Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

Microbiology Services
Food Water and Environmental Microbiology
Standard Method

FNES22 (F19)

Issued by
PHE Microbiology Services
Food, Water & Environmental Microbiology
Methods Working Group
About Public Health England

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## Contents

About Public Health England .......................................................... 2  
Contents .......................................................................................... 3  
Status of Microbiology Services Food, Water and Environmental Microbiology Methods .......... 4  
Amendment history ...................................................................... 5  
Introduction .................................................................................. 6  
  Scope .......................................................................................... 6  
  Background ................................................................................. 6  
1.0 Principle .................................................................................. 9  
2.0 Definitions .............................................................................. 10  
3.0 Safety considerations ............................................................... 10  
  3.1 General safety considerations .................................................. 10  
  3.2 Specific safety considerations .................................................. 11  
  3.3 Laboratory containment .......................................................... 11  
4.0 Equipment .............................................................................. 11  
5.0 Culture media and reagents ...................................................... 12  
6.0 Sample processing ................................................................... 14  
  6.1 Sample preparation, inoculation and incubation for detection ........................................... 14  
  6.2 Sample preparation, inoculation and incubation for enumeration ..................................... 15  
  6.3 Recognition and counting of colonies ............................................................................ 15  
  6.4 Confirmatory tests .................................................................. 16  
7.0 Quality control ......................................................................... 18  
8.0 Calculation of results ............................................................... 18  
  8.1 Calculation of results from routine samples .................................................................. 18  
  8.2 Calculation of results from formal or official control samples ........................................... 18  
  8.3 Estimation of counts in formal or official control sample (low numbers) ............................ 19  
9.0 Reporting of results ................................................................ 20  
  9.1 Detection .............................................................................. 20  
  9.2 Enumeration ......................................................................... 21  
  9.3 Detection and enumeration ......................................................... 22  
10.0 Reference facilities and referral of cultures .................................. 22  
11.0 Acknowledgements and contacts ............................................. 23  
References ...................................................................................... 24  
Appendix: Flowchart showing the process for detection and enumeration of *Listeria monocytogenes* and other *Listeria* species .................................................. 26
Status of Microbiology Services Food, Water and Environmental Microbiology Methods

These methods are well referenced and represent a good minimum standard for food, water and environmental microbiology. However, in using Standard Methods, laboratories should take account of local requirements and it may be necessary to undertake additional investigations.

The performance of a standard method depends on the quality of reagents, equipment, commercial and in-house test procedures. Laboratories should ensure that these have been validated and shown to be fit for purpose. Internal and external quality assurance procedures should also be in place.

Whereas every care has been taken in the preparation of this publication, Public Health England (PHE) cannot be responsible for the accuracy of any statement or representation made or the consequences arising from the use of or alteration to any information contained in it. These procedures are intended solely as a general resource for practising professionals in the field, operating in the UK, and specialist advice should be obtained where necessary. If you make any changes to this publication, it must be made clear where changes have been made to the original document. PHE should at all times be acknowledged.

Citation for this document:
Amendment history

<table>
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<tr>
<th>Amendment Number/ Date</th>
<th>Issue no. Discarded</th>
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<th>Section(s) involved</th>
<th>Amendment</th>
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<tr>
<td>2/21.03.14</td>
<td>HPA F19 1.1</td>
<td>PHE FNES22 (F19) 2</td>
<td>All</td>
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<td>5</td>
<td>Background</td>
<td>Update of ISO reference and tables amended to clarify justification for not testing all samples in duplicate</td>
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<td>8</td>
<td>3.1 General Safety considerations</td>
<td>Information note added</td>
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<td>9</td>
<td>5.0 Media and Reagents</td>
<td>Title update and section update to reflect the use of different biochemical galleries. PCR reagents added</td>
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<td>13</td>
<td>6.4 Confirmatory Tests</td>
<td>Stages separated. Update to include use of API and Microgen biochemical kits. Update to include optional use of PCR for colony confirmation</td>
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<td>14</td>
<td>8.0 Calculation of results</td>
<td>Updated to allow calculation of weighted mean results and estimates and reference to Starlims included</td>
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<td>16</td>
<td>9.0 Reporting of results</td>
<td>Reference to Starlims included</td>
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<td>19</td>
<td>References</td>
<td>Updated</td>
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<td>20</td>
<td>Flowchart</td>
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The amendment history is shown below. On issue of revised or new documents each controlled document should be updated by the copyholder in the laboratory.
Introduction

Scope

The method described is applicable to the detection and enumeration of *Listeria monocytogenes* and other *Listeria* species in all food types including milk and dairy products and in environmental samples.

In general, the lower limit of enumeration of this method is 1 or 2 colony forming units (CFU) per millilitre (mL) of sample for liquid products, or 10/20 CFU per gram (g) of sample for other products.

