United States Department of Agriculture

Food Safety and Inspection Service

MLG 8.14

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

This method describes the laboratory procedure for performing the isolation and identification of *Listeria monocytogenes* from Ready-to-Eat Meat, Poultry, Siluriformes (Fish), Egg Products, and Environmental Samples.

Notice of Change

This method is revised to update the sample preparation instructions and update pictures for processing ready-to-eat samples, egg products, and environmental sponges. A new method flow chart (Page 3) was also created for enhanced analyst understanding of the method.

Figures 1-12 are all new to the document. They have been added primarily to the sample preparation section but can be found throughout the document.

This revision also includes a biosafety chart to further explain safety precautions regarding MLG 8.14.

The requirement to fluoresce HL plates before proceeding to confirmatory testing has been removed. SBA plates will continue to be fluoresced at the confirmatory step before biochemical confirmation.

Analysts may now suspend the *Listeria innocua* control at the end of the presumptive phase instead of carrying forward to biochemical confirmation.

This update will not affect the communication of presumptive positive or confirmed positive *Listeria monocytogenes* results to stakeholders.

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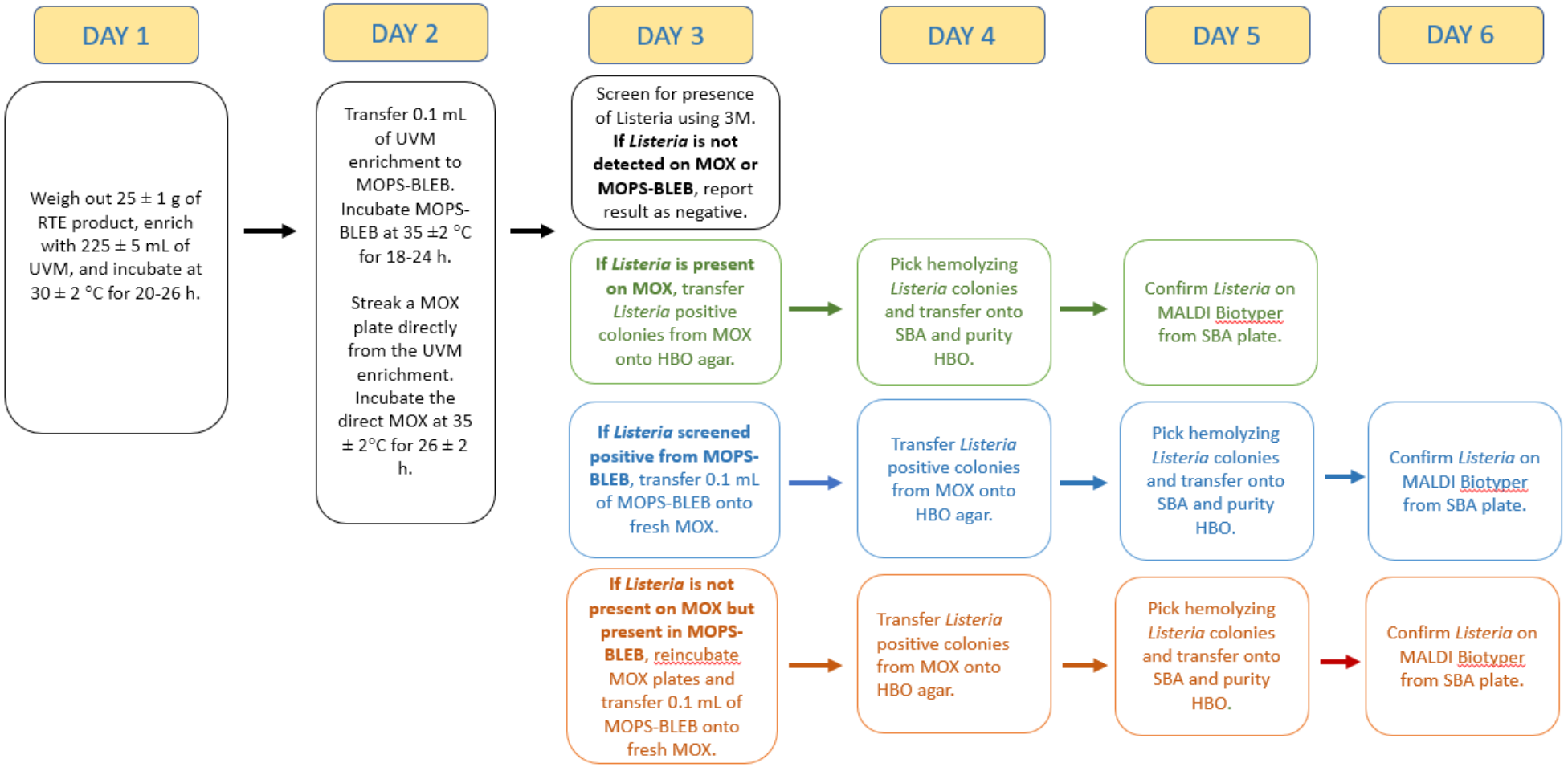
Isolation of Listeria monocytogenes Method Flow Chart



