



United States
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Microbiology Laboratory Guidebook Notice of Change

Chapter new, **revised**, or archived: MLG 8.03

Title: Isolation and Identification of *Listeria monocytogenes* from Red Meat, Poultry, Egg, and Environmental Samples.

Effective Date: 4/29/02

Description and purpose of change(s):

The Microbiology Laboratory Guidebook method chapters are currently under revision. The formatting and is being changed to meet the requirements of the laboratory's document control system. Additional content is being added, i.e. Section 8.1.2 Method Detection Limits, to meet the requirements of ISO 17025. Safety Precautions are also now included in the revised chapters.

Additionally, this chapter has been revised to include use of rapid screen tests such as the procedure described in Chapter MLG 8A.

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8.1 Introduction

8.1.1 General

Listeria monocytogenes is a gram-positive rod-shaped bacterium associated with a variety of environments including soils, water, sewage, silage, as well as plant and animal food

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products. Although reported cases of human foodborne listeriosis are rare, the incidence of serious illness and death in affected individuals is high. Immunocompromised individuals, pregnant women, neonates and the elderly are particularly vulnerable.

Among all species in the genus *Listeria*, only *L. monocytogenes* has been implicated in human foodborne illness. The method described below employs well-established media and tests for the isolation and specific identification of β -hemolytic *L. monocytogenes*. The method is broadly applicable to raw or cooked ready-to-eat red meat and poultry products, including egg products. This protocol supercedes all previous USDA/FSIS protocols for *L. monocytogenes*.

8.1.2 Method Detection Limits

For this method, *L. monocytogenes* detection limits are determined to be better than 1 cfu/g in a 25 g sample.

8.2 Safety Precautions

- a. Laboratory personnel must abide by CDC guidelines for manipulating Biosafety Class II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for aerosolization of pathogens.
- b. Pregnant women and potentially immunocompromised individuals must be prohibited from laboratory rooms or areas where *L. monocytogenes* isolation or identification procedures are in progress. Although a properly sanitized laboratory area should not harbor *L. monocytogenes* or other pathogens, supervisors should use their own discretion in allowing high-risk individuals into these areas when not in use for these activities.

8.3 Quality Control Procedures

The correct performance of all stages of the analysis, including enrichment, screening tests, plating and all confirmatory tests, must be verified through the use of appropriate controls.

- a. For enrichment, screening, plating and verification of tumbling motility, control cultures must be transferred from one medium to the next in the sequence of analysis

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used for the samples. For these stages of analysis, the following requirements apply:

- i. One *L. monocytogenes* positive control and one uninoculated media (negative) control are required for each set of concurrently analyzed samples.
 - ii. For the uninoculated control, use a 225 ml aliquot of UVM broth. For all subsequent uninoculated control tests, use one unit of the medium at the volume specified for the test. Investigate the source of any contaminating organisms.
- b. Specific control requirements for each confirmatory test are addressed in the appropriate sections of this protocol.
 - c. Additional control cultures may be employed for individual tests or the entire sequence of analysis at the discretion of the laboratory.
 - d. *L. monocytogenes* and *L. innocua* control culture inocula are prepared and used as deemed appropriate for the media to be tested. Ideally, an empirically determined standardized quantity of inoculum should be employed.

8.4 Equipment, Supplies, Media, Reagents, and Test Systems

8.4.1 Equipment

- a. Electronic top-loading balance capable of weighing a minimum of 25 ± 0.1 g (500 g capability recommended)
- b. Stomacher™ (model 400 by Tekmar, or comparable model), or blade-type blender, sterilized blender cutting assemblies and jars
- c. Incubator, $30 \pm 2^\circ\text{C}$
- d. Incubator, $35 \pm 2^\circ\text{C}$
- e. Incubator, 20 or $25 \pm 2^\circ\text{C}$
- f. Vortex mixer
- g. Phase-contrast microscope (40X and 100X objectives)
- h. Fluorescent desk lamp or natural spectrum light source

