of tubes: briefly centrifuge the tube strips at 200–600 × *g* for about 20 seconds using a centrifuge with a plate adapter or a benchtop microcentrifuge with an 8-strip PCR tube adapter.

   IMPORTANT! If needed, repeat the vortex/centrifugation steps to ensure complete mixing of the samples with the assay beads.

Load and run the reactions

In the RapidFinder™ Express Software, select **Start Instrument Run** on the main page, select the appropriate run file, and follow the software prompts.

1. Transfer the tubes to the instrument in the same configuration as the run layout.

   Use the 7500 Fast Precision Plate Holder for MicroAmp™ Tube Strips in the instrument.

   Be sure to load empty tube strips as directed by the software (Figure 3).

2. Close the tray to the instrument, and follow the RapidFinder™ Express Software prompts to start the run.

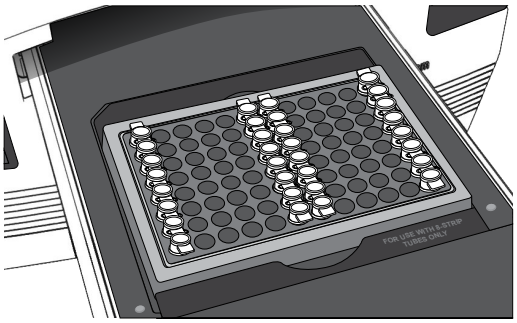


Figure 3 7500 Fast instrument tube layout RapidFinder™ Express Software directs the user to load empty strip tubes in column 1 (far left) and column 12 (far right), if needed. The empty capped 8-tube strips evenly distribute the clamping load applied to the sample tube strips during processing, thereby minimizing the risk of collapsing any tubes.

View results and data analysis

Data analysis is automated by the software.

In the RapidFinder™ Express Software, select **View Results** on the main page, select the appropriate run file, and follow the prompts to view results.

To display a list of results in table format, click **Table View**. Select a sample, then click **View Details** to see replicate information about samples.

5 Recommended confirmation methods

In the context of NF VALIDATION™ by AFNOR™ Certification, all samples identified as positive by the MicroSEQ™ *E. coli* O157:H7 method must be confirmed by one of the following tests:

- Using the conventional tests described in the methods standardized by CEN or ISO from colonies (including the purification step). The confirmation step must start from either BHI Broth or BPW, depending on the MicroSEQ™ protocol followed.
- By streaking 50 µL of the enrichment broth on CT-SMAC or Chrom ID 0157:H7 Agar. Any typical colonies must be plated onto Nutrient Agar, and after a 24-hour incubation, latex agglutination tests specific to both O157 and H7 (Oxoid™ DR0120M) must be performed with isolated colonies. If no characteristic colony is observed on the agar plates, proceed with an immunomagnetic separation (IMS) step prior to streaking 50 µL on selective agar (for example, by using Dynabeads™ anti-*E. coli* O157 (Cat. No. A10714) from 1 mL of the enrichment broth). Then, streak 50 µL onto CT-SMAC or ChromID O157:H7 Agar.
- Using an alternate method certified NF VALIDATION™ by AFNOR™ Certification, the principle of which must be different from the MicroSEQ™ *E. coli* O157:H7 method. The protocol of detection of the second validated method used for the confirmation shall be followed entirely. All steps which are before the step from which the confirmation is started shall be common to both methods. The MicroSEQ™ *E. coli* O157:H7 method and the second validated method must have common first steps.

In the event of discordant results, positive with the alternative method, non-confirmed by one of the means described above, and in particular for the latex test, the laboratory must follow the necessary steps to ensure the validity of the result obtained.

A

Troubleshooting

Observation	Possible cause	Recommended action
A visual difference in PCR beads is observed.	PCR pellets can exhibit differences in morphology.	Ensure thorough pipette mixing followed by vortexing on high speed to confirm pellet is in solution. After PCR, if IPC failure is observed, repeat the reaction.
Bacterial pellet is difficult to avoid during removal of supernatant	The sample was left unattended before removal of the supernatant, causing dissipation of the bacterial pellet.	Remove the supernatant immediately following centrifugation.
	The size of the bacterial pellet is very small and difficult to see.	Remove the supernatant carefully, leaving behind up to 50 µL of supernatant to avoid aspiration of the pellet.
The PCR was inhibited, as indicated by non-detection of the IPC reaction.	Removal of the supernatant was insufficient before addition of Lysis Buffer.	Dilute the sample 1:5 or 1:10 with nuclease-free water to dilute PCR inhibitors. If PCR remains inhibited, repeat the sample preparation.
	Filtrate from the spin column was in the sample.	Centrifuge the sample to separate the filter particulates before transferring sample to the PCR .
	Excess fat was not removed during aspiration of the supernatant.	Apply PrepSEQ™ Rapid Spin extra clean protocol.
	The sample matrix was associated with PCR-inhibitory components.	Pre-wash the bacterial pellet before loading the Rapid Spin column: 1. Transfer 750 µL of sample to a clean microcentrifuge tube. 2. Centrifuge at 12,000–16,000 × *g* for about 3 minutes. 3. Discard supernatant. 4. Resuspend pellet in 650 µL of sterile distilled water. 5. Load the resuspended sample onto the spin column.
In positive control wells, no IPC signal is detected, but target-specific signal is detected.	A high copy number of target DNA existed in the samples, resulting in preferential amplification of the target-specific DNA.	No action is required. The result is considered positive.