Introduction

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. FSIS utilizes the following performance criteria when evaluating the suitability of an alternative laboratory detection method or product for a given analyte and sample matrix pair:

- Sensitivity of 90% or greater
- Specificity of 90% or greater
- Accuracy of 90% or greater
- Positive predictive value of 90% or greater
- Negative predictive value of 90% or greater

Performance criteria are relative to the reference cultural detection method for that analyte and sample matrix as outlined in the corresponding MLG chapter (https://www.fsis.usda.gov/news-events/publications/microbiology-laboratory-guidebook; click on MLG Chapter 1). Method validation is necessary to demonstrate the equivalence of alternative tests as detailed in the document titled "FSIS Guidance for Evaluating Test Kit Performance," Validation Studies Pathogen Detection Methods.pdf (usda.gov)

Unless otherwise stated all measurements cited in this method have a tolerance range of ± 2%.

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

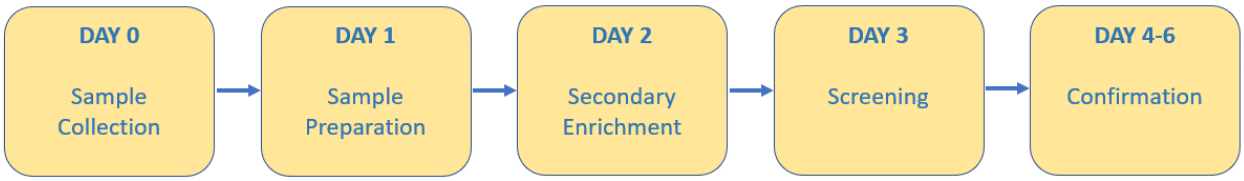


Figure 1: Simple flowchart outlining the daily workflow of the *Listeria monocytogenes* detection method.

Safety Precautions

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

Laboratories are to 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.

QUALITY CONTROL

Lab Quality Control Procedures: *Listeria* controls are to include a positive, fluorescent strain of *Listeria* (*Listeria monocytogenes*, commercially available) and an uninoculated medium control. 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.

The positive control cultures should be inoculated into an appropriate enrichment broth at a low-level inoculum level e.g., by preparing a test organism suspension in broth or saline equivalent in turbidity to a 0.5 McFarland standard. Using a 1µL loop, inoculate the broth or streak the plates to be tested. Alternatively, commercially prepared bacterial pellets will be used. Confirm at least one isolate from the positive control sample. In the absence of a positive test sample, control cultures will be terminated at the same point as the sample analyses.

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.

Each step of the analysis requires the use of appropriate controls to verify that the results are valid. Biochemical kit and rapid test manufacturers will specify control cultures for use with their products. If not specified, quality control procedures for biochemical tests and test media will include cultures that will demonstrate pertinent characteristics of the product.

Additional control cultures may be employed for individual tests or the entire sequence of analysis at the discretion of the laboratory.