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8.4.2 Supplies

- a. Supplies for all samples:
 - i. Sterile sample preparation supplies: scalpels, chisels, knives, scissors, spatulas, forceps, disposable or reusable dishes, pans or trays
 - ii. Stomacher[®] Strainer 400[™] sterile filter bags or non-filtered bags or equivalent
 - iii. Non-filter plastic bags for double-bagging sample homogenates (optional)
 - iv. Pipets (e.g. 1 ml)
 - v. Pipettor and sterile disposable tips for dispensing 100 µl
 - vi. Microscope slides, cover slips and immersion oil
 - vii. Disposable plastic and/or platinum inoculating needles and loops
 - viii. Wax or waterproof ink marker
 - ix. Sterile cotton-tipped applicators (i.e. swabs)

- b. Additional supplies necessary for environmental samples:
 - i. 0.45 µm hydrophobic grid membrane filter system (e.g. SELeCT 56 mm/100-ml 0.45-µm Monitor, cat# BPM9261, BioPath Inc., 2611 Mercer Ave., W. Palm Beach, FL 33401, 800-645-2302, or equivalent)
 - ii. Vacuum flask/hose and vacuum source
 - iii. Sterile disposable scalpels
 - iv. Sterile forceps
 - v. Non-bactericidal sampling sponges (e.g. Nasco cat # B01299WA, or equivalent)

8.4.3 Media

Refer to the MLG Media Appendix for formulations and preparation instructions.

- a. Media required for enrichment, plating and preliminary confirmation tests

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- i. Modified University of Vermont broth (UVM, also known as UVM1)
 - ii. Fraser broth (FB)
 - iii. Modified Oxford agar (MOX)
 - iv. Horse blood overlay agar (HL, also known as HBO)
 - v. Trypticase soy agar with 5% sheep blood (TS-SBA, also known as CAMP test agar)
 - vi. Brain heart infusion broth (BHI broth)
- b. Additional media for environmental samples
- Dey-Engley (D/E) neutralizing broth (e.g. Nasco cat# B01256WA, or equivalent)
- c. Optional media
- Trypticase Soy Agar-Yeast Extract (TSA-YE)

8.4.4 Reagents and Test Systems

- a. Reagents and materials required for routine tests
 - i. β -lysin CAMP factor discs (Remel #21-120, or equivalent)
 - ii. Biochemical test panel (MICRO-ID[®] *Listeria*, api[®]-*Listeria* system, or equivalent)
- b. Reagents for supplemental tests
 - i. Ribosomal RNA-based test system (i.e. GenProbe Accuprobe[®] *L. monocytogenes*-specific test, GeneTrak[®] *L. monocytogenes*-specific test, or equivalent)

8.4.5 Commercially Available Screening Tests

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Any screening method under consideration for *L. monocytogenes* testing must be validated for the intended use and must be at least as sensitive as the culture method described in this procedure.

8.4.6 Cultures

- a. At least one *L. monocytogenes* positive control strain is required. Appropriate cultures include ATCC 19111, NCTC 7973 or other *L. monocytogenes* cultures validated to perform in an equivalent manner.
- b. At least one *L. innocua* negative control culture is required. Appropriate cultures include ATCC 33090 or other *L. innocua* validated to perform in an equivalent manner.
- c. Other *Listeria* spp., such as *L. seeligeri*, *L. grayi* and *L. ivanovii*, may be necessary as controls for additional confirmatory testing.
- d. If the β -lysin CAMP factor test is not employed, *Staphylococcus aureus* ATCC 25923 or ATCC 49444, and *Rhodococcus equi* ATCC 6939 are required to perform the traditional CAMP test.

8.5 Detection and Isolation Procedures

8.5.1 Sample preparation

- a. Meat, poultry and egg products: A 25 ± 1.0 g portion is used for raw and processed red meat, poultry and egg product testing. The test portion should be a composite representative of the entire sample or available samples common to a specific lot.
 - i. Intact retail packages must be disinfected at the incision sites immediately prior to incision for sampling. Appropriate disinfectants include but are not limited to ca. 3% hydrogen peroxide, ca. 70% ethanol or ca. 70% isopropanol. If the package does not appear to be clean, scrub gently using soapy water and rinse thoroughly prior to disinfection. A sterile scalpel may be

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helpful for cutting the packaging. Aseptically pull the packaging away to expose the product for sampling.

