Public Health England

Protecting and improving the nation's health

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

National Infection Service
Food Water and Environmental Microbiology
Standard Method

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Contents

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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.

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Amendment history

Controlled document reference	**FNES22 (F19)**
Controlled document title	**Standard Method for Detection and Enumeration of *Listeria monocytogenes* and other *Listeria* species.**

The amendments that have occurred since the previous version of this document are shown below. On issue of revised or new documents each controlled document should be updated by the copyholder in the laboratory.

Page	Section(s) involved	Amendment
6	Background	ISO references and table of amendments updated
8	Principle	Updated with revised incubation times (CR12022)
10	3.2 Specific H&S Considerations	Updated to include those with compromised immunity as a result of a medical condition or treatment (CR12022)
10	4.0 Equipment	Fridge added (CR12022)
13	6.1 Sample Preparation, Inoculation and Incubation for Detection	Pre-warming of ½ Fraser Broth included (CR12022). References to pooling of samples where evidence is available to show that this does not affect the results included (CR12022). Information note added to enable storage for up to 72 h prior to sub-culture (CR12022) Incubation time for ½ Fraser broth changed from 24±3 h to 25±1 h. Incubation time for Fraser broth changed from 48±3 h to 48±2 h .Incubation time for OCLA/Oxford agar changed from 24±3 h followed by a further 24±3 h to 24±2 h followed by 24±2 h (CR12022).
14	6.2 Sample Preparation, Inoculation and Incubation for Enumeration	Pre-warming of BPW and ½ Fraser Broth added. ½ Fraser broth can now be used as a diluent for the *Listeria* enumeration test (CR12022) Incubation time for OCLA agar changed from 24±3 h and a further 24±3h to 24±2 h and a further 24±2 h (CR12022).
15	6.3 Recognition and colony counting	Safety note update to include those with compromised immunity.(CR12022) 6.3.1 Colony recognition section update to include additional species that produce blue colonies in Annex C of ISO (CR12022).
16	6.4 Confirmatory Tests	Updated to make it clear that at least one of the optional confirmatory procedures must be used (CR9966) Updated to replace initial sub-culture of 5 colonies with one colony and further sub-culture up to a maximum of 5. (CR12022) Colony Morphology and presence of Haemolysis section updated to include 24±2 h incubation (CR12022)
18	7.0 Quality control	Updated to include NCTC strains and WDCM numbers as described in ISO 11133 (CR12022)
20	9.0 Reporting	Information note added to specify the need for including a report based comment if PCR has been used as the sole confirmatory procedure.
25	References	Updated and re-ordered (CR12709)
27-28	Appendix	Updated

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*[1] 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[2] 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: 2017[3] and BS EN ISO 11290-2:2017 [4]. These are internationally recognised horizontal methods for the detection and enumeration of *L. monocytogenes* and other *Listeria* species. 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. However, the opaque halo may not always be visible as it can take up to four days to develop in some strains of *L. monocytogenes*.

This method differs from the current EN ISO 11290-1:2017[3] and EN ISO 11290-2:2017[4] in a number of minor ways.

These differences in methodology are described in the tables below:

	PHE method F19	**EN ISO 11290-1:2017**	**Justification for variation**
Culture media	Media formulation specified with option for use of ALOA or OCLA (ISO)	ALOA specified in ISO with ability to use other formulations if validated	OCLA (ISO) has the same formulation as ALOA.
Incubation of chromogenic plates	Requires 48 h incubation	Requires 24h with a further 24h if growth is weak or no colonies. Recommends incubation of plates for up to 4 days to enable identification of strains that are slow to produce opaque halos	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 for confirmation regardless of halo development and this would enable identification of atypical *L.monocytogenes* strains
Sub-culture of enrichment broths	Volume sub-cultured to the plates is defined as a minimum of 10µL.	Subculture from enrichment broths using a 3mm loop (ie 1µL)	High levels will have been produced during enrichment. The volume sub-cultured using the PHE method is spread to ensure discrete colonies.
	PHE method F19	**BS EN ISO 11290-2:2017**	**Justification for variation**
Enumeration	0.5mL or 0.5mL in duplicate for official control work	0.1 mL per plate in duplicate or 1mL on a 140mm plate in duplicate or over 3 90mm plates in duplicate.	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

	PHE method F19	EN ISO 11290-1:2017	Justification for variation
			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
Enumeration	Spiral plater used	Above procedures used for serial dilutions.	Spiral plater widely used in PHE procedures

Confirmations	Horse BA used	Sheep, calf or bovine BA used	No evidence based on IQC EQA result that the procedure is adversely 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.
Reference testing	All isolates of *L.monocytogenes* are sent for serotyping and further epidemiological characterisation.	Isolate may be sent for definitive confirmation.	PHE surveillance requirement.

