



## Laboratory Guidebook Notice of Change

Chapter new, **revised**, or archived: MLG 8.10

Title: Isolation and Identification of *Listeria monocytogenes* from Red Meat, Poultry, Ready-To-Eat Siluriformes (Fish) and Egg Products, and Environmental Samples

Effective Date: 01/02/2017

Description and purpose of change(s):

FSIS Laboratories validated ready-to-eat Siluriformes (fish) as a matrix for the isolation and identification of *Listeria monocytogenes* method.

A note was added to allow a more dilute food to broth ratio for dehydrated foods.

**The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use. Method validation is necessary to demonstrate the equivalence of alternative tests as detailed in the document titled “FSIS Guidance for Evaluating Test Kit Performance” available on the FSIS website.**

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### 8.1 Introduction

*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 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 *Listeria monocytogenes* is typically implicated in human foodborne illness. The method described below employs well-established media

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and tests for the isolation and specific identification of  $\beta$ -hemolytic *Listeria monocytogenes*. The method is broadly applicable to raw or cooked ready-to-eat (RTE) red meat, poultry products, egg products, RTE Siluriformes (fish) and environmental sponge samples.

## 8.2 Safety Precautions

CDC guidelines for the handling of BioSafety Level 2 organisms should be followed whenever live cultures of *Listeria* are used. The Safety Data Sheet (SDS) may be obtained from the manufacturer for the media, chemicals, reagents, and microorganisms used in the analysis. The personnel who will handle the material should read the SDS prior to startup.

Laboratories should develop a policy and inform pregnant women and potentially immunocompromised individuals of the risk from working in laboratory rooms or areas where *Listeria monocytogenes* isolation or identification procedures are in progress.

## 8.3 Quality Control Procedures

The correct performance of all stages of the analysis must be verified through the use of appropriate controls. One *Listeria monocytogenes* positive control and one uninoculated media control are required for each set of concurrently analyzed samples. In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses.

- a. The positive control culture should be inoculated into an appropriate matrix at a low inoculum level, e.g., by preparing a test organism suspension in broth or saline equivalent in turbidity to a McFarland 0.5 standard. Using a 1ul loop, inoculate the broth or streak the plates to be tested. Alternatively, commercially prepared bacterial pellets may be used. The laboratory may use tagged cultures such as those that visibly fluoresce under ultraviolet (UV) light to differentiate QC strains from true contaminants if available. Once the control cultures are started, incubate the controls along with the samples, and analyze them in the same manner as the samples.
- b. For the uninoculated control, use an 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.

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- c. Specific control requirements for each confirmatory test are addressed in the appropriate sections of this protocol.
- d. Additional control cultures may be employed for individual tests or the entire sequence of analysis at the discretion of the laboratory.

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

### **8.4.1 Equipment**

- a. Electronic top-loading balance capable of weighing a minimum of  $25 \pm 0.1$  g (500 g capability recommended)
- b. Paddle blender or blade type blender with 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

### **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. Sterile, filter or non-filter bags
  - 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  $\mu\text{l}$
  - 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)

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- b. Additional supplies necessary for environmental sponge samples:  
  
Non-bactericidal sampling sponges, polyurethane or cellulose
- c. Additional supplies necessary for environmental aqueous chilling solutions:
  - i. 0.45 µm hydrophobic grid membrane filter system
  - ii. Vacuum flask/hose and vacuum source
  - iii. Sterile disposable scalpels
  - iv. Sterile forceps
  - v. Whirl pak filter bag
  - vi. 2.7µm Glass fiber filter
  - vii. 500 ml graduated polypropylene beaker with handle
  - viii. Sterile 500 - 1000 ml non-polystyrene sample container

### **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:
  - i. Modified University of Vermont broth (UVM, also known as UVM1)
  - ii. Morpholinepropanesulfonic acid-buffered *Listeria* enrichment broth (MOPS-BLEB) or
  - iii. Fraser broth (FB)
  - iv. Modified Oxford agar (MOX)
  - v. Horse blood overlay agar (HL, also known as HBO)
  - vi. Trypticase soy agar with 5% sheep blood (TSA-SB, also known as CAMP test agar)
  - vii. Brain heart infusion broth (BHI broth)

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- b. Additional media for environmental samples:

Dey-Engley (D/E) neutralizing broth

- c. Optional media:

- i. Trypticase Soy Agar-Yeast Extract (TSA-YE)
- ii. Brain Heart Infusion (BHI) agar