- ii. Using a sterile scalpel, knife, spoon, chisel or other tool cut small pieces from representative sites on the sample and/or a variety of same-lot samples to prepare a composite sample.
 - iii. Place 25 ± 1.0 g of the composite sample in a sterile Stomacher™ bag (i.e. filter bag recommended). If necessary, double-bag with a non-filter bag prior to stomaching (e.g. zip-lock bag).
 - iv. If analysis of the test portion is not to be initiated within 1 h, store at $\leq -10^{\circ}\text{C}$. Do not dilute the sample until ready to initiate analysis.
 - v. For analysis, proceed to Section 8.5.2.
- b. Outbreak/recall samples: Some samples or sample lots, particularly those implicated in foodborne illness outbreaks, may require analysis of up to thirteen 25 g test portions. The test portion should be a composite representative of the entire sample or available samples common to a specific lot. The need for multiple subsample analyses must be determined on a case-by-case basis, or by client requirements.

8.5.2 Primary Enrichment in UVM broth

- a. For all meat, poultry and egg product samples (including outbreak/recall samples):

To the 25 ± 1.0 g test portion, dispense 225 ± 5 ml (or 225 ± 5 g) of UVM broth. Stomach or blend for 2 ± 0.2 minutes. If blended, aseptically transfer the homogenate to an appropriate sterile container. Incubate the homogenate at $30 \pm 2^{\circ}\text{C}$ for 22 ± 2 h. Proceed to Section 8.5.3.
- b. For environmental sponge samples:

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Add 200 ± 5 ml of UVM broth to each bagged sponge sample and stomach 2 ± 0.2 min.

Incubate at $30 \pm 2^\circ\text{C}$ for 22 ± 2 h. Proceed to Section 8.5.3.

- c. For environmental aqueous chilling solutions and surface rinse solutions:

Aqueous chilling solutions may include water, brine and propylene glycol solutions.

- i. Filter an appropriate volume (100 ± 2 ml recommended if available) of sample solution through one or more $0.45 \mu\text{m}$ hydrophobic grid membrane filters. These filters can be easily clogged by particulates. Therefore, more than one filter may be necessary to filter the entire test portion. Pre-sieving may remove a significant number of *Listeria* cells from the sample and should be applied only if absolutely necessary.
- ii. Using sterile forceps, aseptically remove the membrane from the plastic housing and transfer to a stomacher bag. A sterile scalpel may be used to cut around the circumference of the hydrophobic grid membrane to excise it from the filter housing (e.g. SELeCT[®] system). All membranes associated with a given sample may be combined in a single enrichment bag.
- iii. Add 200 ± 5 ml or additional volume of UVM broth sufficient to completely cover the filters. Stomach 2 ± 0.2 minutes. Incubate the homogenate at $30 \pm 2^\circ\text{C}$ for 22 ± 2 h. Proceed to Section 8.5.3.

8.5.3 Secondary Enrichment in FB and Direct Plating of UVM

- a. Transfer 0.1 ± 0.02 ml of the UVM enrichment to 10 ± 0.5 ml of FB. As per media preparation instructions, be sure that appropriate supplements have been added to the FB prior to inoculation. Incubate inoculated FB tubes at $35 \pm 2^\circ\text{C}$ for 26 ± 2 h.

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- b. Streak a MOX plate. Streak a loopful or a drop approximating 0.1 ml of the UVM over the surface of the plate. Alternatively, dip a sterile cotton-tipped applicator or equivalent into the UVM and swab 25-50% of the surface of a MOX plate. Use a loop to streak for isolation from the swabbed area onto the remainder of the plate. Incubate the MOX at $35 \pm 2^\circ\text{C}$ for 26 ± 2 h.

