AS 5013.26:2020

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Food microbiology

Method 26: Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Escherichia coli* O157 (ISO 16654:2001/Amd.1:2017, MOD)

AS 5013.26:2020

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The following are represented on Committee FT-035:

ACT Health Directorate
Australian Institute of Food Science and Technology
Australian Society for Microbiology
CSIRO
Dairy Industry Association of Australia
Department of Agriculture, Water and the Environment
Department of Health and Human Services, Tas.
Meat and Livestock Australia
National Association of Testing Authorities Australia
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Method 26: Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Escherichia coli* O157 (ISO 16654:2001/Amd.1:2017, MOD)

Originated as AS 5013.26—2009.
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Preface

This Standard was prepared by the Standards Australia Committee FT-035, Food Microbiology, to supersede AS 5013.26—2009, *Food microbiology, Method 26: Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of Escherichia coli O157 (ISO 16654:2001, MOD).*

The objective of this Standard is to specify a horizontal method for the detection of *Escherichia coli* serogroup O157.

This Standard is an adoption with national modifications, and has been reproduced from, ISO 16654:2001, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of Escherichia coli O157* and its Amendment 1 (2017), which has been added at the end of the source text.

Appendix ZZ lists the variations to ISO 16654:2001 for the application of this Standard in Australia.

As this document has been reproduced from an International Standard, the following applies:

(a) In the source text "this International Standard" should read "this Australian Standard".

(b) A full point substitutes for a comma when referring to a decimal marker.

Australian or Australian/New Zealand Standards that are identical adoptions of international normative references may be used interchangeably. Refer to the online catalogue for information on specific Standards.

The terms "normative" and "informative" are used in Standards to define the application of the appendices or annexes to which they apply. A "normative" appendix or annex is an integral part of a Standard, whereas an "informative" appendix or annex is only for information and guidance.

Contents

Foreword

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

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

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

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 16654 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

Annex A forms a normative part of this International Standard.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this International Standard so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

NOTES

Australian Standard®

Food microbiology

Method 26: Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Escherichia coli* O157 (ISO 16654:2001/Amd.1:2017, MOD)

WARNING — *Escherichia coli* O157 can cause severe life-threatening illness and has a low infective dose. Laboratory-acquired infections have been reported.

In order to safeguard the health of laboratory personnel, it is essential that the whole of this method be carried out only by skilled personnel using good laboratory practices and preferably working in a containment facility. Relevant national Health and Safety Regulations relating to this organism must be adhered to.

Care must be taken in the disposal of all infectious materials.

1 Scope

This International Standard specifies a horizontal method for the detection of *Escherichia coli* serogroup O157.

Subject to the limitations discussed in the introduction, this International Standard is applicable to products intended for human consumption or for animal feeding stuffs.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*.

ISO 7218, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*.

3 Term and definition

For the purposes of this International Standard, the following term and definition applies.

3.1
***Escherichia coli* O157**
***E. coli* O157**
microorganisms which form typical colonies on the surface of the plating-out medium used in this International Standard, and which produce indole and agglutinate specifically with antiserum against the O157 antigen

Note 1 to entry: Sorbitol-positive *E. coli* O157 strains are not detected on CT-SMAC (5.2) media.

Note 2 to entry: Some indole-negative mutations have been found.

4 Principle

The detection of *Escherichia coli* O157 necessitates four successive stages (see annex A).

a) **Enrichment** of the test portion homogenized in modified tryptone soya broth containing novobiocin (mTSB + N) with incubation at 41,5 °C ± 1 °C for 6 h and subsequently for a further 12 h to 18h.

b) **Separation and concentration** of microorganisms by means of immunomagnetic particles coated with antibodies to *E. coli* O157.

c) **Isolation** by subculture of the immunomagnetic particles with adhering bacteria onto cefixime tellurite sorbitol MacConkey agar (CT-SMAC) and the user's choice of a second selective isolation agar.

d) **Confirmation** of sorbitol-negative colonies from CT-SMAC and colonies typical of *E. coli* O157 on the second isolation agar, by indole production and agglutination with *E. coli* O157 antiserum.

NOTE Further characterization, by for example pathogenic markers, of the positive isolates can be obtained by forwarding them to an appropriate reference laboratory.

5 Culture media, reagents and antisera

For current laboratory practices, see ISO 7218.

5.1 Enrichment medium: Modified tryptone soya broth with novobiocin (mTSB + N)

See reference [1].

5.1.1 Modified tryptone soya broth (mTSB)

5.1.1.1 Composition

Enzymatic digest of casein	17,0 g
Enzymatic digest of soya	3,0 g
D(+)-glucose	2,5 g
Bile salts No. 3	1,5 g
Sodium chloride	5,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	4,0 g
Water	1 000 ml

5.1.1.2 Preparation

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

Adjust the pH, using the pH-meter (6.6), if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Dispense the medium in appropriate amounts in flasks or bottles (6.7).

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.1.2 Novobiocin solution

5.1.2.1 Composition

Novobiocin	0,45 g
Water	100 ml

5.1.2.2 Preparation

Dissolve the novobiocin in the water and sterilize by membrane filtration.