Equipment, Reagents, Media, and Cultures

Table 1: Equipment and supplies for MLG 8

Equipment	Supplier	Purpose
Sterile spoons, scissors, forceps, and knives	General lab supplier	Sample preparation
0.45 µm hydrophobic grid membrane filter system	General lab supplier	Environmental aqueous chilling solutions preparation
Vacuum flask/hose and vacuum source	General lab supplier	Environmental aqueous chilling solutions preparation
Sterile disposable scalpels	General lab supplier	Sample preparation
Whirl-Pak® filter bags	General lab supplier	Sample preparation
2.7 µm Glass fiber filter	General lab supplier	Environmental aqueous chilling solutions preparation
500 mL graduated polypropylene beaker with handle	General lab supplier	Environmental aqueous chilling solutions preparation
Sterile 500 - 1000 mL non-polystyrene sample container	General lab supplier	Environmental aqueous chilling solutions preparation
Balance, sensitivity to at least 0.1 g	General lab supplier	Weigh samples
Blending/mixing equipment: Paddle blender or equivalent	General lab supplier	Homogenize samples
Stomacher bags	General lab supplier	Primary bag for preparation and enrichment
Vortex Mixer	General lab supplier	Vortex/mix samples
Incubators, 30 ± 2°C	General lab supplier	Incubation of primary enrichment
Incubators, 35 ± 2°C	General lab supplier	Incubation of secondary enrichment and plating media
Refrigerator (2 - 8°C)	General lab supplier	Store media and sample reserves
Heating Block, (100 ± 1°C)	General lab supplier	Prepare sample DNA
Cooling Block (20 ± 25°C)	General lab supplier	Prepare sample DNA
3M™ Molecular Detection System	3M, Model # MDS100	Screen secondary enrichment
Transfer pipet (plastic)	General lab supplier	Transfer to secondary enrichment
Sterile inoculating loops	General lab supplier	Spread/streak plates
Pipettor and sterile filter tips for 20 µL volume	General lab supplier	Add and mix reagents
UV lamp, blue light excitation 475-495 nm light	General lab supplier	Detect fluorescence in blood plates
Bruker® MALDI Biotyper	Bruker® Inc.	Proteomic Confirmation

Table 2: Kits and Reagents for MLG 8

Kits and Reagents	Supplier	Purpose
3M™ Molecular Detection Assay 2 - *Listeria monocytogenes*	3M, Catalog # MDA2LMO96	Screen secondary enrichment for *Listeria* analyses
Bruker® MALDI Biotyper reagents	Bruker Inc. or General supplier	Proteomic Confirmation
Biochemical test kit and system, GN cards VITEK® 2 System	*bioMérieux* Vitek, Inc.	Biochemical Identification (alternative confirmation technique)

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)
iii. Modified Oxford agar (MOX)
iv. Horse blood overlay agar (HL, also known as HBO)
v. Trypticase soy agar with 5% sheep blood (SBA)
vi. Brain Heart Infusion agar slants (BHI)

Media formulations are available in MLG Appendix 1, Media and Reagents.

Cultures and Controls

Analyze a positive *Listeria* control and an uninoculated media control with each sample set. Appropriate cultures include *L. monocytogenes* Microbiologics 01248UV-V (requires blue light excitation 475-495 nm light), ATCC® 19111, NCTC 7973 or other *Listeria monocytogenes* cultures validated to perform in an equivalent manner. Confirm at least one isolate from the positive control sample. In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analysis. For more information, refer to the section entitled "Lab Quality Control Procedure".

Listeria innocua negative control strain, such as ATCC® 33090, is required for HL plates.

KEY FACT

Listeria monocytogenes is a member of the *Listeria* genus, which includes other *Listeria* species such as *L. ivanovii, L. seeligeri, L. innocua,* and *L. welshimeri.* Only *L. ivanovii* and *L. monocytogenes*, both virulent strains, causes disease in animals. However, only *L. monocytogenes* typically causes disease in humans, which can be life-threatening. *L. monocytogenes* contains four evolutionary lineages (I, II, III, and IV), although most clinical and foodborne isolates of *L. monocytogenes* come from lineages I and II. Lineages III and IV are most commonly isolated from animal origins due to a difference in phenotypic and genetic characteristics between lineages I/II and III/IV. These differences also directly impact each lineage's ability to cause foodborne human illness, a primary concern of the Food Safety and Inspection Service.

Listeria monocytogenes has an optimal growth temperature range of 30-37ºC, but it can also withstand freezing. At refrigeration temperatures, *Listeria monocytogenes* forms a biofilm to protect itself from environmental stress. This biofilm also protects the pathogenic organism from many common cleaners and disinfectants, making it difficult to fully eradicate from an environment, such as in a food processing plant. For this reason, the Food Safety and Inspection Service routinely tests environmental samples from food processing establishments to monitor for any instances of *L. monocytogenes* in the establishments or on food contact surfaces.