1. 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 25 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 50 h. Sub-culture to 2 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 other appropriate decimal dilutions of the test sample. *Listeria* chromogenic agar plates are incubated at 37°C for up to 52 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. 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 of food or dairy product or environmental sample swab/cloth/other).

3. Safety considerations

3.1 General safety considerations

Normal microbiology laboratory precautions apply [5]. All laboratory activities associated with this SOP must be risk assessed to identify hazards[6-7]. Appropriate controls must be in place to reduce the risk to staff or other groups. Staff must be trained to perform 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.

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 and those with compromised immunity as a result of a medical condition or treatment 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 or any other condition or treatment likely to increase the health risk to staff and adjustments made to enable these 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. Equipment

- top pan balance capable of weighing to 0.1g
- gravimetric diluter (optional)
- stomacher
- vortex mixer
- fridge 5± 3^{o}C
- 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 method FNES43 (M2)[8]

5. 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

pH 7.0 ± 0.2 at 25°C

Buffered peptone water

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 dihydrogen phosphate	1.5 g
Water	1L

pH 7.0 ± 0.2 at 25°C

Half Fraser and Fraser broth

	Half Fraser	**Fraser**
Proteose peptone	5.0 g	5.0 g
Tryptone	5.0 g	5.0 g
Meat extract	5.0 g	5.0 g
Yeast extract	5.0 g	5.0 g
Sodium chloride	20.0 g	20.0 g
di-Sodium hydrogen phosphate	12.0 g	12.0 g
Potassium di-hydrogen phosphate	1.35 g	1.35 g
Aesculin	1.0g	1.0g
Lithium chloride	3.0 g	3.0 g
Ferric ammonium citrate	0.5 g	0.5 g
Nalidixic acid	10 mg	20 mg
Acriflavine hydrochloride	12.5 mg	25 mg
Water	1 L	1 L

pH 7.2 ± 0.2 at 25°C

Horse blood agar

Columbia agar with 5 % horse blood

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 FNES43 (M2)[8] and FNES44 (M3)[9] are used

Information note: *Additional diluents may be required for dairy products please refer to SOP FNES26 (F2)[10] for media formulations.*

6. 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 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 pre-warmed to room temperature 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). When more than one 25g test portion from a specified product is to be examined and when evidence is available that combining test portions does not affect the result for that particular food, the test portions can be pooled.

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 25 ± 1 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 ± 2 h and also using a 10µL loop sub-culture the primary enrichment (half Fraser) broth to Listeria chromogenic agar and Oxford agar in order to achieve single colonies.

Information note*: Following incubation cultures can be stored at 5 ± 3°C for 72 h before sub-culture.*

After incubation at 37 ± 1°C for 48 ± 2 h and using a 10µL loop, 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 ± 2 h and a further 24 ± 2 h.

6.2 Sample Preparation, Inoculation and Incubation for Enumeration

Following the procedure described in Standard Method FNES26 (F2)[10] – Preparation of Samples and Dilutions prepare a 10^{-1} homogenate of the sample in pre-warmed BPW or pre-warmed ½ Fraser broth and prepare further decimal dilutions as required in PSD. For swabs refer to Standard Method FNES4 (E1)- Detection and Enumeration of Bacteria in Swabs and Other Environmental Materials[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 2 Listeria chromogenic agar plates. If counts are expected to be high, plate further dilutions or use a spiral plater to inoculate 50 µL of the 10^{-1} 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 ± 2 h and then for a further 24 ± 2 h.

6.3 Recognition and counting of colonies

Safety note
Pregnant staff or those with compromised immunity should not be involved further.

6.3.1 Colony recognition

Plates must be examined at 24 ± 2h and after a further 24 ± 2h.

Listeria chromogenic agar
Colonies of *Listeria* appear blue or blue-green. Typical colonies of *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 *L. ivanovii* also develop an opaque halo, but within 48 h. Other species of *Listeria* do not develop a halo. Blue colonies may also be formed by other bacteria such as *Bacillus* spp., *Carnobacterium* spp., *Cellulosimicrobium funkei*, *Enterococcus* spp., *Kocuria kristinae, Marinilactibacillus psychrotolerans, Rothia terrae, Staphylococcus* spp. and *Streptococcus* spp.