Prepare on the day of use.

5.1.2.3 Preparation of the complete medium

Immediately before use, add 1 ml or 4 ml of novobiocin solution (5.1.2) to either 225 ml or 900 ml of cooled mTSB (5.1.1).

The final concentration of novobiocin is 20 mg per litre of mTSB.

5.2 First selective isolation medium: Cefixime tellurite sorbitol MacConkey agar (CT-SMAC)

See reference [2].

5.2.1 Base medium

5.2.1.1 Composition

Enzymatic digest of casein	17,0 g
Enzymatic digest of animal tissues	3,0 g
Sorbitol	10,0 g
Bile salts No. 3	1,5 g
Sodium chloride	5,0 g
Neutral Red	0,03 g
Crystal Violet	0,001 g
Agar	9 g to 18 g [a]
Water	1 000 ml
[a] Depending on the gel strength of the agar.	

5.2.1.2 Preparation

Dissolve the basic components or the complete dehydrated base in the water by boiling. Adjust the pH (6.6), if necessary, so that after sterilization it is 7,1 ± 0,2 at 25 °C.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.2.2 Potassium tellurite solution

5.2.2.1 Composition

Potassium tellurite for bacteriological use	0,25 g
Water	100 ml

5.2.2.2 Preparation

Dissolve the potassium tellurite in the water and sterilize by membrane filtration.

This solution may be stored at room temperature for up to 1 month, but discard it if a white precipitate forms.

5.2.3 Cefixime solution

5.2.3.1 Composition

Cefixime	5,0 mg
Water	100,0 ml

5.2.3.2 Preparation

Dissolve the cefixime in the water and sterilize by membrane filtration.

NOTE Cefixime may need to be dissolved in ethanol.

This solution may be stored at 3 °C ± 2 °C for 1 week.

5.2.4 Complete medium

5.2.4.1 Composition

Base medium (5.2.1)	1 000 ml
Potassium tellurite solution (5.2.2)	1,0 ml
Cefixime solution (5.2.3)	1,0 ml

5.2.4.2 Preparation

Either cool the freshly sterilized base medium (5.2.1) to between 44 °C and 47 °C (6.5), or melt it by steaming the previously sterilized and solidified base medium, then cool to between 44 °C and 47 °C.

Add 1 ml of the tellurite solution and 1 ml of the cefixime solution to 1 000 ml of the base medium. Mix and pour about 15 ml amounts into sterile Petri dishes (6.15). Allow to solidify.

The final concentration of tellurite is 2,5 mg/l and cefixime 0,05 mg/l.

Immediately before use, dry the agar plates, preferably with the lids removed and with the agar surfaces facing downwards, in an oven set at a temperature between 25 °C and 50 °C (6.2), until the droplets have disappeared from the surface of the medium. Do not dry them any further. The agar plates may also be dried in a laminar-flow safety cabinet for 30 min with half-open lids, or overnight with the lids in place.

If prepared in advance, the undried plates may be stored in the dark in plastic bags or other moisture-retentive containers, in a refrigerator at 3 °C ± 2 °C for up to 2 weeks.

5.3 Second selective isolation medium

Use any other solid selective medium, at the choice of the laboratory, complementary to CT-SMAC agar and especially appropriate for the isolation of *Escherichia coli* O157.

Immediately before use, dry the agar plates, preferably with the lids removed and with the agar surfaces facing downwards, in an oven set at a temperature between 25 °C and 50 °C (6.2), until the droplets have disappeared from the surface of the medium. Do not dry them any further. The agar plates may also be dried in a laminar-flow safety cabinet for 30 min with half-open lids, or overnight with the lids in place.

If prepared in advance, the undried plates may be stored in the dark in plastic bags or other moisture-retentive containers, in a refrigerator at 3 °C ± 2 °C for a time that causes no change to its performance.

5.4 Nutrient agar

5.4.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Agar	9 g to 18 g [a]
Water	1 000 ml

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

5.4.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary. Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Transfer the medium into flasks or bottles (6.7) of appropriate capacity.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.4.3 Preparation of nutrient agar plates

Transfer about 15 ml of the molten, cooled medium (5.4.2) at between 44 °C and 47 °C (6.5) to Petri dishes and allow to solidify.

Immediately before use, dry the agar plates, preferably with the lids removed and with the agar surfaces facing downwards, in an oven set at a temperature between 25 °C and 50 °C (6.2), until the droplets have disappeared from the surface of the medium. Do not dry them any further. The agar plates may also be dried in a laminar-flow safety cabinet for 30 min with half-open lids, or overnight with the lids in place.

If prepared in advance, the undried plates may be stored in the dark, in plastic bags or other moisture-retentive containers, in a refrigerator at 3 °C ± 2 °C for up to 2 weeks.

5.5 Tryptone/tryptophan medium

5.5.1 Composition

Tryptone	10,0 g
Sodium chloride	5,0 g
DL-Tryptophan	1,0 g
Water	1 000 ml

5.5.2 Preparation

Dissolve the components in the water by boiling if necessary. Adjust the pH (6.6) so that after sterilization it is 7,5 ± 0,2 at 25 °C.