Preparing Samples with Primary Enrichment

This procedure outlines the steps to screen and isolate *Listeria* from RTE meat, poultry, pasteurized egg products, and environmental sponges. Enriched samples are screened through 3M™ Molecular Detection System, a rapid screen technology. Samples that test positive are isolated through selective enrichment and plating media. Isolates are then confirmed by Bruker® MALDI Biotyper.

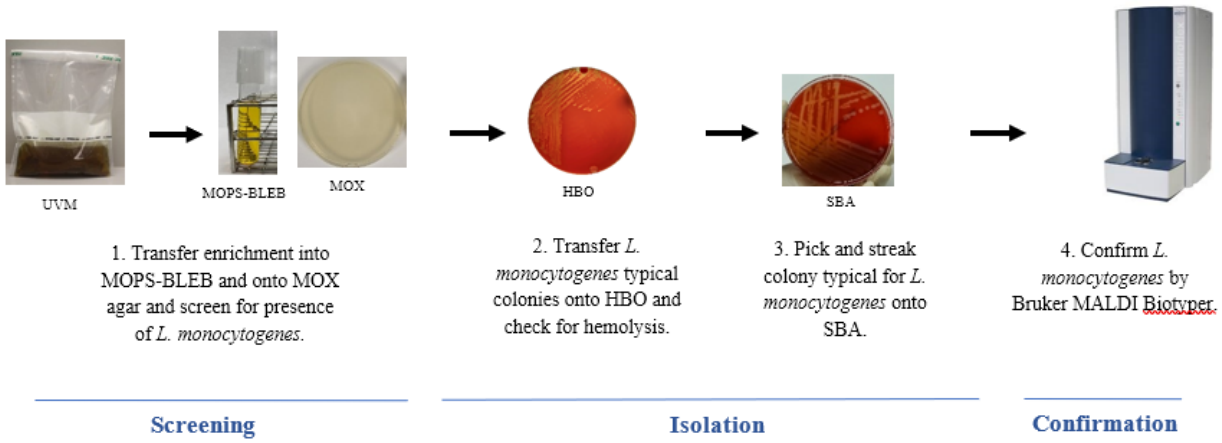


Figure 2: Overview of the steps for isolating *L. monocytogenes* from laboratory samples (Photo Credit: Leo Gude and Sherre Chambliss).

- Intact retail packages must be disinfected at the incision sites immediately prior to incision for sampling using an appropriate disinfectant, e.g., 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 or equivalent will be used for cutting the packaging. Aseptically pull the packaging away to expose the product for sampling.
- For ready-to-eat (RTE) sausages in casing, such as products involved in surface sampling, the shell/casing is an integral part of the sample and must be free of pathogens and toxins. The casing is not to be disinfected since some casings are permeable and the disinfectant can be introduced into the core of the product. 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.
    - 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.

KEY FACT

Listeria monocytogenes is commonly found in the environment and can cross-contaminate food contact surfaces and foods. It is a particular hazard of concern in RTE products that are exposed to the environment after lethality treatment. Improper sanitation, product handling, and employee practices at processing establishments can lead to the transfer of *Listeria monocytogenes* from the environment to the surface of the product.

RTE Meat, Poultry, Siluriformes Products and Pasteurized Egg Products

Follow additional program requirements for preparing sample and subsample composites. Outbreak samples will require a different sample preparation. Follow customer specifications.

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

For multi-component RTE products, follow the appropriate sample preparation instructions listed below:

- If the meat, or poultry 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 portion separate from any vegetable/dessert component, or fajita kits with meat/poultry, onions/peppers, and tortillas in three separate internal packages/bags within an outer package.
- Analysts are to prepare the *Listeria monocytogenes* enrichment sample by maximizing available surface area of the product. *Listeria* enrichment is primarily used to verify for post lethality cross-contamination, hence why an analyst should maximize a product's available surface area.
- When meat, or poultry, is combined with other ingredients to form the product (e.g., beef stew containing vegetables, potatoes, etc.), analyze representative meat/poultry portions in combination with other ingredients.
- Weigh the composite sample into a large sterile bag.
- Add ambient temperature sterile UVM. Stomach approximately two minutes.
- Incubate at 30 ± 2°C for 20 – 26 h.
- Proceed to the section entitled "Rapid Screening *Listeria monocytogenes* Test Procedure" for use of the rapid screen or refer to the section entitled "Examination of Direct MOX and MOX Plating of MOPS-BLEB" to continue the cultural analysis.