Oxford agar
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. Subtle differences in the size, zone size and colour of colonies may be evident. If colonies with zones are present after 24 h perform a count to enable differential counting of colonies at 48 h. Zones may also 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. Calculations occur automatically if using StarLims.

6.4 Confirmatory tests

Sub-culture one presumptive *Listeria* colony of each morphological type to horse blood agar from Listeria chromogenic agar(s) and perform confirmatory tests as described below. If after sub-culture of one characteristic zone bearing colonies, *L.monocytogenes* has not been identified sub-culture and confirm further colonies up to a total of 5.

If *L. monocytogenes* has **not** been found (either through absence of typical colonies or through confirmatory tests yielding *Listeria* species other than *L. monocytogenes)* following enumeration tests and detection in 25g has been performed, sub-culture one colony 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 colony type. If after sub-culture of one characteristic zone bearing colony *L.monocytogenes* has not been identified sub-culture and confirm further colonies up to a total of 5.

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.

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 needs to 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 ± 2 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.*

If colonial morphology appears atypical perform a Gram stain. Select pure cultures of different morphological colony types for confirmation. Wearing gloves and safety glasses perform a Gram stain 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.

Following initial confirmation, further confirmation must be performed using at least one of the procedures described below.

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 Microgen 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)
If colonies are being confirmed from enumeration plates a separate PCR tube must be set up for each colony. If colonies are being confirmed from enrichment broths, up to 5 colonies can be emulsified in the same PCR tube. Following method FNES44 (M3)[9] heat treat the PCR grade water with emulsified colonies at 95^{o}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 FNES43 (M2)[8]. The positive control described in Standard Method FNES45 (M4)[12] should be included in each real time PCR assays.

7. 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 10527;	WDCM 00021
	or NCTC 7973;	WDCM 00109
Listeria innocua	NCTC 11288;	WDCM 00017
Negative control:		
Enterococcus faecalis	NCTC 775;	WDCM 00009
	or NCTC 12697;	WDCM 00087
Escherichia coli	NCTC 12923;	WDCM 00012
	or NCTC 12241;	WDCM 00013

8. Calculation of results

Calculations occur automatically in the StarLims system as described in Method FNES6 (Q12) Sample processing and result entry in StarLims[13] . 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:

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

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:

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

when:

- Σa is the sum of the colonies counted on all the plates retained from 2 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 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 2 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 2 parallel plates using the following equation:

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

when:

Σa 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 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

9. Reporting of results

All results are reported using the StarLims system as described in method FNES17 (Q13) Technical Validation and release of result in StarLims[14]. 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.

Information note: *If PCR is used as the only confirmatory method this must be included as a result based comment in the test report*.

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 25mL 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 25g or 25mL 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 x 10^b 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 10. Round counts up if the last figure is 5 or more, round counts down if the last figure is 4 or less.

eg: 1920 CFU per g = 1.9 x 10^3 CFU per g
235,000 CFU per g = 2.4 x 10^5 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”.

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 FNES4 (E1)- Detection and Enumeration of Bacteria in Swabs and other Environmental Materials[11].

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. Reference facilities and referral of cultures

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

A request form for referral to reference facilities can be obtained using the following link: assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/648460/L4_gastrointestinal_bacteria_culture_referral_form.pdf

11. Acknowledgements and contacts

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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 pre-warmed half Fraser broth

Environmental swabs
Immerse in pre-warmed half Fraser broth (approximately 1:9 ratio)
⇩
Homogenise or mix
⇩
Incubate at 30°C for 25 ±1h
⇩
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 ±2h
⇩
Sub-culture to selective agar plates
⇩
Incubate Listeria chromogenic and Oxford agar plates at 37°C for up to 52 h in aerobic conditions
⇩
Examine at 24 ±2h and again after a further 24 ±2h
⇩
Subculture up to 5 presumptive colonies from each plate to horse blood agar
⇩
Incubate at 37°C for 24±2h
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

Enumeration

Prepare a 10^{-1} dilution of sample in pre-warmed BPW or ½ Fraser Broth
⇩

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 52 h in aerobic conditions
⇩

Examine at 24 ±2h and after a further 24 ±2h.
⇩

Sub-culture up to 5 presumptive colonies onto blood agar
⇩

Incubate at 37°C for 24 ±2h
⇩

Identify using biochemical gallery or PCR
⇩

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