Dispense in 5 ml amounts into test tubes or bottles (6.7) of appropriate capacity.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.6 Kovac's indole reagent

5.6.1 Composition

4-Dimethylaminobenzaldehyde	5,0 g
2-Methylbutan-1-ol or pentan-1-ol	75,0 ml
Hydrochloric acid (ρ_{20} 1,18 g/ml to 1,19 g/ml)	25,0 ml

5.6.2 Preparation

Dissolve the 4-dimethylaminobenzaldehyde in the alcohol, by warming if necessary in a water bath (6.5) maintained at between 44 °C and 47 °C. Cool to room temperature and add the hydrochloric acid.

Protect from light in a brown glass bottle and store at 3 °C ± 2 °C.

The reagent shall be light yellow to light brown and free of precipitate.

5.7 Anti-*Escherichia coli* O157 immunomagnetic particles

These are immunomagnetic particles coated with specific antibodies against *E. coli* O157 for concentration and separation of these microorganisms.

NOTE They are available from commercial sources. The manufacturer's instructions should be followed precisely regarding their preparation for use.

5.8 Wash buffer: Modified phosphate buffer, 0,01mol/l, of pH 7,2

5.8.1 Composition

Sodium chloride	8,0 g
Potassium chloride	0,2 g
Disodium hydrogen phosphate (anhydrous)	1,44 g
Potassium dihydrogen phosphate (anhydrous)	0,24 g
Polyoxyethylene sorbitan monolaurate (Tween 20 syrup)	0,2 ml
Water	1 000 ml

5.8.2 Preparation

Dissolve the components in water. Adjust the pH (6.6), if necessary, to 7,2 ± 0,2 at 25 °C.

Dispense in bottles or flasks (6.7) in appropriate volumes for use

Sterilize for 15 min in the autoclave (6.1) set at 121 °C. The solution may appear cloudy but becomes clear on standing.

Commercially available phosphate buffer with the same composition and the same performance may be used.

5.9 Normal saline solution

5.9.1 Composition

Sodium chloride	8,5 g
Water	1 000 ml

5.9.2 Preparation

Dissolve the sodium chloride in the water. Dispense in bottles or flasks (6.7) in appropriate volumes for use.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.10 *Escherichia coli* O157 antiserum, available either from specialist laboratories or from commercial sources as separate somatic "O" 157.

The antiserum shall be tested with positive and negative controls prior to use on unknown isolates.

6 Apparatus and glassware

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

6.1 Apparatus for dry sterilization (oven) and/or wet sterilization (autoclave)

See ISO 7218.

6.2 Drying cabinet or **incubator,** capable of being maintained between 25 °C and 50 °C.

6.3 Incubator, capable of being maintained at 37 °C ± 1 °C.

6.4 Incubator, capable of being maintained at 41,5 °C ± 1 °C.

6.5 Water bath, capable of being maintained at between 44 °C and 47 °C.

6.6 pH-meter, capable of being read to the nearest 0,01 pH unit at 25 °C, enabling measurements to be made which are accurate to ± 0,1 pH unit.

6.7 Test tubes, flasks or **bottles,** of appropriate capacity, for sterilization and storage of culture media and incubation of liquid media.

6.8 Measuring cylinders, of appropriate capacity, for preparation of dilutions and complete media.

6.9 Total-delivery graduated pipettes, of nominal capacities 1 ml and 10 ml, graduated in 0,1 ml and 0,5 ml divisions, respectively.

6.10 Loops and wires, made of platinum/iridium or nickel/chrome or **Pasteur pipettes** or **single-use loops**.

6.11 Mechanical air-displaced pipettors, sterile, with an operating range from 20 µl to 200 µl with 10 µl divisions, or similar.

6.12 Magnetic separator with **magnetic rack,** for concentration of immunomagnetic particles, for use with Eppendorf-type plastic tubes (6.13).

6.13 Eppendorf-type plastic tubes, with screw caps, sterile, disposable, centrifuge type, of 1,5 ml capacity to fit the magnetic rack.

Avoid the creation of aerosols when opening.

6.14 Rotary mixer (windmill type, blood sample mixer), capable of rotating at 15 r/min to 20 r/min.

6.15 Petri dishes, of diameter 90 mm and 140 mm.

6.16 Vortex mixer

7 Sampling

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

It is recommended to cool the sample quickly before storage.

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

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure (see annex A)

9.1 Test portion and initial suspension

See ISO 6887-1 and any specific International Standard appropriate to the product concerned.

NOTE Further parts of ISO 6887 are in preparation, see bibliography.

In general, to prepare the initial suspension, add a test portion of *x* g or *x* ml to 9*x* ml or 9*x* g of modified tryptone soya broth with novobiocin (mTSB + N) (5.1), pre-warmed in the incubator (6.4) to 41,5 °C to obtain a ratio of test portion to mTSB + N of 1/10 (mass to volume, or volume to volume).