Meat, Poultry, RTE Siluriformes, and Egg Products

Figure 3: Example of 25 g of Lm product in a sterile bag (Photo Credit: Leo Gude and Sherre Chambliss).

Figure 4: Liquid whole egg products (Photo Credit: Leo Gude and Sherre Chambliss).

Since the *L. monocytogenes* sample is prepared by maximizing the surface area of the product, it is to be prepared before the *Salmonella* sample, which uses the interior of the product.

Weigh 25 ± 1 g of the product into a sterile polypropylene bag. Add a small portion of the ambient temperature sterile UVM and mix to obtain a homogeneous suspension. Add the remainder of the 225 ± 5 mL UVM. Mix until a lump-free suspension is obtained.

Incubate at 30 ± 2°C for 20 – 26 h.

Multi-Component RTE Products

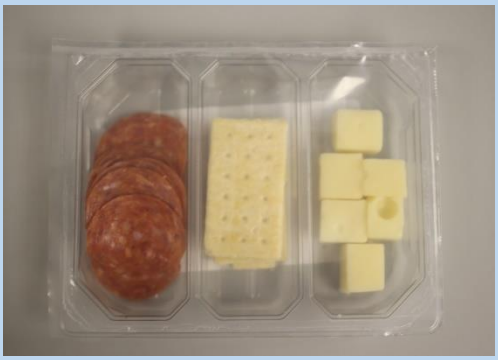
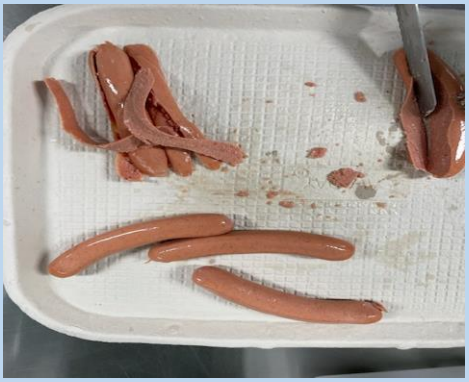


Figure 5: A multi-component RTE product (Photo Credit: Leo Gude and Sherre Chambliss).

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.

Surface Sampling



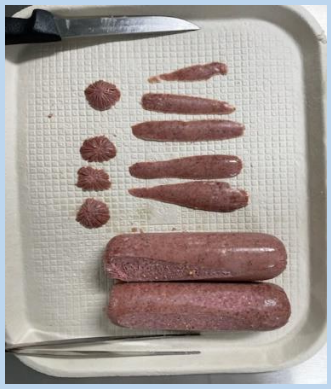


Figure 6: Examples of RTE products that require surface sampling (Photo Credit: FSIS Western Lab).

For intact RTE samples (examples: whole prosciutto, whole deli products, sausages, and hotdogs), cut thin slices from the surface of the product to maximize surface area sampled. Generally, for longer food products, slices cut longitudinally rather than horizontally will achieve maximum surface area. Do not cut deep into the tissue since *Listeria* is typically a post processing contamination event. Reserve the interior product for the *Salmonella* analysis which verifies under processing where interior product is more important.

Incubate at 30 ± 2°C for 20 – 26 h.

Environmental Aqueous Chilling Solutions Samples

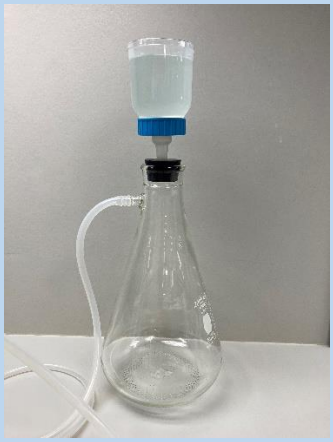


Figure 7: Example photos of how to set up for a brine sample (Photo Credit: Leo Gude and Sherre Chambliss).

For environmental aqueous chilling solutions and surface rinse solutions such as water, brine, and propylene glycol solutions, see below instructions.

Briefly:

Pour 500 ± 2 mL of sample solution into a sterile filter bag. Filter the solution by pouring it through a glass fiber filter and a 0.45 µm hydrophobic grid membrane filter in a vacuum filter system. When the sample has been completely filtered, aseptically remove both the glass fiber filter and the hydrophobic membrane filter and transfer them back to the used filter bag. These filters can be easily clogged by particulates. Therefore, it may be necessary to replace the filters during the filtration process. If more than one filtration is required, transfer all filters used into original filter bag.