#### **8.4.4 Reagents and Test Systems**

- a.  $\beta$ -lysin CAMP factor discs
- b. Biochemical test panel (MICRO-ID<sup>®</sup> *Listeria*, *Listeria* API<sup>®</sup> system, VITEK<sup>®</sup> 2 Compact System or equivalent)
- c. Ribosomal RNA-based test system (GenProbe Accuprobe<sup>®</sup> *L. monocytogenes*-specific test, GeneTrak<sup>®</sup> *L. monocytogenes*-specific test) or equivalent *Listeria monocytogenes*-specific genetic test system

#### **8.4.5 Cultures**

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

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## **8.5 Detection and Isolation Procedures**

### **8.5.1 Sample preparation**

- a. Meat, poultry, and RTE Siluriformes products: A  $25 \pm 1$  g portion or a  $125 \pm 5$  g portion is analyzed for raw and processed red meat and poultry product testing. A  $25 \pm 1$  g portion is used for pasteurized egg products testing.

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

Note: For RTE sausages in casing, the shell/casing is an integral part of the sample and should be free of pathogens and toxins. The casing is not to be disinfected since some casings are permeable and the disinfectant may be introduced into the core of the product. In addition consumers often slice through an inedible casing and then remove it thus any contamination on the surface of the casing could be transferred to the edible core of the product.

- ii. For meat and poultry products, weigh representative portions from each submitted package to achieve a  $25 \pm 1$  g or  $125 \pm 5$  g total sample portion. For egg products, weigh representative portions from each submitted package to achieve a  $25 \pm 1$  g total sample portion.

Using a sterile scalpel, knife, spoon, chisel or other tool cut small pieces from representative sites on the sample to prepare a composite sample. While multiple packages of a product are usually submitted, for large products a single package may be submitted.

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For multi-component ready-to-eat products, follow the appropriate sample preparation instructions listed below:

If the meat, poultry, or Siluriformes component is separate and distinct from other non-meat ingredients, analyze only the representative meat/poultry portion of the RTE product. Examples include products with the meat/poultry/Siluriformes portion separate from any vegetable/dessert component, or fajita kits with meat/poultry/Siluriformes, onions/peppers, and tortillas in three separate internal packages/bags within an outer package.

When meat/poultry/Siluriformes is combined with other ingredients to form the product (e.g., beef stew containing vegetables, potatoes, etc.), analyze representative meat/poultry/Siluriformes portions in combination with other ingredients.

- iii. Place the test portion in a sterile 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.



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- c. Most Probable Numbers (MPN) Determination: Follow MPN instructions given in the specific program protocol or see MLG Appendix 2, Most Probable Number Procedure and Tables.

### 8.5.2 Primary Enrichment in UVM broth

Table 1. Sample Preparation and Enrichment Guide

Product	Sample Preparation		Incubation
	Portion Size	Enrichment Amount Determined by volume or weight	Cultural or PCR rapid screen
Meat, poultry, Siluriformes, and egg products	25 ± 1 g	225 ± 5 ml UVM broth.	30 ± 2°C for 20 - 26 h.
For programs allowing compositing of five product subsamples:	125 ± 5 g	1125 ± 25 ml UVM broth	30 ± 2°C for 23 - 26 h
Environmental Sponge samples	1 single sponge	225 ± 5 ml UVM broth	30 ± 2°C for 20 - 26 h
	multiple sponges	100 ± 2 ml of UVM <u>per sponge</u>	30 ± 2°C for 20 - 26 h
Environmental aqueous chilling solutions and surface rinse solutions	500 ± 2 ml of sample solution filtered	225 ± 5 ml UVM broth	30 ± 2°C for 20 - 24 h

- a. For all meat, poultry, Siluriformes, and egg product samples (including outbreak/recall samples):  
To a 25 ± 1 g test portion, dispense 225 ± 5 ml (or 225 ± 5 g) of UVM broth.  
To a 125 ± 5 g test portion, dispense 1125 ± 25 ml (or 1125 ± 25 g) of UVM broth. Stomach or blend for 2 ± 0.2 minutes. If blended, aseptically transfer the homogenate to an appropriate sterile container. Incubate 25 ± 1 g test portions at 30 ± 2°C for 20 - 26 h. Incubate 125 ± 5 g test portions at 30 ± 2°C for 23 - 26 h. Proceed to Section 8.5.3.

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Note: Dried products such as soup mixes may require a sample/broth ratio greater than 1:10 because of physical difficulties encountered by absorption of broth by dehydrated product.

b. For environmental sponge samples:

Add  $225 \pm 5$  ml (or  $225 \pm 5$  g) of UVM broth to each bagged single sponge sample or add  $100 \pm 2$  ml of UVM per sponge to each bagged composite sponge sample that contains up to five sponges. For example, a composite of five sponge samples would require  $500 \pm 10$  ml of UVM. Stomach for  $2 \pm 0.2$  min. Hand mixing is an acceptable alternative for stomaching. To hand mix, briefly massage each sponge to expel the collection broth into the UVM broth.