It is recommended to use stomacher bags with mesh inserts to reduce the interference of food particles with immunocapture kits (9.3).

9.2 Enrichment

Incubate (6.4) the initial suspension, prepared in accordance with 9.1, at 41,5 °C for 6 h, and subsequently for a further 12 h to 18 h (i.e. to a total elapsed time of 18 h to 24 h).

A 6-h incubation followed by immunomagnetic separation and plating onto selective agars can yield a presumptive positive result which can become negative after a further 18-h incubation.

9.3 Immunomagnetic separation (IMS)

9.3.1 General

IMS should be carried out after 6 h and again, if necessary, after 12 h to 18 h of incubation.

The instructions below are for general guidance and may not be complete in all details. Therefore the manufacturer's instructions should be followed concerning the procedure and method for the use of immunocapture kits and the equipment needed.

9.3.2 Immunocapture

WARNING — Use aseptic techniques to avoid any external contamination and the creation of aerosols. Perform this protocol in a containment safety cabinet, if available. Wear gloves.

Using the magnetic separator (6.12) and antibody-coated immunomagnetic particles (5.7), carry out the following capture/separation procedure.

Mix the enrichment culture (9.2) and allow any coarse food materials to settle out. To an Eppendorf-type plastic tube (6.13), add 20 µl of the prepared immunomagnetic particles (5.7) at room temperature. Take 1 ml of the upper liquid from the enrichment culture, avoiding if possible the transfer of any food particles or fatty materials, and transfer to the Eppendorf-type plastic tube.

Mix the suspension on the rotary mixer (6.14) set at about 12 r/min to 20 r/min for 10 min.

9.3.3 Separation

Place each Eppendorf-type plastic tube (see 9.3.2) in the magnetic rack (6.12) and allow the magnetic particles to congregate against the magnet by gently rocking the rack through 180°. Open the cap carefully without disturbing the particles on the wall of the tube. Using a new sterile Pasteur pipette (6.10) for each sample and with the tube still in the magnetic rack, remove the liquid by sucking slowly from the bottom of the tube. Add 1 ml of sterile wash buffer (5.8) and replace the cap. Remove the magnet from the rack. Mix the contents of the tubes by gentle inversion of the rack through 180° and then return the magnet to the rack.

Take care to avoid cross contamination when adding fresh buffer.

Proceed as above to remove the wash liquid with a new Pasteur pipette for each sample. Repeat the washing procedure several times.

Remove from the magnetic separator and add 100 µl of sterile wash buffer (5.8) to the tube and re-suspend the magnetic particles.

NOTE This procedure could be difficult to apply to fat products or fresh cheese.

9.4 Plating out onto selective agars and identification of *E. coli* O157 colonies

9.4.1 Plating out

Using a mechanical-type pipettor (6.11), transfer 50 µl of the washed and re-suspended magnetic particles (9.3.3) to a pre-dried plate of cefixime tellurite sorbitol MacConkey agar (5.2) and also 50 µl to a pre-dried plate of the second isolation medium (5.3).

Streak out the particles using a sterile loop (6.10) to obtain many well-isolated colonies over the agar.

Incubate (6.3) the CT-SMAC (5.2) at 37 °C for 18 h to 24 h, and incubate the second selective agar at its recommended temperature and specified time.

Depending on the type of food sample and its microbial flora, incubation of the enrichment broth for 20 h to 24 h may give rise to a heavy growth of other bacteria on the selective agar plates so that colonies of *E. coli* O157 are difficult to find. Inoculation of selective agars with dilutions of the IMS preparation or volumes less than 50 µl per plate can increase the chance of gaining separated colonies of *E. coli* O157 but note that this can increase the detection limit as well.

9.4.2 Recognition of typical *E. coli* O157 colonies

On CT-SMAC agar, typical colonies are transparent and almost colourless with a pale yellowish-brown appearance and a diameter of approximately 1 mm.

Examine the second selective isolation agar for typical colonies of *E. coli* O157 following the manufacturer's instructions.

9.5 Confirmation

NOTE Commercially available miniaturized biochemical identification kits that permit the identification of sorbitol-negative and indole-positive *E. coli* and latex agglutination kits for *E. coli* O157 may be used, provided appropriate tests with known positive and negative strains are carried out to confirm performance.

9.5.1 Selection of colonies

Take five typical colonies from each plate, as selected in 9.4. If an agar plate contains less than 5 typical colonies, all the colonies shall be examined.

Streak each selected colony onto a plate of nutrient agar (5.4) to allow well-separated colonies to develop.

Incubate (6.3) the plates for 18 h to 24 h at 37 °C.

Use only pure cultures from the nutrient agar plate for the tests described in 9.5.2 and 9.5.3.

9.5.2 Biochemical confirmation: Indole formation

Inoculate one colony from the pure culture on nutrient agar (9.5.1) into a tube of tryptone/tryptophan medium (5.5).

Incubate (6.3) at 37 °C for 24 h.

Add 1 ml of Kovac's reagent (5.6) and allow to stand at room temperature for 10 min.