Background

European legislation containing microbiological food safety criteria for *L. monocytogenes* either specify absence in 25 g of sample or a level below 100 CFU per g at any point in the shelf life of the ready-to-eat food. *L. monocytogenes* results exceeding the food safety criteria are judged to be legally unsatisfactory. There is also a requirement for producers of ready-to-eat foods that may pose a *L. monocytogenes* risk to public health to sample the food processing areas and equipment for the presence of *L. monocytogenes* as part of their sampling scheme.

Current guidelines for assessing the microbiological safety of ready-to-eat foods contain guideline criteria for total *Listeria* species and *Listeria monocytogenes*. The presence of species of *Listeria* other than *L. monocytogenes* is used to indicate the likelihood that *L. monocytogenes* may also be present in other parts of the batch of food or food processing environment. Samples containing more than 100 CFU per g of *Listeria* species are considered unsatisfactory and their presence above this level requires investigation. The presence of more than 100 CFU per g of *L.monocytogenes* is considered to be potentially injurious to health and requires immediate investigation.

In order to assess the level of contamination in these foods direct enumeration of the organism is carried out on solid selective media. In some ready-to-eat foods such as soft ripened cheeses, pâtés and vacuum or modified atmosphere packed cooked meats with a long assigned shelf life, the very presence of *Listeria* is significant due to the organism’s ability to multiply to significant levels during refrigerated storage. For these foods, an enrichment procedure is also required to determine presence or absence in a defined quantity of food.

The method described is based on EN ISO 11290-1: 1996+A1:2004 and BS EN ISO 11290-2:1998+A1:2004. These are internationally recognised horizontal methods for the detection and enumeration of *L. monocytogenes*. A *Listeria* chromogenic isolation medium is used that results in the formation of blue-green colonies by *Listeria* species due to the β-glucosidase activity of these bacteria. Further distinction between the species is obtained by the inclusion of phosphatidylinositol which is hydrolysed by the phospholipase enzyme produced by *L. monocytogenes* and *L. ivanovii* but not other *Listeria* species to produce an
opaque halo around the colony. Some strains of *L. monocytogenes* can take up to four days to develop an opaque halo.


These differences in methodology are described in the tables below:

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Whole Method</td>
<td>All <em>Listeria</em> species including <em>L. monocytogenes</em></td>
<td><em>L. monocytogenes</em> only</td>
<td>Other species act as indicators of the potential for the presence of <em>L. monocytogenes</em> and are discussed in HPA RTE Guidelines 2009³</td>
</tr>
<tr>
<td>Definitions</td>
<td>Includes a definition of <em>Listeria</em> species</td>
<td>Only covers a definition for <em>Listeria monocytogenes</em></td>
<td><em>Listeria</em> species are important indicators of poor practices if detected in foods.</td>
</tr>
<tr>
<td>Sample preparation</td>
<td>Preparation of suspension in PSD and BPW</td>
<td>Preparation of suspension in BPW or ½ Fraser Broth without supplements</td>
<td>This ISO is out of step with other ISO methods and does not comply with ISO 6887-1:1999 for preparation of samples.</td>
</tr>
<tr>
<td>Sample preparation for enumeration</td>
<td>1 h resuscitation in BPW not indicated</td>
<td>Requires a 1 h resuscitation in BPW at 20°C</td>
<td>No other enumeration tests require this resuscitation and <em>Listeria</em> are harder than other organisms sought by enumeration. ISO 6887 specifies inoculation within 45 minutes of preparation of the suspension.</td>
</tr>
<tr>
<td>Culture media</td>
<td>Media formulation specified with option for use of ALOA or OCLA(ISO)</td>
<td>ALOA specified in ISO with ability to use other formulations if validated</td>
<td>OCLA (ISO) has the same formulation as ALOA.</td>
</tr>
<tr>
<td>Incubation of chromogenic plates</td>
<td>Requires 48 h incubation</td>
<td>Requires 24 h with a further 24 h if growth is week or no colonies.</td>
<td>Rare strains may not produce an opaque halo until they have been incubated for 4 days. The PHE method however takes all blue green colonies forward regardless of halo development and this would enable identification of atypical <em>L. monocytogenes</em> strains</td>
</tr>
</tbody>
</table>

³ HPRA RTE Guidelines 2009
Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

<table>
<thead>
<tr>
<th>Environmental samples</th>
<th>PHE method F19</th>
<th>EN ISO 11290-1:1996+A1:2004</th>
<th>Justification for variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Describes the processing of environmental swabs as part of the method.</td>
<td>Only applicable to products intended for human or animal consumption</td>
<td>Detection of <em>Listeria</em> species is considered to be an important tool in monitoring the food processing environment</td>
</tr>
</tbody>
</table>