8.5.4 Examination of UVM-streaked MOX and Interpretation/Plating of 26-h FB

- a. Examine the UVM-streaked MOX for colonies with morphology typical of *Listeria* spp. At ca. 24 h, suspect colonies are typically small (ca. 1 mm) and are surrounded by a zone of darkening due to esculin hydrolysis.
 - i. If suspect colonies are present on MOX, transfer suspect colonies to HL agar as described in Section 8.5.6. Then, proceed to step b below.
 - ii. If no suspect colonies are evident, re-incubate the MOX plate until a total incubation time of 48 ± 2 h has been achieved.
- b. After 26 ± 2 h of incubation, examine the FB for the potential presence of *L. monocytogenes*, by visual examination of the broth for darkening due to esculin hydrolysis.
 - i. If any degree of FB darkening is evident, aseptically dispense 0.1 ± 0.02 ml of the FB onto a MOX plate. Swab or streak 25-40% of the surface of the MOX plate with the FB inoculum. Use a loop to streak for isolation from the initial swab/streak quadrant onto the remainder of the plate. Incubate the MOX plate at $35 \pm 2^\circ\text{C}$ for a minimum of 24 h. Proceed to Section 8.5.5.
 - ii. If no FB darkening is evident, re-incubate the FB at $35 \pm 2^\circ\text{C}$ until a total incubation time of 48 ± 2 h has been achieved. Proceed to Section 8.5.5.b.

8.5.5 Examination of MOX Plates and Interpretation/Plating of 48 h FB

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- a. Examine and select suspect colonies from any MOX agar plate pending analysis (i.e. MOX plates streaked from 26 ± 2 h FB and/or UVM) as described in Section 8.5.4.a.
- b. Re-examine the FB for evidence of darkening after 48 ± 2 h of total incubation.
 - i. If any degree of darkening is evident, swab, streak and incubate a MOX plate as described in Section 8.5.4.i.
 - ii. If no darkening of FB is evident and no suspect MOX and/or HL colonies have been demonstrated, the sample is considered negative for *L. monocytogenes*.

8.5.6 Isolation and Purification Procedures

- a. If suspect colonies are present on MOX from any source, use a loop or equivalent sterile device to contact a minimum of 20 (if available) suspect colonies and collectively streak for isolation on one or more HL agar plates. Alternatively, a swipe of suspect growth representing at least 20 colonies may be used. Incubate the streaked HL at $35 \pm 2^\circ\text{C}$ for 22 ± 4 h.
- b. After incubation, examine the HL plate(s) against backlight for translucent colonies surrounded by a small zone of β -hemolysis.
 - i. If at least one suspect colony is clearly isolated, proceed to confirmatory testing (Section 8.6 below). Hold all HL plates containing suspect colonies (room temperature or refrigeration) until confirmatory testing is complete.

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- ii. If suspect colonies or β -hemolytic growth are present on HL but not clearly isolated, re-streak representative suspect colonies/growth onto one or more fresh HL plates and incubate per section 8.5.6.a.
- iii. If no suspect isolates are present on HL, pursue follow-up of MOX and/or HL isolates from other branches of analysis (e.g. FB follow-up vs. UVM direct streak follow-up). If no branch of the analysis produces suspect β -hemolytic colonies on HL, the sample may be reported as negative for *L. monocytogenes*.

8.6 Confirmation and Other Identification Procedures

Confirmatory identification of *L. monocytogenes* consists of preliminary confirmation tests (Section 8.6.1) followed by biochemical tests (Section 8.6.2). The CAMP/CAMP factor test (Section 8.6.3) and genetic tests (Section 8.6.4) may be required in certain circumstances. All confirmatory identification tests require a pure culture.

8.6.1 Preliminary Confirmation Tests for *Listeria* spp.

- a. Inoculation of preliminary confirmation test media

Use one isolated HL colony only to inoculate, in order, a BHI broth aliquot and (optionally) a fresh HL plate to confirm purity. In addition, media required for inoculation of biochemical test systems (e.g. HL, BHI agar, TSA-YE, TSA-SBA or equivalent as described in Section 8.4.3) must be inoculated from the same colony or growth subcultured from that colony. A minimum of one colony must be confirmed. If the first selected suspect HL colony does not confirm as *L. monocytogenes*, confirmation must be attempted for additional suspect HL colonies, if available, until at least three isolates from the test portion have failed confirmation.

Incubate BHI broth at 18-25°C for 16-18 h. Proceed to step 8.6.1.c.

Incubate the HL purity streak plate at 35 ± 2°C for 22 ± 4 h. Proceed to Section 8.6.2.