The formation of a red colour indicates a positive reaction. A yellow/brown colour indicates a negative reaction.

9.5.3 Serological identification

9.5.3.1 General

Only examine indole-positive colonies for their serological reaction with antiserum to *E. coli* O157.

9.5.3.2 Elimination of auto-agglutinating isolates

Place a drop of saline solution (5.9) onto a cleaned glass slide.

Using a loop (6.10), mix into this drop one colony from the nutrient agar plate (9.5.1) so as to obtain a homogeneous and turbid suspension.

Rock the slide gently for 30 s to 60 s. Observe the result against a dark background and, if necessary, with the aid of a magnifying lens.

If the suspension has formed visible clumps, the strain is considered to auto-agglutinate and shall not be tested further, as the reaction with the specific antiserum is not possible.

9.5.3.3 Reaction with *E. coli* O157 antiserum

Using a pure colony from the nutrient agar (9.5.1), suspend it in a fresh drop of saline as in 9.5.3.2 and add a small drop of *E. coli* O157 antiserum (5.10).

If agglutination occurs within 1 min, the reaction is positive.

9.5.3.4 Positive identification

Consider as positive isolates those that are indole positive and react with either O157 antiserum or O157 plus H7 antisera, if available.

9.6 Further characterization

For further identification of positive colonies for the detection of flagellar antigens and for pathogenic characteristics, cultures should be sent to a Reference Laboratory.

10 Quality assurance

10.1 Test strains for quality assurance purposes

Strains of *E. coli* O157 that do not carry the virulence factors attributed to pathogenicity are available from national or international culture collections. These are recommended for quality assurance testing of media and antisera.

10.2 Culture method

To check the ability of the laboratory and media to detect low numbers of *Escherichia coli* O157 in the food samples under test by the method described in this International Standard, reference samples of a low inoculum of a non-pathogenic *E. coli* O157 and a large inoculum of another strain of *E. coli* should be run in parallel with the test sample.

11 Expression of results

In accordance with the interpretation of the results, report the presence or absence of *Escherichia coli* O157 in the test portion, specifying the mass in grams or the volume in millilitres of the sample tested.

12 Test report

The test report shall specify the following:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard;

d) the temperature of incubation;

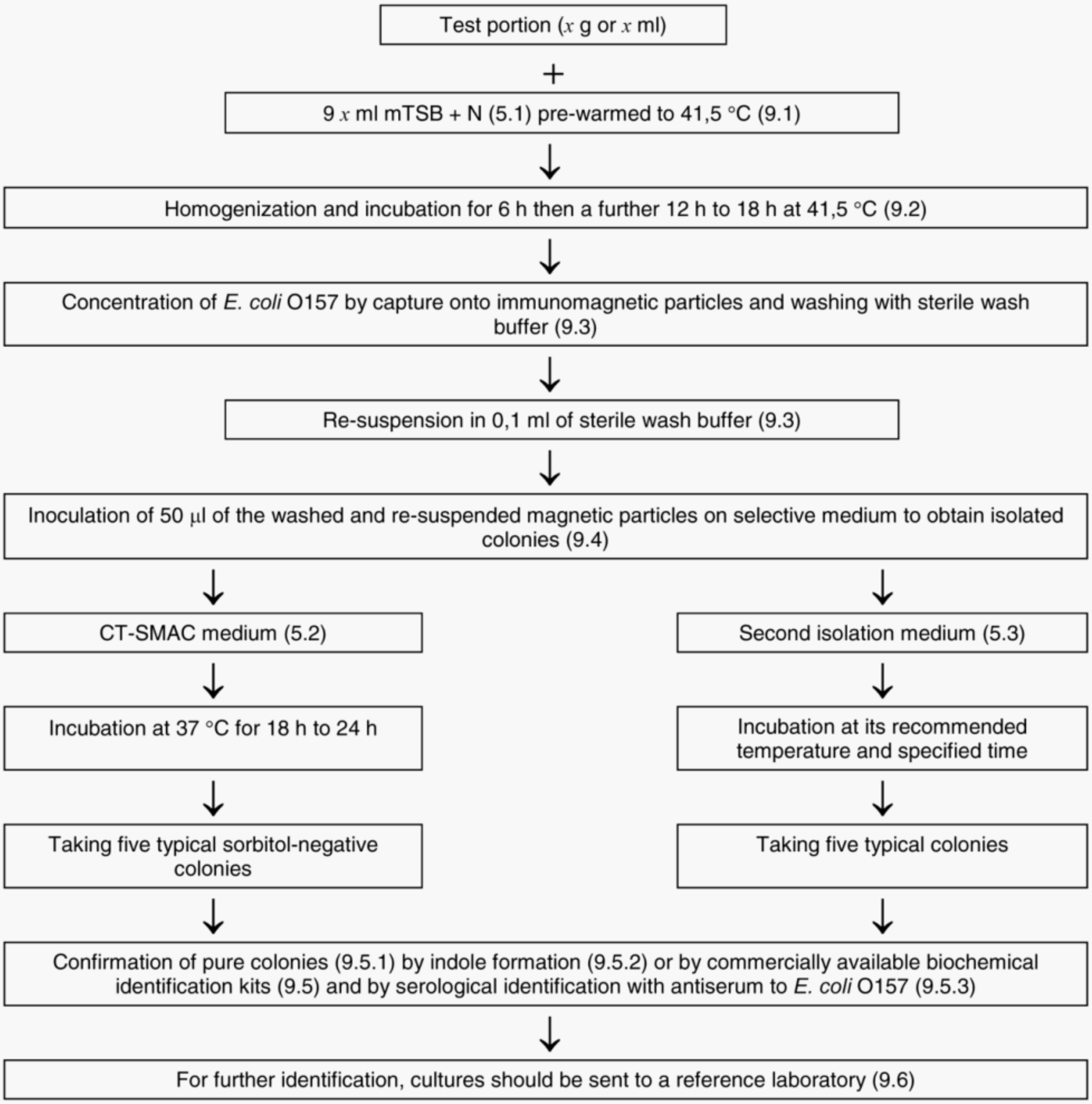
e) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the results;