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<tbody>
<tr>
<td>Volume sub-cultured to the plates is defined as a minimum of 10µL.</td>
<td>Subculture from enrichment broths using a 3mm loop (ie 1µL)</td>
<td>High levels will have been produced during enrichment. The volume sub-cultured using the PHE method is spread to ensure discrete colonies.</td>
<td></td>
</tr>
</tbody>
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</thead>
<tbody>
<tr>
<td>Sub-cultures are made to Columbia Blood Agar.</td>
<td>Sub-culture to TSBYE is recommended</td>
<td>Use of BA permits detection of haemolysis at an earlier stage and a pure culture for biochemical tests is still produced.</td>
<td></td>
</tr>
</tbody>
</table>

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<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Enumeration 0.5mL or 0.5mL in duplicate for official control work</td>
<td>0.1 mL per plate in duplicate or 1mL on a 140mm plate in duplicate or over 3 90mm plates in duplicate.</td>
<td>The use of duplicate plates at each dilution to achieve a weighted mean is not considered essential where the focus is on identifying bacterial levels that pose a risk to public health. The impact of plating variation is addressed by determining method uncertainty. Official control samples that have been submitted strictly in accordance with sampling plans and formal samples are tested in duplicate and weighted mean counts determined because the methodology used in these circumstances is liable to challenge in a court of law</td>
</tr>
</tbody>
</table>

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</thead>
<tbody>
<tr>
<td>Spiral plater used</td>
<td>Above procedures used for serial dilutions.</td>
<td>Spiral plater widely used in PHE procedures</td>
</tr>
</tbody>
</table>

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<tbody>
<tr>
<td>Horse BA used</td>
<td>Sheep BA used</td>
<td>No evidence based on IQC EQA result that the procedure is adversely</td>
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Effective Date 21.03.14
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Page 8 of 27
Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

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<tbody>
<tr>
<td></td>
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<td>affected by use of horse blood as an alternative to sheep blood. Horse blood agar is readily available commercially while sheep blood agar is not.</td>
</tr>
<tr>
<td>Confirmations</td>
<td>Commercial gallery permitted</td>
<td>Galleries not used</td>
<td>Many labs used these for biochemical identification of bacteria. General permission to use these galleries by ISO.</td>
</tr>
<tr>
<td>Confirmations</td>
<td>PCR may be performed</td>
<td>PCR not included</td>
<td>PCR confirmation using a UKAS accredited method allow confirmation on same day.</td>
</tr>
<tr>
<td>Confirmation</td>
<td>Options for confirmation include the use of biochemical galleries or PCR</td>
<td>Catalase test, Gram stain test, Motility test, Camp test and specific sugars utilisation tests.</td>
<td>Biochemical galleries in combination with colony morphology characteristics are sufficient to give additional assurance of isolate identity. Gram stain is only performed when colony morphology on CBA is atypical for Listeria.</td>
</tr>
<tr>
<td>Reference testing</td>
<td>All isolates of <em>L.</em> <em>monocytogenes</em> are sent for serotyping and further epidemiological characterisation.</td>
<td>Isolate may be sent for definitive confirmation.</td>
<td>PHE surveillance requirement.</td>
</tr>
</tbody>
</table>

### 1.0 Principle

In foods or swabs that require presence/absence testing or where low numbers of organisms in foods may be significant, detection of *L. monocytogenes* and other *Listeria* species necessitates a primary enrichment at 30°C for 24 h in a selective enrichment broth containing half the normal concentration of nalidixic acid and acriflavine. This is followed by secondary enrichment in the same selective enrichment broth containing the full concentration of selective agents with incubation at 37°C for up to 48 h. Sub-culture to two selective agar media are made from both enrichment stages. The selective agars are examined for the presence of typical colonies and identification of the species by means of morphological, biochemical or molecular tests.

The enumeration of *L. monocytogenes* and other *Listeria* species by this method involves inoculation of the surface of a selective agar media with a specified volume of a 10⁻¹ and
Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

other appropriate decimal dilutions of the test sample. Listeria chromogenic agar plates are incubated at 37°C for up to 48 h. Calculation of the number of CFU per gram (g) or millilitre (mL) of sample for either *L. monocytogenes* or total *Listeria* species is made from the number of typical colonies obtained on the selective media, and subsequently confirmed by morphological, biochemical or molecular tests.

### 2.0 Definitions

For the purpose of this method, the following definitions apply:

*Listeria species*
Micro-organisms which form typical colonies on solid selective media, and which display the morphological and biochemical characteristics described in this method or confirm by molecular testing.

*Listeria monocytogenes*
Micro-organisms that conform to the above definition for *Listeria* species, usually display β-haemolysis on horse blood agar, gives rise to an acceptable profile with a *Listeria* biochemical gallery or molecular test kit.

*Detection of L. monocytogenes and other Listeria species*
Determination of the presence or absence of these micro-organisms in a defined weight or volume of food or dairy product or in an environmental sample.