Incubate at  $30 \pm 2^\circ\text{C}$  for 20 -26 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. Pour  $500 \pm 2$  ml of sample solution into a filter bag. Filter the solution by pouring it through a glass fiber filter and a  $0.45 \mu\text{m}$  hydrophobic grid membrane filter in a vacuum filter system. These filters can be easily clogged by particulates. Therefore, more than one filter may be necessary to filter the entire test portion. When the sample has been filtered, aseptically remove the glass filter and transfer it back to the filter bag for enrichment.
- ii. Aseptically remove the membrane from the membrane filter plastic housing and transfer to the same filter bag as above. A sterile scalpel may be used to cut around the circumference of the hydrophobic grid membrane to excise it from the filter housing. All membranes associated with a given sample are combined in the single enrichment bag.

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- iii. Add  $225 \pm 5$  ml (or  $225 \pm 5$  g) of UVM broth. Stomach  $2 \pm 0.2$  minutes. Ensure that the filters are submerged. Incubate the homogenate at  $30 \pm 2^\circ\text{C}$  for 20 - 24 h. Proceed to Section 8.5.3.

**8.5.3 Secondary Enrichment in FB or MOPS-BLEB and Primary Enrichment Plating of UVM**

- a. Transfer  $0.1 \pm 0.02$  ml of the UVM enrichment to  $10 \pm 0.5$  ml of FB or MOPS-BLEB. 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  $26 \pm 2$  h or inoculated MOPS-BLEB tubes at  $35 \pm 2^\circ\text{C}$  for 18-24 h.
- 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  $26 \pm 2$  h.

**8.5.4 Examination of UVM-streaked MOX, Interpretation/Plating of 26-h FB, and Plating of MOPS-BLEB**

- a. Examine the UVM-streaked MOX for colonies with morphology typical of *Listeria* spp. At  $26 \pm 2$  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.
  - ii. If no suspect colonies are evident, re-incubate the MOX plate for an additional  $26 \pm 2$  hour.
  - iii. Proceed to step b below for FB or step c for MOPS-BLEB.

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- b. After  $26 \pm 2$  h of incubation, examine the FB for the potential presence of *Listeria monocytogenes*, by visual examination of the broth for darkening due to esculin hydrolysis.
  - i. If any degree of FB darkening is evident, aseptically dispense a drop approximating  $0.1 \pm 0.02$  ml of 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  $26 \pm 2$  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  $48 \pm 2$  h has been achieved. Proceed to Section 8.5.5.b.
- c. After 18-24 h, streak a MOX plate using a loopful of the MOPS-BLEB, or by streaking a drop approximating 0.1 ml, or aseptically dip a sterile cotton-tipped applicator or equivalent into the MOPS-BLEB 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  $26 \pm 2$  h. Proceed to Section 8.5.5.a.

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

- a. Examine and select suspect colonies from any MOX agar plate pending analysis (i.e. MOX plates streaked from  $26 \pm 2$  h FB, 18-24 h MOPS-BLEB, and/or UVM) as described in Section 8.5.4.a.i and ii.
- b. Re-examine the FB for evidence of darkening after  $48 \pm 2$  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 *Listeria monocytogenes*.

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### **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  $22 \pm 4$  h.
- b. After incubation, examine the HL plate(s) against backlight for translucent colonies surrounded by a small zone of  $\beta$ -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.
  - ii. If suspect colonies or  $\beta$ -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 Primary Enrichment streak follow-up). If no branch of the analysis produces suspect  $\beta$ -hemolytic colonies on HL, the sample may be reported as negative for *L. monocytogenes*.

Note: Removal of a few colonies may assist the analyst in observing medium clearing for weakly hemolytic strains.

### **8.6 Confirmation and 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.

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### 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-SB 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 optional HL purity streak plate at 35 ± 2°C for 22 ± 4 h. Proceed to Section 8.6.2.

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 (optional):

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*.

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- 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

Using a pure culture, perform confirmatory biochemical tests. Commercially available test systems (MICRO-ID<sup>®</sup> *Listeria*, *Listeria* API<sup>®</sup> test system or VITEK<sup>®</sup> 2 Compact System) or validated equivalent systems, including well-established schemes involving traditional tube biochemical media (e.g. Compendium of Methods for the Microbiological Examination of Foods, Bacteriological Analytical Manual), may be employed. However, exercise caution in interpreting the identification of atypical *Listeria* spp. isolates when using biochemical systems. Cultures identified as “*L. monocytogenes/innocua*” or any beta-hemolytic *Listeria* spp. that is biochemically indeterminate or identified as *L. innocua* must be further characterized using a ribosomal RNA-based test system as described in Section 8.6.4.a.