Observation	Possible cause	Recommended action
In positive control wells, no target-specific signal is detected.	Positive control was omitted (pipetting error).	Repeat the assay. Make sure to pipette the positive control into all positive control wells.
In negative control wells, no IPC signal is detected, but a target-specific signal is detected	Carryover contamination caused target signal in negative control wells. Additionally, no IPC signal in negative control wells could be caused by: • A high copy number of target DNA existed in the samples, resulting in preferential amplification of the target-specific DNA. • A problem occurred with IPC amplification.	To correct carryover contamination, repeat the assay using fresh aliquots of all reagents and clean pipetting equipment. To determine whether IPC amplification is a problem, examine unknown wells for an IPC signal. If an IPC signal is present, IPC amplification is not a problem.
In negative extraction control wells, target-specific signal is detected. The result is considered invalid by the software.	Carryover contamination occurred.	1. Repeat the assay using fresh aliquots of all reagents, fresh enrichment, and clean pipetting equipment. 2. If the negative extraction control continues to show contamination, repeat the assay using a new kit. 3. If the negative extraction control continues to show contamination, contact Technical Support.
In unknown wells, no IPC or target-specific signal is detected.	Inhibition of PCR occurred.	Dilute the sample 1:5 with nuclease-free water to dilute PCR inhibitors, then repeat the assay. If PCR remains inhibited, repeat the sample preparation. Refer to other troubleshooting suggestions for removal of particulates from the DNA sample.
In unknown wells, no IPC signal is detected, but target-specific signal is detected.	A high copy number of target DNA existed in the samples, resulting in preferential amplification of the target-specific DNA.	No action is required. The result is considered positive.

Observation	Possible cause	Recommended action
Multicomponent plot signals for FAM™, VIC™, and ROX™ detectors increase/decrease during cycles 1–15, but the amplification curve and result are not affected (this observation applies to **View in SDS** mode).	Incomplete mixing and dissolution of the lyophilized bead with sample or control occurred.	After adding 30 µL of sample or Pathogen Negative Control to the bead and capping the tubes: 1. Vortex strips at high speed for about 10 seconds, then centrifuge the strips at 200–600 × *g* for about 10 seconds. 2. Vortex the strips again on high speed for about 10 seconds, then centrifuge the strips at 200–600 × *g* for about 1 minute. Ensure that all liquid is at the bottom of the tubes and the beads are fully dissolved before proceeding.
Replicate results for a sample are inconsistent.	All replicate wells for a sample did not have the same result.	If more than two replicates yield the same result (for example, 2 of 3 replicates are negative, but 1 replicate is positive), refer to your laboratory protocol to determine whether to repeat the assay using fresh samples and reagents. If only 2 replicates were run and the results are not consistent, repeat the assay using fresh samples and reagents.

Observation	Possible cause	Recommended action
Amplicon contamination.	• Contamination was introduced into the PCR clean area from post-amplification reaction tubes that were either opened in the clean area or brought into the PCR clean area from contaminated gloves or solutions. • Contamination was introduced into the real-time PCR instrument from crushed and broken PCR reaction tubes.	To confirm amplicon contamination, perform the following experiment: Prepare negative control samples using at least one 8-tube strip of MicroSEQ™ Assay Beads. 1. Divide the assay beads into two sets. a. To the first set of assay beads, add 30 µL of nuclease-free water. b. To the second set of assay beads, add 29 µL of nuclease-free water plus 1 µL of 1 U/µL Uracil DNA Glycosylase (Cat. No. 18054-015). 2. Run samples on the 7500 Fast Real-Time PCR Instrument using SDS software, then select **Fast 7500** run mode. 3. Under the instrument tab: • Select **Add Step** to stage 1 of the PCR cycle that consists of 10 minutes at 50°C. • Extend the 95°C step from 20 seconds to 10 minutes. Amplicon contamination is indicated by target-specific signal in the –UNG samples and no target-specific signal in +UNG samples. If the instrument block was contaminated, consult the *Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Getting Started Guide: Absolute Quantitation using Standard Curve* (Pub. No. 4347825) and/or contact a service representative to clean the instrument.