*Enumeration of L. monocytogenes and other Listeria species*
Determination of the number of these micro-organisms per gram or mL food or dairy product.

### 3.0 Safety considerations

#### 3.1 General safety considerations

Normal microbiology laboratory precautions apply. All laboratory activities associated with this SOP must be risk assessed to identify hazards. Appropriate controls must be in place to reduce the risk to staff or other groups. Staff must be trained to performed the activities described and must be provided with any personal protective equipment (PPE) specified in this method. Review of this method must also include a review of the associated risk assessment to ensure that controls are still appropriate and effective. Risk assessments are site specific and are managed within safety organiser.
Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

**Information Note:** Throughout this method hazards are identified using red text. Where a means of controlling a hazard has been identified this is shown in green text.

### 3.2 Specific safety considerations

Pregnant women should not be allowed to handle cultures of *L. monocytogenes*. Women known to be pregnant or who think that they may be should be excluded from working with known cultures of *Listeria monocytogenes*. Zym B is toxic and may impair fertility and cause harm to the unborn child. Infection caused by *L. monocytogenes* in pregnancy is rare but can result in complications including miscarriage and neonatal infection depending on the trimester when infection occurs. A specific risk assessment must be performed in the event of notification of pregnancy and adjustments made to enable pregnant staff to avoid exposure to these organisms and Zym B reagent.

### 3.3 Laboratory containment

All samples and cultures are handled in a containment level 2 (CL2) laboratory.

### 4.0 Equipment

- Top pan balance capable of weighing to 0.1g
- Gravimetric diluter (optional)
- Stomacher
- Vortex mixer
- Incubator: 30 ± 1°C
- Incubator: 37 ± 1°C
- Colony Counter (optional)
- Spiral plater (optional)
- Stomacher bags (sterile)
- Automatic pipettors and associated sterile pipette tips capable of delivering up to 10 mL and 1 mL volumes (optional)
- 10µL loops or cotton tipped swabs
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)
- Light microscope: x 40 objective
- PCR equipment as specified in method M2\(^{15}\).
5.0 Culture media and reagents

Equivalent commercial dehydrated media may be used; follow the manufacturer’s instructions.

**Peptone saline diluent (Maximum recovery diluent)**

Peptone 1.0 g  
Sodium chloride 8.5 g  
Water 1L  
PpH 7.0 ± 0.2 at 25°C

**Buffered peptone water (ISO formulation)**

Enzymatic digest of casein 10.0 g  
Sodium chloride 5.0 g  
Disodium hydrogen phosphate dodecahydrate 9.0 g  
or anhydrous disodium hydrogen phosphate 3.5 g  
Potassium di-hydrogen phosphate 1.5 g  
Water 1L  
PpH 7.0 ± 0.2 at 25°C

**Half Fraser and Fraser broth**

<table>
<thead>
<tr>
<th></th>
<th>Half Fraser</th>
<th>Fraser</th>
</tr>
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<tbody>
<tr>
<td>Proteose peptone</td>
<td>5.0 g</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>5.0 g</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Meat extract</td>
<td>5.0 g</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0 g</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>20.0 g</td>
<td>20.0 g</td>
</tr>
<tr>
<td><em>d</em>-Sodium hydrogen phosphate</td>
<td>12.0 g</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Potassium di-hydrogen phosphate</td>
<td>1.35 g</td>
<td>1.35 g</td>
</tr>
<tr>
<td>Aesculin</td>
<td>1.0g</td>
<td>1.0g</td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>3.0 g</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.5 g</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>10 mg</td>
<td>20 mg</td>
</tr>
<tr>
<td>Acriflavine hydrochloride</td>
<td>12.5 mg</td>
<td>25 mg</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
<td>1 L</td>
</tr>
</tbody>
</table>

pH 7.2 ± 0.2 at 25°C

**Horse blood agar**

**Columbia agar with 5 % horse blood**
Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

*Listeria Chromogenic Agar* (ALOA or OCLA ISO Formulation)

- Enzymatic digest of animal tissues: 18.0 g
- Enzymatic digest of casein: 6.0 g
- Yeast extract: 10.0 g
- Sodium pyruvate: 2.0 g
- Glucose: 2.0 g
- Magnesium glycerophosphate: 1.0 g
- Magnesium sulphate (anhydrous): 0.5 g
- Sodium chloride: 5.0 g
- Lithium chloride: 10.0 g
- *di*-Sodium hydrogen phosphate (anhydrous): 2.5 g
- L-α-Phosphatidylinositol: 2.0 g
- 5-Bromo-4-chloro-3-indolyl-β-D-glucopyranoside: 0.05 g
- Amphotericin B: 0.01 g
- Nalidixic acid sodium salt: 0.02 g
- Ceftazidime: 0.02 g
- Polymixin B sulphate: 76,700 IU
- Agar: 12 - 18.0 g
- Water: 1 L

pH 7.2 ± 0.2 at 25°C

*Listeria selective agar* (Oxford agar)