- a. MICRO-ID<sup>®</sup> *Listeria* test system
  - i. Follow the instructions provided by the manufacturer for inoculation and interpretation of the test panel.
  - ii. TSA-YE, BHI, TSA-SB 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.

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- iii. A CAMP/CAMP factor test (Section 8.6.3) must be performed to augment MICRO-ID<sup>®</sup> results.
  - iv. At minimum, one *L. monocytogenes* positive control must be analyzed concurrently with sample isolates.
- b. *Listeria* API<sup>®</sup> 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. At a minimum, one *L. monocytogenes* positive control must be analyzed concurrently with sample isolates.
- c. VITEK<sup>®</sup> 2 Compact System
- i. Follow the instructions provided by the manufacturer for inoculation and loading the VITEK<sup>®</sup> 2 Compact System.
  - 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. 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<sup>®</sup> test results, or to resolve the hemolytic capability of indeterminate strains. Either of two test options may be employed:

- a.  $\beta$ -lysin CAMP factor test



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

- i. Aseptically place a  $\beta$ -lysin disc in the approximate center of a TS-SBA plate. (A  $9 \pm 1$ -ml TS-SBA plate is easier to interpret than thicker plates and is recommended.)
  - ii. Individually and aseptically streak up to eight sample isolates per plate 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  $35 \pm 2$  °C for  $24 \pm 2$  h.
  - iv. An arrowhead-shaped zone of  $\beta$ -hemolysis surrounding the inoculum line proximal to the disc indicates a positive CAMP factor reaction. *L. monocytogenes* and *L. seeligeri* are CAMP factor-positive by this test. Although *L. ivanovii* does have clearing near the disc it is not the “enhanced hemolysis” required to consider the test positive. Non-hemolytic *Listeria* spp. are CAMP factor-negative.
  - v. If a suspected  $\beta$ -hemolytic *Listeria* spp. does not produce a CAMP factor-positive reaction at  $24 \pm 2$  h, continue to incubate the culture at  $35 \pm 2$  °C until a total incubation time of  $48 \pm 2$  h has been achieved. Re-examine as before. If a CAMP factor-positive reaction is still not evident at  $48 \pm 2$  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  $\beta$ -lysin discs are not available, perform the following test:

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- i. For the traditional culture CAMP test, apply single-line streaks of *S. pseudintermedius* ATCC<sup>®</sup> 49444 or *S. aureus* ATCC<sup>®</sup> 25923 and *R. equi* (ATCC<sup>®</sup> 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  $24 \pm 2$  h at  $35 \pm 2^\circ\text{C}$ .
- iv. Examine the test culture streaks for enhanced  $\beta$ -hemolysis at both ends proximal to the reference cultures. The zone of enhanced  $\beta$ -hemolysis may resemble an arrowhead, circle or rectangle. The presence of this zone indicates a CAMP-positive reaction. Absence of enhanced  $\beta$ -hemolysis indicates a CAMP-negative reaction. *L. monocytogenes* and *L. seeligeri* are CAMP-positive to the *Staphylococcus* 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 *Staphylococcus* reference strains.
- v. If a suspected  $\beta$ -hemolytic *Listeria* spp. does not produce a CAMP-positive reaction with either reference culture at  $24 \pm 2$  h, continue to incubate the culture at  $35 \pm 2^\circ\text{C}$  until a total incubation time of  $48 \pm 2$  h has been achieved. Re-examine as before.
- vi. If the culture does not produce a CAMP-positive reaction with the *Staphylococcus* culture, but has or does clearly demonstrate  $\beta$ -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 or equivalent *L. monocytogenes* specific genetic tests

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Ribosomal RNA-based tests may be performed as a confirmatory test for all biochemically identified *L. monocytogenes* strains or on typical HL colonies (see Section 8.5.6) prior to conducting confirmatory procedures on positive colonies. 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, rhamnose-negative  $\beta$ -hemolytic *L. monocytogenes* and phospholipase C-negative, weakly hemolytic *L. monocytogenes* can be misidentified as *L. innocua* on biochemical testing. And  $\beta$ -hemolytic *L. innocua* strains exist. Several *L. monocytogenes*-specific ribosomal RNA-based tests are commercially available.

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*.

## **8.7 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<sup>®</sup> 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 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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