B

Supplemental information

NF VALIDATION™ by AFNOR™ Certification

Go to thermofisher.com/foodsafety for a complete list of workflows for detection of *E. coli* (Pub. No. MAN0009419).

Table 9 NF VALIDATION™ by AFNOR™ Certification of the workflow

Certification	Expiration
 ABI 29/03 – 03/11 ALTERNATIVE ANALYTICAL METHODS FOR AGRIBUSINESS http://nf-validation.afnor.org/en/	For more information about the end of validity of the NF VALIDATION™ by AFNOR™ Certification, refer to the certificate, ABI 29/03 – 03/11, available at nf-validation.afnor.org/en or thermofisher.com/foodsafety.

The MicroSEQ™ E. coli O157:H7 Detection Kit has been certified by NF VALIDATION™ by AFNOR™ Certification according to EN ISO 16140 standard. The certified workflow described in this user guide includes:

- Enrichment in BHI or BPW
- The PrepSEQ™ Rapid Spin Sample Preparation Kit
- The MicroSEQ™ E. coli O157:H7 Detection Kit
- The Applied Biosystems™ 7500 Fast Real-Time PCR Instrument
- RapidFinder™ Express Software Version 2.0 or higher
- Confirmation testing as described in Chapter 5, “Recommended confirmation methods”.

Table 10 Validated matrices

Reference method	Matrix
EN ISO 16654:2001	Raw beef meat Raw vegetables

General remarks and recommendations:

- In the context of NF VALIDATION™ by AFNOR™ Certification, samples weighing more than 25 grams have not been tested.
- Comply with Good Laboratory Practices (GLP; refer to EN ISO 7218 standard).
- For preparation of initial suspensions, follow instructions of EN ISO 6887 and EN ISO 16654 standards.

  The short protocols of detection are sensitive to conditions of incubation. It is required to respect scrupulously the conditions of temperature indicated in the technical specification. Notably, you must verify that the temperature of pre-warming of the enrichment broth reaches the required temperature. The time of preparation of samples, delay between the end of the step of pre-warming of the enrichment broth and the beginning of the step of incubation of the food sample, does not exceed 45 minutes. Using a ventilated incubator during incubation is recommended.

Good laboratory practices for PCR

Note: Spin tubes/plates before performing PCR. Spinning of PCR tubes is most easily accomplished by using a centrifuge designed for PCR tubes or plates. Follow manufacturer instructions for loading tubes/plates.

To avoid amplicon contamination of samples, follow these guidelines when preparing or handling samples for PCR amplification:

- Wear clean gloves and a clean lab coat (not previously worn while handling amplified products or used during sample preparation).
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Do not open reaction tubes after PCR.
- Do not autoclave reaction tubes after PCR.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA*Zap*™ Solutions (Cat. No. AM9890) according to the Thermo Fisher Scientific PCR Decontamination Protocol. After cleaning with bleach we recommend a rinse with distilled water or an ethanol solution because bleach will rust stainless steel. Note that minor discoloration of metal parts may occur.

For additional information, refer to EN ISO 22174:2005 or **www.thermofisher.com/us/en/home/life-science/pcr/real-time-learning-center/real-time-pcr-basics.html**.

C

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

Documentation and support

Food safety support

Website: **https://www.thermofisher.com/us/en/home/industrial/food-beverage/food-microbiology-testing.html** or **thermofisher.com/foodsafety**

Support email:

- Europe, Middle East, Africa: **microbiology.techsupport.uk@thermofisher.com**
- North America: **microbiology@thermofisher.com**

Phone: Visit **thermofisher.com/support**, select the link for phone support, then select the appropriate country from the dropdown list.

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Related documentation

Document	Publication number
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407
SimpliAmp™ Thermal Cycler User Guide	MAN0009889
SimpliAmp™ Thermal Cycler Installation and Operation Quick Reference	A24827

Limited product warranty

Life Technologies Corporation and its affiliates warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have questions, contact Life Technologies at **www.thermofisher.com/support**.

References

EN ISO 16654:2001. Microbiology of food and animal feeding stuffs—Horizontal method for the detection of *E. coli* O157.

EN ISO 22174:2005. Microbiology of food and animal feeding stuffs—Polymerase chain reaction (PCR) for the detection of food-borne pathogens—General requirements and definitions.

EN ISO 7218:2007. Microbiology of food and animal feeding stuffs—General requirements and guidance for microbiological examinations.

EN ISO 6887-1:1999. 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.

EN ISO 6887-2:2003. 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.

EN ISO 6887-3:2003. 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.

EN ISO 6887-4:2003. 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.

EN ISO 6887-5:2010. 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.