- Columbia blood agar base: 39.0 g
- Aesculin: 1.0 g
- Ferric ammonium citrate: 0.5 g
- Lithium chloride: 15.0 g
- Cycloheximide: 0.4 g
- or Amphotericin B: 0.01 g
- Colistin sulphate: 20.0 mg
- Acriflavine: 5.0 mg
- Cefotetan: 2.0 mg
- Fosfomycin: 10.0 mg
- Water: 1 L

pH 7.0 ± 0.2 at 25°C

**Gram stain reagents**

*Biochemical gallery* eg *BioMerieux API Listeria* or equivalent validated test kit

**PCR testing reagents**

*Reagents as specified in M2\textsuperscript{15} and M3\textsuperscript{14} are used*

**Note:** Additional diluents may be required for dairy products please refer to SOP D1\textsuperscript{11} for media formulations.
6.0 Sample processing

6.1 Sample preparation, inoculation and incubation for detection

Enrichment is necessary for presence/absence testing of environmental swabs and food samples such as pâté, vacuum or modified atmosphere packed products with extended shelf-life and foods intended for infants. Enrichment should also be considered for any ready-to-eat foods that are able to support the growth of *L. monocytogenes* if they have been sampled from a processing premises where there is no evidence that shelf-life testing has been done to confirm that less than 100 CFU per g is maintained throughout the products shelf-life. Samples of products likely to be served to vulnerable groups including products from premises supplying foods to healthcare settings or where there is a Public Health concern should also be tested.

Using sterile instruments and aseptic technique, weigh a representative 25 g sample of food into a sterile stomacher bag. Add nine times that weight or volume of half Fraser broth and homogenise for between 30 seconds and 3 minutes in a stomacher. The homogenisation time required will depend on the manufacturer instructions and the type of sample being examined. Record the weight of sample and the weight or volume of half Fraser broth used. If the amount of food product available is less than 25 g or mL maintain the sample to diluent volume ratio at 1:9 (ie 10^{-1} dilution).

For environmental swabs, ensure that the swab is completely immersed in half Fraser broth, such that an approximate 1 in 10 dilution is achieved. Vortex or stomach to bring the organisms into suspension. Transfer the homogenate or swab suspension into a container capable of closure.

Place the primary enrichment (half Fraser) broth in an incubator at 30 ± 1°C for 24 ± 3 h.

Sub-culture 0.1 mL of the incubated primary enrichment (half Fraser) broth to 10 mL of secondary enrichment (Fraser broth) and place in an incubator at 37 ± 1°C for 48 ± 3 h and also sub-culture the primary enrichment (half Fraser) broth to Listeria chromogenic agar and oxford agar in order to achieve single colonies.

After incubation at 37 ± 1°C for 48 ± 3 h sub-culture the secondary enrichment (Fraser broth) cultures to Listeria chromogenic agar and Oxford agar plates in order to achieve single colonies.

Invert the inoculated plates so that the bottom is uppermost and place them in an incubator at 37 ± 1°C for 24 ± 3 h and a further 24 ± 3 h.
6.2 Sample preparation, inoculation and incubation for enumeration

Following the procedure described in Standard Method F2\textsuperscript{10} – Preparation of Samples and Dilutions prepare a $10^{-1}$ homogenate of the sample in either peptone saline diluent (PSD) or buffered peptone water (BPW) and further decimal dilutions as required in PSD. – Preparation of Samples and Decimal Dilutions. For swabs refer to Standard Method E1- Detection and Enumeration of Bacteria in Swabs and Other Environmental Materials\textsuperscript{11}.

Homogenise for between 30 seconds and 3 minutes in a stomacher. The homogenisation time required will depend on the manufacturer instructions and the type of sample being examined.

Inoculate 0.5 mL of the $10^{-1}$ homogenate onto the surface of a Listeria chromogenic agar plate. Carefully spread the inoculum as soon as possible over the surface of the plate using a sterile spreader without touching the sides of the plate with the spread. If the sample has been collected for the purpose of Official Control, as part of a formal investigation or is associated with an outbreak of infection inoculate 0.5 mL of the $10^{-1}$ homogenate to the surface of two Listeria chromogenic agar plates. If counts are expected to be high use a spiral plater to inoculate 50 $\mu$L of the $10^{-7}$ and $10^{-3}$ dilutions onto Listeria chromogenic agar plates.

Plating of the medium with the test portion must be performed within 45 minutes of preparation of the sample homogenate.

Leave the plates on the bench for approximately 15 minutes to allow absorption of the inoculum into the agar. Invert the inoculated plates so that the bottom is uppermost and place them in an incubator at 37 ± 1°C for 24 ± 3 h and then after a further 24 ± 3 h.