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b. Purity streak test (optional)

Examine the re-streaked HL plate for consistent morphological characteristics. The colonies on this second HL plate should represent a single clone. If the culture appears to be mixed, repeat Steps 8.5.6.b.ii. A pure culture is required for all confirmatory tests.

c. Tumbling motility test

After 16-18 h of incubation of BHI broth at 18-25°C, prepare a wet-mount. Using the 100X oil immersion objective (phase contrast microscopy recommended), examine the wet-mount culture for small rods that exhibit an active end-over-end tumbling/rotating movement characteristic of *Listeria* spp.

- i. If cell morphology and motility is not characteristic of *Listeria* spp., and the culture appears pure, report the sample as negative for *L. monocytogenes*.
- ii. If a mixture of typical *Listeria* cells and cells that do not have the characteristic *Listeria* morphology (i.e. small rods) are present, streak a loopful of the contaminated BHI broth onto fresh HL agar for further purification (Step 8.5.6.b.ii).
- iii. If no growth is evident at 16-18 h, re-incubate at 18-25°C until growth is evident or up to a total of 48 h.
- iv. If cell morphology is typical, tumbling motility is evident and the culture appears pure, proceed with biochemical confirmation (Section 8.6.2 below).

8.6.2 Biochemical Tests

If the suspect culture is pure and demonstrates a cell morphology and tumbling motility characteristic of *Listeria* spp., confirmatory biochemical tests must be performed. Either of two commercially available test systems (MICRO-ID[®] *Listeria* or api[®]-*Listeria*) or validated equivalent systems, including well-established schemes involving traditional tube

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biochemical media (e.g. Compendium of Methods for the Microbiological Examination of Foods, Bacteriological Analytical Manual), may be employed.

- a. MICRO-ID[®] *Listeria* test system
 - i. Follow the instructions provided by the manufacturer for inoculation and interpretation of the test panel.
 - ii. TSA-YE, BHI, TSA-SBA or HL agar inoculated and incubated as described in Section 8.6.1.a may be used for preparing the inoculum suspension. All growth on the agar of choice must represent the same clone.
 - iii. A CAMP/CAMP factor test (Section 8.6.3) must be performed to augment MICRO-ID[®] results.
 - iv. At minimum, one *L. monocytogenes* positive control must be analyzed concurrently with sample isolates.

Exercise caution in interpreting the identification of atypical *Listeria* spp. isolates when using the MICRO-ID[®] *Listeria* system. Be aware that rhamnase-negative *L. monocytogenes* do exist as well as β -hemolytic *L. innocua*. Therefore, all β -hemolytic CAMP-positive *Listeria* spp. identified as *L. innocua* by MICRO-ID[®], must be analyzed using a ribosomal RNA-based test system as described in Section 8.6.4.a.

- b. api[®]-*Listeria* test system
 - i. Follow the instructions provided by the manufacturer for inoculation and interpretation of the test panel.
 - ii. TSA-YE, BHI, TSA-SBA or HL agar may be used for preparing the inoculum suspension. All growth on the agar of choice must represent the same clone.
 - iii. Cultures identified by api[®]-*Listeria* as "*L. monocytogenes/innocua*" must be further characterized using a ribosomal RNA-based test

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system as described in Section 8.6.4.a.

- iv. At a minimum, one *L. monocytogenes* positive control must be analyzed concurrently with sample isolates.

8.6.3 CAMP/CAMP Factor Test

A CAMP/CAMP factor test is required to augment traditional biochemical or MICRO-ID[®] test results, or to resolve the hemolytic capability of indeterminate strains. Either of two test options may be employed:

- a. β -lysin CAMP factor test

This test system may provide results that are easy to interpret compared to the traditional CAMP test. Therefore, the β -lysin CAMP factor test is recommended over the traditional test.