f) the test results obtained.

The test report shall also state if further tests are to be carried out by a reference laboratory or, if done, what the results were.

Annex A
(normative)

Diagram of procedure



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

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

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

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

Amendment 1

Page 8, 9.1

Replace “NOTE” with “NOTE 1”.

Page 8, 9.1

Add the following at the end of the subclause.

This document has been validated for test portions of a specific weight or volume for each food matrix. A smaller test portion may be used, without the need for additional validation/verification, providing that the same ratio between (pre-) enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used, if a validation/verification study has shown that there are no adverse effects on the detection of *E. coli* O157.

NOTE 2 Validation can be conducted in accordance with the appropriate documents of ISO 16140 (all parts). Verification for pooling samples can be conducted in accordance with the protocol described in ISO 6887-1:2017, Annex D (verification protocol for pooling samples for qualitative tests).

Page 11, after Clause 11

Add the following as Clause 12 and renumber the previous Clause 12 “Test report” as Clause 13 “Test report”.

12 Performance characteristics of the method

12.1 Interlaboratory study

The performance characteristics of the method were determined in interlaboratory studies to determine the specificity, sensitivity and, when possible, the LOD_{50} of the method. The data are summarized in Annex B. The values derived from the interlaboratory studies may not be applicable to food types other than those given in Annex B.

12.2 Sensitivity

The sensitivity is the ability of the method to detect the analyte. It is defined as the number of samples found positive divided by the number of samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample.

12.3 Specificity

The specificity is the ability of the method to correctly identify the absence of the analyte in negative samples. It is defined as the number of samples found negative divided by the number of blank samples tested.

12.4 LOD_{50}

The LOD_{50} (level of detection) is the concentration (cfu/sample) for which the probability of detection is 50 %.

The LOD_{50} has been calculated in the CEN Mandate studies (see Annex B) for the matrix sprouts only as the other study, on milk, returned no negative results for both the levels of contamination.

The LOD_{50} was 8,4 cfu/g (6,5 cfu/g to 10,9 cfu/g confidence limit, 95 % confidence interval).

Page 13, after Annex A

Add the following as Annex B.

Annex B
(informative)

Results of interlaboratory studies

B.1 Results of interlaboratory studies of an NMKL study

An international interlaboratory study was organized in 1999 by the Nordic Committee on Food Analysis on the following food matrices: minced meat, raw milk and lettuce (see Reference [1]) on method assessed as equivalent to the method ISO 16654:2001.

The values of the performance characteristics derived from this interlaboratory study are shown per type of sample in Tables B.1 to B.3.

Table B.1 — Results of data analysis obtained with 25 g minced meat samples

Performance characteristic	Minced meat (blank)	Minced meat (low level, 17 cfu/sample)	Minced meat (high level, 80 cfu/sample)
Year of interlaboratory test	1999	1999	1999
Number of laboratories having returned results	14	14	14
Number of samples per laboratory	2	2	2
Number of excluded laboratories	0	0	0
Number of laboratories retained after the exclusion	14	14	14
Number of accepted samples	28	28	28
Sensitivity in %	–	100	100
Specificity in %	92,8	–	–

Table B.2 — Results of data analysis obtained with 25 ml raw milk samples

Performance characteristic	Raw milk (blank)	Raw milk (low level, 17 cfu/sample)	Raw milk (high level 78 cfu/sample)
Year of interlaboratory test	1999	1999	1999
Number of laboratories having returned results	14	14	14
Number of samples per laboratory	2	2	2
Number of excluded laboratories	0	0	0
Number of laboratories retained after the exclusion	14	14	14
Number of accepted samples	28	28	28
Sensitivity in %	–	92,8	100
Specificity in %	85,7	–	–

Table B.3 — Results of data analysis obtained with 25 g lettuce samples

Performance characteristic	Lettuce (blank)	Lettuce (low level, 10 cfu/sample)	Lettuce (high level, 33 cfu/sample)
Year of interlaboratory test	1999	1999	1999
Number of laboratories having returned results	14	14	14
Number of samples per laboratory	1	2	2
Number of excluded laboratories	0	0	0
Number of laboratories retained after the exclusion	14	14	14
Number of accepted samples	14	28	28
Sensitivity in %	–	100	100
Specificity in %	85,7	–	–

B.2 Results of interlaboratory studies of a CEN Mandate study

B.2.1 Study on milk

A second international interlaboratory study was run in 2012 in the framework of the CEN Mandate M/381.