6.3 Recognition and counting of colonies

\textbf{Safety Note}
\textit{Pregnant staff should not be involved further.}

6.3.1 Colony recognition
Plates must be examined at 24 ± 3h and after a further 24 ± 3h.

\textit{Listeria chromogenic agar}
Colonies of \textit{Listeria} appear blue or blue-green. Typical colonies of \textit{L. monocytogenes} are surrounded by an opaque halo after 24 h; this halo may be weak or slow to develop if the organism is stressed, particularly acid-stressed. Strains of \textit{L. ivanovii} also develop an opaque halo, but within 48 h. Other species of \textit{Listeria} do not develop a halo. Blue colonies may also be formed by other species such as \textit{Bacillus}, \textit{Carnobacterium}, staphylococci and streptococci.

\textit{Oxford agar}
Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

After 24 h colonies of *Listeria* appear small, 1 mm in diameter, greyish surrounded by black halos (aesculin positive). After 48 h colonies become darker, sometimes with a greenish sheen, and are about 2 mm in diameter with black halos and often sunken centres.

6.3.2 Counting of colonies from the enumeration method

For enumeration, use plates containing up to 150 colonies (if possible). If more than one colonial type is present on enumeration plates perform a differential count. If colonies with zones are present after 24 h perform a count as zones may increase in size during further incubation making counting difficult.

*Spiral Plating*

A minimum of 20 colonies must be counted in each segment. Count the number of colonies on the plates either manually in conjunction with a viewing grid or using an automated colony counter.

If counting manually, centre the plate over the counting grid. Choose any segment and count the colonies from the outer edge into the centre until 20 colonies have been counted. Continue to count the remaining colonies in the subdivision of the segment containing the twentieth colony. For colonies on the dividing line count the colonies on the outermost line of the segment and on one side only. Record this count together with the number assigned to the subdivision of the segment. Count in the same area on the opposite side of the plate and record the count. Calculate the count per g or mL of dilution plated by adding together the counts from the two segments and dividing the total by the volume constant for the segment counted. Alternatively, use the tables supplied by the manufacturer.

6.4 Confirmatory tests

Sub-culture up to five presumptive *Listeria* colonies of each morphological type to horse blood agar from *Listeria chromogenic agar(s)* (or all colonies if less than five are present) and perform confirmatory tests as described below.

If *L. monocytogenes* has not been detected (either through absence of typical colonies or through confirmatory tests yielding *Listeria* species other than *L. monocytogenes*), sub-culture five colonies (or all if less than five are present) from each of the sub-culture plates made from the primary and secondary enrichment broths. Examine carefully for different morphological appearances; if present sub-culture at least one representative of each type.

Where the *Listeria* chromogenic agar or the Oxford agar is overgrown colonies for further confirmation must be taken from the plate that is not overgrown. Where both media types are overgrown the test must be reported as void.
Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

If none of these colonies are confirmed as *L. monocytogenes*, but some or all of them are confirmed as *Listeria* species, a final count of *Listeria* species can be calculated.

If the presence of *L. monocytogenes* has already been confirmed by enumeration, no further work need be carried out from the enrichment broth sub-culture plates unless an epidemiological investigation is being carried out in which a specific strain is being sought.

**Colony morphology and presence of haemolysis**

Sub-culture each presumptive *Listeria* colony selected to horse blood agar by first performing a single stab inoculation (to facilitate haemolysis detection) followed by separate streaking to demonstrate purity and to give discrete colonies. If confirming using PCR the loop is then carefully emulsified in 0.5 mL of PCR grade water. Incubate plates at 37 ± 1°C for 24 ± 3 h and examine for purity, colonial morphology and presence of β-haemolysis. Record the haemolysis results. Almost all strains of *L. monocytogenes* are haemolytic. Strains of *L. ivanovii* are strongly haemolytic and *L. seeligeri* are weakly haemolytic. Other species are non-haemolytic including *L. innocua*.

**Information Note:** Some strains of *L. monocytogenes* appear non-haemolytic on horse blood agar and if found should be sent to a reference laboratory with the result of the biochemical or molecular test.

Select pure cultures of different morphological colony types for confirmation. If colonial morphology appears atypical perform a gram stain. Wearing gloves and safety glasses perform a Gram stain if required to verify the nature of the isolates. *Listeria* species are Gram-positive, pleomorphic, non-sporing slender rods that are non-pigmented on horse blood agar.

**Biochemical confirmation (optional)**

Wearing gloves and safety glasses and For each morphological type perform biochemical testing with a biochemical gallery eg API Listeria identification system or Microgen Listeria ID following the manufacturer’s instructions.