- i. Aseptically place a β -lysin disc in the approximate center of a TS-SBA plate. (A 9 ± 1 -ml TS-SBA plate is easier to interpret than thicker plates and is recommended.)
- ii. Individually and aseptically streak four to eight isolates each as straight lines radiating away from the disc. The inoculation line should almost but not quite touch the disc. Be sure to include positive and negative control cultures. A non-hemolytic *L. innocua* is an appropriate negative control.
- iii. Incubate at 35 ± 2 °C for 24 ± 2 h.
- iv. An arrowhead-shaped zone of β -hemolysis surrounding the inoculum line proximal to the disc indicates a positive CAMP factor reaction. *L. monocytogenes*, *L. seeligeri* and *L. ivanovii* are CAMP factor-positive by this test. However, *L. ivanovii* demonstrates relatively intense β -hemolysis distal to the disk and, therefore, can be distinguished from the other two species. Non-hemolytic *Listeria* spp. are CAMP factor-negative.

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- v. If a suspected β -hemolytic *Listeria* spp. does not produce a CAMP factor-positive reaction at 24 ± 2 h, continue to incubate the culture at 35 ± 2 °C until a total incubation time of 48 ± 2 h has been achieved. Re-examine as before. If a CAMP factor-positive reaction is still not evident at 48 ± 2 h, ribosomal RNA-based testing is required to determine the genetic identity of the isolate (Section 8.6.4.a).

b. Traditional CAMP test

If the CAMP test is necessary and β -lysin discs are not available, perform the following test:

- i. For the traditional culture CAMP test, apply single-line streaks of *S. aureus* (ATCC 4944 or ATCC 25923) and *R. equi* (ATCC 6939) reference cultures on a TS-SBA plate in parallel and 3-4 cm apart.
- ii. Streak test cultures between and perpendicular to the two reference cultures (i.e. like rungs of a ladder). The test culture streak must be 2-4 mm from each reference culture streak. Test and reference cultures must not touch or be cross contaminated in any manner.
- iii. Incubate 24 ± 2 h at 35 ± 2 °C.
- iv. Examine the test culture streaks for enhanced β -hemolysis at both ends proximal to the reference cultures. The zone of enhanced β -hemolysis may resemble an arrowhead, circle or rectangle. The presence of this zone indicates a CAMP-positive reaction. Absence of enhanced β -hemolysis indicates a CAMP-negative reaction. *L. monocytogenes* and *L. seeligeri* are CAMP-positive to the *S. aureus* reference strain and CAMP-negative to *R. equi*. In contrast, *L. ivanovii* is CAMP-positive to the *R. equi* reference strain and CAMP-negative to the *S. aureus* strains.
- v. If a suspected β -hemolytic *Listeria* spp. does not produce a CAMP-

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positive reaction with either reference culture at 24 ± 2 h, continue to incubate the culture at $35 \pm 2^\circ\text{C}$ until a total incubation time of 48 ± 2 h has been achieved. Re-examine as before.

- vi. If the culture does not produce a CAMP-positive reaction with the *S. aureus* culture, but has or does clearly demonstrate β -hemolysis on HL agar, ribosomal RNA-based testing is required to determine the genetic identity of the isolate (Section 8.6.4.a).

8.6.4 Genetic Identification Tests

- a. Ribosomal RNA-based tests

A ribosomal RNA-based test is required to resolve the identity of any atypical suspected *L. monocytogenes* strain. In some circumstances, available phenotypic tests cannot clearly distinguish strains of *L. monocytogenes* from *L. innocua*. In particular, rhamnase-negative β -hemolytic *L. monocytogenes* can be misidentified as *L. innocua*. The following *L. monocytogenes*-specific ribosomal RNA-based tests are commercially available:

- i. GenProbe AccuProbe[®] *L. monocytogenes*-specific test
- ii. GENE-TRAK[®] *L. monocytogenes*-specific test

Follow the test kit instructions provided by the manufacturer for performing and interpreting these tests.

A *L. monocytogenes* positive control, a *L. innocua* negative control and an uninoculated sterility control must be analyzed concurrently with sample isolates.

A positive ribosomal RNA-based test result from either of the above test systems indicates that atypical phenotypes are confirmed *L. monocytogenes*. A negative result indicates that atypical phenotypes are not *L. monocytogenes*.