In the preliminary steps of the process, the method ISO 16654:2001 was evaluated as being equivalent to the NMKL method N. 164 (See resolution 297 taken at the ISO/TC 34/SC 9 meeting in Prague in June 2006 for reference). Given the equivalence assessment between the two methods, at the 14th meeting of CEN/TC 275/WG 6 (23 to 28 April 2007, Cairo, Egypt) it was agreed to perform the validation study of the method ISO 16654:2001 in a reduced form, including only one epidemiologically relevant food matrix (milk).

National reference laboratories (NRLs) for *E. coli*, and laboratories involved in the official controls of foodstuffs of EU Member States were selected based on whether they were accredited according to ISO 17025, had the method ISO 16654:2001 accredited and had expertise in IMS-based isolation of food-borne bacteria.

In total, 15 laboratories participated in the inter-laboratory study, which was based on the analysis, with the method ISO 16654:2001, of raw milk samples artificially contaminated with *E. coli* O157 at three different levels of contamination: blank (0 cfu/ml), low (25 cfu/ml, combined uncertainty (*Uc* = 7 cfu/ml) and high (140 cfu/ml, *Uc* = 38 cfu/ml). The samples contained naturally present background microflora, and eight blind replicates of each level of contamination were sent to each laboratory, for a total of 24 samples examined. The samples contained glycerol and were sent to the laboratories as frozen specimens (–70 °C in dry ice).

The values of the performance characteristics derived from this collaborative test is shown in Table B.4.

Table B.4 — Results of data analysis obtained with 10 ml milk samples

Performance characteristic	Milk (blank)	Milk (low level, 250 cfu/sample)	Milk (high level, 1 400 cfu/sample)
Year of interlaboratory test	2012	2012	2012
Number of laboratories having returned results	15	15	15
Number of samples per laboratory	8	8	8
Number of excluded laboratories	0	0	0
Number of laboratories retained after the exclusion	15	15	15
Number of accepted samples	120	120	120
Sensitivity in %	–	100	100
Specificity in %	94,4	–	–

B.2.2 Study on sprouts

A third international interlaboratory study was organized and run in 2013 involving laboratories enrolled according to the same principles used for the first CEN mandate study on milk.

Fourteen laboratories received a total of 24 samples consisting of 8 blind replicates of sprouts, purchased at retail, spiked with 3 different concentrations of *E. coli* O157: 0 cfu/g, 10 cfu/g and 100 cfu/g. The extended uncertainty of measurement associated with the inoculum was 0,27 log cfu/ml (calculated according to the ISO/TS 19036). The sprout samples each contained a mixture of alfalfa (90 %) and watercress (10 %), with an endogenous microflora estimated to be 10^5 cfu/g to 10^7 cfu/g and have been sent refrigerated to the participating laboratories.

The values of the performance characteristics derived from this collaborative test are shown in Table B.5.

Table B.5 — Results of data analysis obtained with 25 g sprout samples

Performance characteristic	Sprouts (blank)	Sprouts (low level, 250 cfu/sample)	Sprouts (high level, 2 500 cfu/sample)
Year of interlaboratory test	2014	2014	2014
Number of laboratories having returned results	14	14	14
Number of samples per laboratory	8	8	8
Number of excluded laboratories	0	0	0
Number of laboratories retained after the exclusion	14	14	14
Number of accepted samples	112	112	112
Sensitivity in %	–	75,9	96,4
Specificity in %	99,1	–	–

Bibliography

Add the following reference.

[7] NMKL N. 164:1999, *Escherichia coli* O157. Detection in food and feeding stuffs

Appendix ZZ
(normative)

Variations to ISO 16654: 2001 for Australia

ZZ.1 Scope

This Appendix lists the normative variations to ISO 16654: 2001.

ZZ.2 Variations

The following modifications are required for Australian conditions:

Element	Instruction / New text
Cl 2	1 After the first paragraph, *add* the following: The Australian Standards listed below are modified adoptions of, or not equivalent to, the ISO normative references and are required for the application of this Standard. All references in the source text to those ISO normative references shall be replaced by references to the corresponding Australian Standards. Australian or Australian/New Zealand Standards that are identical adoptions of international normative references may be used interchangeably. 2 *Delete* "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*" and *replace* with the following: AS 5013.11.1, *Food microbiology, Method 11.1: Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — General rules for the preparation of the initial suspension and decimal dilutions (ISO 6887-1:2017, MOD)* 3 *Delete* "ISO 7218, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*" and *replace* with the following: AS 5013.14.1, *Food microbiology, Method 14.1: Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations (ISO 7218:2007, MOD)* 4 After "AS 5013.14.1, *Food microbiology, Method 14.1: Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations (ISO 7218:2007, MOD)*", *add* the following: AS 5140, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media (ISO 11133:2014, MOD)*
Cl 6.10	*Delete* text and *replace* with the following: **Loops (of diameter approximately 3 mm) and wires**, made of platinum/iridium or nickel/chromium, or **glass rods**, or equivalent sterile **disposable loops** or **inoculating needles**.
Cl 6.15	*Delete* text and *replace* with the following: **Petri dishes**, for example, of diameter 90 mm.
Cl 7	*Delete* text and *replace* with the following: Sampling is not part of the method specified in this document. Refer to the specific International Standard dealing with the product concerned. Where there is no specific International Standard dealing with the sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject. Recommended sampling techniques are given in the following:

Element	Instruction / New text
	(a) ISO/TS 17728 for food and animal feed. (b) ISO 13307 for primary production stage. (c) ISO 17604 for carcasses. It is recommended to cool the sample quickly before storage. It is important that the laboratory receives a sample that is truly representative and has not been damaged or changed during transport or storage.
Cl 9.1	*Delete* NOTE and *replace* with the following: NOTE AS 5013.11.2, AS 5013.11.3, AS 5013.11.4, AS 5013.11.5 and ISO 6887-6 cover the preparation of the initial suspension and decimal dilutions for a range of products.
Cl 9.5	1 Delete "NOTE" and *replace* with "NOTE 1". 2 After Note 1, *add* the following: NOTE 2 Other procedures may be used to confirm and characterize *E. coli* O157, provided that the suitability of the alternative procedure has been validated (refer to AS 5013.14.1).
Cl 10.1	After paragraph, *add* the following: The following reference cultures shall be used: (a) *Escherichia coli* O157:H7 WDCM 00014 (non-toxigenic). (b) *Staphyloccus aureus* WDCM 00032 or 00034. (c) *Escherichia coli* WDCM 00012 or 00013. Reference cultures shall be subjected to the test procedure at the same time as the test samples. A test shall be regarded as invalid unless the reference culture gives the appropriate results.
Cl 10.3 (new)	**10.3 Performance testing for the quality assurance of the culture medium** Table 1 shows the performance criteria of cefixime tellurite sorbitol MacConkey agar (CT-SMAC). Refer to AS 5140 for the definitions of selectivity and productivity.

Table 1 — Performance Criteria of Cefixime Tellurite Sorbitol MacConkey Agar (CT-SMAC)

Medium	Type	Function	Incubation	Control strains	Strain number	Ref media	Method of control	Criteria[c]	Characteristic reactions
CT-SMAC	Solid	Productivity	(21 ± 3) h/ (37 ± 1)°C	*Escherichia coli* O157:H7	WDCM[a] 00014 (non-toxigenic strain)	—	Qualitative	Good growth (2)	Transparent colonies with a pale yellowish-brown appearance and diameter ~1 mm
		Selectivity		*Staphylococcus aureus*	WDCM[a] 00032 [b] or 00034[b]	—	Qualitative	Total inhibition (0)	—
				Escherichia coli	WDCM[a] 00012 [b] or 00013[b]	—		Partial inhibition (1)	Growth of some pink colonies

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

[b] Either strain may be chosen; one of the strains shall be used as a minimum.

[C] Growth is categorized as: 0 - No growth; 1 - Weak growth; 2 - Good growth.

Cl 13	*Delete* list and *replace* with the following:

(a) all information necessary for the complete identification of the sample;

(b) the sampling method used, if known;

(c) the test method used, with reference to this document, i.e. AS 5013.26;

(d) the size of the test portion;

(e) any deviations from the method (e.g. in the media or the incubation conditions used);

(f) all operating details not specified in this Standard, or regarded as optional, together with details of any incidents which may have influenced the results; and

(g) the test results obtained.

Bibliography *Delete* text and *replace* with the following:

[1] DOYLE M.P. and SCHOENI J.L. Appl. Environ. Microbiol., 53, 1987, pp. 2394-2396

[2] ZADIK P.M., CHAPMAN P.A. and SIDDONS C.A. J. Med. Microbiol., 39, 1993, pp. 155-158

[3] AS 5013.11.2, *Food Microbiology, Method 11.2: Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of meat and meat products*

[4] AS 5013.11.3, *Food microbiology, Method 11.3: Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of fish and fishery products (ISO 6887-3:2003, MOD)*

[5] AS 5013.11.4, *Food Microbiology, Method 11.4: Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of miscellaneous products (ISO 6887-4:2017, MOD)*

[6] AS 5013.11.5, *Food microbiology, Method 11.5: Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of milk and milk products (ISO 6887-5:2010, MOD)*

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

[8] ISO 13307, *Microbiology of food and animal feed — Primary production stage — Sampling techniques*

[9] ISO 17604, *Microbiology of the food chain—Carcass sampling for microbiological analysis*

[10] ISO/TS 17728, *Microbiology of the food chain — Sampling techniques for microbiological analysis of food and feed samples*

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