**Information Note:** If using API Listeria, Zym B reagent is sensitive to light and deteriorates rapidly, leading to false positive reactions. Store under refrigerated conditions, protect the reagent from light and minimise the length of time that the reagent is held at ambient temperature. Do not exceed the shelf life recommended by the manufacturer.

**Information Note:** If performing the Micogen Listeria ID assay, it is recommended that isolates that look typical for *L. monocytogenes* on OCLA (ie halo) that confirm as *L. innocua* (ie non-haemolytic) should be sent to the reference laboratory for further investigation.

**Colony confirmation using PCR (optional)**

Following method M312 heat treat the PCR grade water with emulsified colonies at 95°C for 15 minutes allow to cool and add 30 µL of heat-treated bacterial suspension to lyophilised
real time PCR assay tubes as described in Standard Method M2. The positive control described in Standard Method M4 should be included in each real time PCR assays.

7.0 Quality control

Further quality control of media and internal quality assurance checks should be performed according to in-house procedures using the following test strains:

Positive control:
- *Listeria monocytogenes* NCTC 11994
- *Listeria innocua* NCTC 11288

Negative control:
- *Enterococcus faecalis* NCTC 775

8.0 Calculation of results

Calculations occur automatically in the STARLIMS system as describe in Method FNES6 (Q12) Sample processing and result entry in STARLIMS. Calculations are performed as described below.

8.1 Calculation of results from routine samples

Calculate the number of *Listeria* spp per gram or mL as follows:

\[
\text{Count per g/mL} = \frac{\text{No. of colonies confirmed}}{\text{No. of colonies tested}} \times \frac{\text{Presumptive count}}{\text{Volume tested x dilution}}
\]

8.2 Calculation of results from formal or official control samples

For a result to be valid, it is considered necessary to count at least one dish containing a minimum of 15 colonies. Calculate the confirmed count for each plate as described in 8.1 above.

Use these confirmed counts to calculate N, the confirmed *Listeria* spp present in the test sample per millilitre or per gram, as the weighted mean from two successive dilutions using the following equation:
Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

\[
N = \frac{\Sigma a}{V (n_1 + 0.1n_2) d}
\]

when:
- \(\Sigma a\) is the sum of the colonies counted on all the plates retained from two successive dilutions, at least one of which contains a minimum of 15 CFU.
- \(n_1\) is the number of plates counted at the first dilution.
- \(n_2\) is the number of plates counted at the second dilution.
- \(d\) is the dilution from which the first counts were obtained \([d = 1\text{ in the case (liquid products) where the directly inoculated test sample is retained}]\).
- \(V\) is the volume of the inoculum, in millilitres, applied to each plate.

Round the results to two significant figures.

### 8.3 Estimation of counts in formal or official control sample (low numbers)

If both dishes at the level of the first retained dilution contain less than 15 confirmed colonies, calculate \(N_E\), the estimated number of confirmed *Listeria* spp present in the test sample, as the arithmetical mean from two parallel plates using the following equation:

\[
N_E = \frac{\Sigma a}{V \cdot n \cdot d}
\]

when:
- \(\Sigma a\) is the sum of the confirmed colonies counted on the two plates.
- \(n\) is the number of plates retained.
- \(d\) is the dilution factor to the initial suspension or the first inoculated or retained dilution \([d = 1\text{ in the case of liquid products where the directly inoculated test sample is retained}]\).
- \(V\) is the volume of the inoculum, in millilitres, applied to each plate.
Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

### 9.0 Reporting of results

All results are reported using the STARLIMS system as described in method Q13 Technical Validation and release of result in STARLIMS\(^6\). The test report specifies the method used, all details necessary for complete identification of the sample and details of any incidents that may have influenced the result. Report all *Listeria* organisms including *L. monocytogenes* as *Listeria* spp (total). If *L.monocytogenes* is detected report this separately.

#### 9.1 Detection

If *Listeria* species are not isolated by detection report as:

*Listeria* species (total) Not Detected in 25 g or 25 mL or sample.

If *Listeria* species are isolated by detection but enumeration has not been performed report as:

*Listeria* species (total) DETECTED in 25 g or 25 mL or sample.

Also report the identity of the species.

Listeria identified as *L.* (insert species name).

If *L.monocytogenes* is not found, also report this separately as described below.

* *L.monocytogenes* Not Detected in 25 g or 25 mL or sample.

If any colonies are confirmed as *Listeria monocytogenes* report as:

*L. monocytogenes* DETECTED in 25 g

**Information Note:** Where enrichment culture has been performed the actual weight of sample examined must be reported, for example, 10g, 25g or 100g.
9.2 Enumeration

If Listeria species are not detected by enumeration report as follows:

**Liquid products**
Where plates have been prepared from the undiluted \(10^0\) product are found to contain no colonies, report the result as

**Listeria species (total) Not Detected per mL.**

**Solid food products**
Where plates have been prepared from the \(10^{-1}\) dilution of the product contain no colonies report the result as

**Listeria species (total) Less than 10 CFU per g or mL**
(2 x 0.5 ml surface spread using a \(10^{-1}\) dilution)

OR

**Listeria species (total) Less than 20 CFU per g or mL**
(1 x 0.5 ml surface spread using a \(10^{-1}\) dilution)

If *Listeria* species including *L. monocytogenes* are found by enumeration, report the total count as *Listeria* species (total) CFU per g or mL. Also report the count of *L. monocytogenes* separately as a CFU per g or mL.