- b. Pulsed-field gel electrophoresis (PFGE) (optional)

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PFGE is a highly discriminative and reliable means of subtyping *L. monocytogenes* strains. The pulsotype (i.e. “genetic fingerprint”) derived from PFGE analysis is used in tandem with epidemiologic evidence to link clinical and food isolates implicated in foodborne illness. Standardized PFGE methodology and internet-based exchange of federal and state laboratory pulsotype data (i.e. PulseNet) facilitate epidemiologic and traceback investigations.

8.7 Enumeration: Most Probable Number (MPN)

- a. Stomach or blend 25 ± 1 g of sample with 225 ml of UVM as specified in Section 8.5.2.
- b. Dispense 10 ± 0.5 ml of the undiluted UVM/sample homogenate into each of three sterile culture tubes. Each tube represents a 1.0 g test portion.
- c. Dispense 1 ± 0.05 ml of the undiluted UVM/sample homogenate into each of three tubes containing 9-10 ml of UVM broth. Each tube represents a 0.1 g test portion.
- d. Dispense 0.1 ± 0.005 ml (i.e. 100 ± 5 μ l) of the undiluted UVM/sample homogenate into each of three tubes containing 9-10 ml of UVM broth. Each tube represents a 0.01 g test portion.
- e. If an additional dilution is necessary, prepare two successive ca. 1:10 dilutions (i.e. total dilution of ca. 1:100) of the undiluted UVM/sample homogenate in fresh UVM. Dispense 1 ± 0.05 ml of the second dilution (1:100) into a 9 ± 0.5 ml aliquot of UVM. Repeat for two additional tubes representing a 0.001 g test portion. For additional dilutions, prepare additional ca. 1:10 dilutions of the ca. 1:100 dilution above and dispense as before.
- f. Incubate all nine UVM tubes and the remainder of the undiluted UVM/sample homogenate (i.e. the qualitative test) as specified in Section 8.32. Perform isolation and identification of *L. monocytogenes* for each of the ten individual test aliquots as specified in Sections 8.5.3 through 8.6. The following procedures may be employed to simplify and expedite analysis:
 - i Direct streaking of UVM MPN tubes to MOX agar (Section 8.5.3.b) may not

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be necessary under some circumstances. If qualitative analysis was performed on a given sample prior to enumeration, and direct plating of UVM to MOX was necessary for isolation of *L. monocytogenes*, direct plating of UVM is required for all MPN tubes. If qualitative analyses have not been performed prior to enumeration, direct plating is required for the concurrent qualitative analysis only and is optional for the UVM MPN tubes. However, if direct streaking of UVM is ultimately found to be necessary for isolation of *L. monocytogenes* from the sample, the sample should be re-enumerated by MPN and direct streaking must be performed from all UVM MPN tubes.

- ii As with the qualitative procedure, at least one colony must be confirmed from three available suspect isolates on the HL plate(s) representing each positive FB in the MPN series, including that from the undiluted UVM/sample homogenate. A ribosomal RNA-based test (Section 8.6.4) may be employed to supplant biochemical/CAMP testing and expedite confirmation of most isolates. However, both biochemical/CAMP testing and ribosomal RNA-based testing must be used in tandem to characterize at least one suspect isolate representing the highest dilution found to contain suspects.

- g. Record the number of tubes per dilution that were ultimately found to be *L. monocytogenes* positive. Use a 3-tube MPN table (e.g. MLG MPN Appendix) to determine the estimated number of *L. monocytogenes* per gram of product. If all tubes are negative but the undiluted UVM/sample homogenate (i.e. the qualitative test) is positive, report the sample as positive at an enumerated level less than the lowest value on the table.

8.8 Culture Storage and Shipment

- a. BHI or TSA-YE slants may be used for short-term storage of *Listeria* spp. The culture should be stabbed into the agar using an inoculating needle. Tubes should be sealed with Parafilm[®] or equivalent to prevent desiccation and stored at 2-8°C. Under these conditions, *Listeria* spp. can remain viable for many months.

- b. For long-term storage (i.e. for more than one year) or to assure that the genetic character of the strain does not change over time (e.g. lose plasmids or other unstable genetic elements), cultures should be lyophilized and/or frozen

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at -20 to -80° C. Fetal calf serum or commercially available cryobead products are appropriate media for frozen storage of *Listeria* spp.

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