If the count is 100 or more, report counts with one figure before and one figure after the decimal point in the form of:

\[ a \times 10^b \text{ CFU per g or mL} \]

where \(a\) is never less than 1.0 or greater than 9.9 and \(b\) represents the appropriate power of ten. Round counts up if the last figure is 5 or more, round counts down if the last figure is 4 or less.

\[ \text{e.g.: } 1920 \text{ CFU per g} = 1.9 \times 10^3 \text{ CFU per g} \]
\[ 235,000 \text{ CFU per g} = 2.4 \times 10^5 \text{ CFU per g} \]

If there are only plates containing more than 150 typical Listeria colonies report as greater than the upper limit for the test dilution used with the comment “Count too high to be estimated at the dilution used”.

Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

**Swabs and cloths**
The lower limit of detection may vary, depending on the quantity of diluent used in the preparation of the sample. Care must be taken when reporting these results to ensure that the appropriate dilution factor is used in the calculation of results. Guidance on the calculation for results from swabs and other materials can be obtained from Standard Method FNESX4 (E1)- Detection and Enumeration of Bacteria in Swabs and other Environmental Materials.

9.3 Detection and enumeration

If *Listeria* species (total) are not isolated by enumeration but are isolated by detection report as:

*Listeria* species (total) DETECTED in 25 g or 25 mL or sample.

Also report the identity of the species and the limit of the enumeration test used.

Listeria identified as *L.*(insert species name) (Less than 10 or 20 CFU per g).

If any colonies are confirmed as *Listeria monocytogenes* separately report as:

*L. monocytogenes* DETECTED in 25 g (Less than 10 or 20 CFU per g)

10.0 Reference facilities and referral of cultures

All isolates of *L. monocytogenes* (haemolytic and non-haemolytic), regardless of the level should be sent to the Food and Environmental Pathogens Reference Unit (FEPRU), Laboratory of Gastrointestinal Pathogens, PHE Colindale for serotyping and further epidemiological characterisation.

A request form for referral to reference facilities can be obtained using the following link

http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947408749
11.0 Acknowledgements and contacts

This Standard Method has been developed, reviewed and revised by Microbiology Services, Food, Water and Environmental Microbiology Methods Working Group.

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References


6. 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


Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species


Appendix: Flowchart showing the process for detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

**Detection**

**Primary enrichment**

- Foods and Dairy Products
  - Weigh or measure 25 g or mL of sample and add 225 mL of half Fraser broth

- Environmental swabs
  - Immerse in half Fraser broth (approximately 1:9 ratio)
  - Homogenise or mix
  - Incubate at 30°C for 24 ±3h

Sub-culture to selective agars and confirm isolates as described below for secondary enrichment

**Secondary enrichment**

- Inoculate 0.1 mL of incubated half Fraser broth culture into 10 mL of Fraser broth
- Incubate at 37°C for up to 48 ±3h
- Sub-culture to selective agar plates
- Incubate Listeria chromogenic and Oxford agar plates at 37°C for up to 48 h in aerobic conditions
- Examine at 24 ±3h and again after a further 24 ±3h
- Subculture 5 presumptive colonies from each plate to horse blood agar
- Incubate at 37°C for 24±3h
- Select appropriate morphological colony types for confirmation
- Identify using biochemical gallery or PCR

Report as *Listeria* species (total) with identification or *L. monocytogenes* per 25g or mL or sample.
Detection and enumeration of *Listeria monocytogenes* and other *Listeria* species

**Enumeration**

Prepare a $10^{-1}$ dilution of sample

Homogenise or mix

Prepare further dilutions if required in peptone saline diluent

Surface spread 0.5 mL of $10^{-1}$ dilution onto one or two Listeria chromogenic plates

If high counts are expected, also inoculate 50 µL of a $10^{-1}$ and $10^{-3}$ dilution on to Listeria chromogenic agar media using a spiral plater

Incubate Listeria chromogenic agar plates at 37°C for up to 48 ±3h in aerobic conditions

Examine at 24 ±3h and after a further 24 ±3h.

Sub-culture 5 presumptive colonies onto blood agar

Incubate at 37°C for 24 ±3h

Identify using biochemical gallery or PCR

Calculate and report the counts of *Listeria* species (total) (and *L. monocytogenes* if present) per gram or mL