Add 225 ± 5 mL (or 225 ± 5 g) of UVM broth to the bag containing the filters. Stomach 2 ± 0.2 minutes. Ensure that the filters are submerged.

Incubate the homogenate at 30 ± 2°C for 20 – 24 h.

Routine Listeria Sampling (RLm) & Intensified Verification Testing (IVT) Contact Sponges/IVT Environmental Sponges

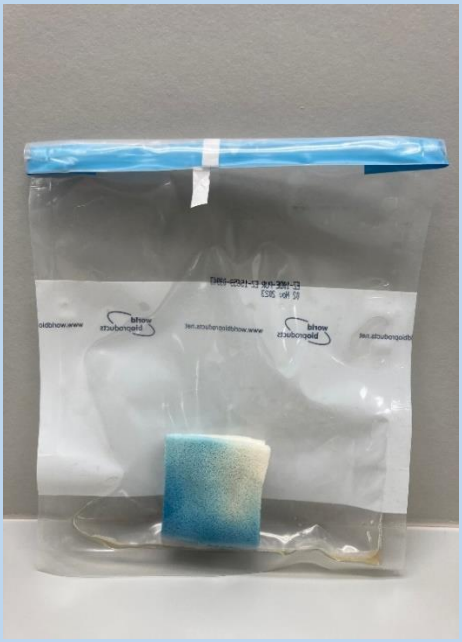


Figure 8: An RLm contact sponge (Photo Credit: Leo Gude and Sherre Chambliss).

For RLm and IVT food contact sponge samples: Add 225 ± 5 mL (or 225 ± 5 g) of UVM broth to each bagged single sponge sample.

For IVT environmental sponge samples, the sponges are not composited. Each sponge is a separate sample. Add 225 ± 5 mL (or 225 ± 5 g) of UVM broth to each bagged single sponge sample.

Incubate at 30 ± 2°C for 20 – 26 h.

For RLm Products: Add 1125 ± 5 mL (or 1125 ± 5 g) of UVM broth and 125 g product sample in a sterile WhirlPak bag.

Incubate RLm Products at 30 ± 2°C for 23 – 26 h.

RLm Environmental Sponges

Figure 9: An environmental composite sample (Photo Credit: Leo Gude and Sherre Chambliss).

For environmental composite sponge samples: Add 100 ± 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 ± 10 mL of UVM.

Incubate at 30 ± 2°C for 20 – 26 h.

Secondary Enrichment in MOPS-BLEB and Direct MOX Plating of UVM

Transfer 0.1 ± 0.02 mL of the UVM enrichment to 10 ± 0.5 mL of MOPS-BLEB. Incubate inoculated MOPS-BLEB tubes at 35 ± 2°C for 18 – 24 h.

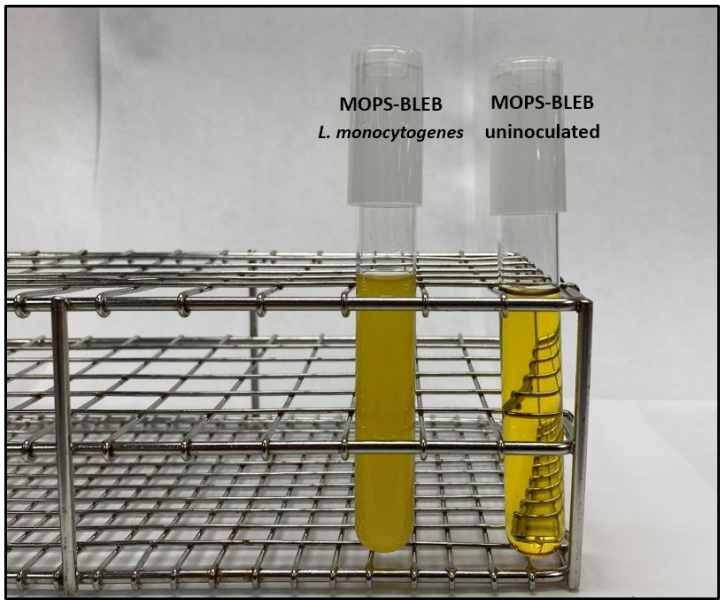


Figure 10: Morpholinepropanesulfonic acid-buffered *Listeria* enrichment broth (MOPS-BLEB) is a selective liquid medium used for the isolation and identification of *Listeria monocytogenes* from food (Photo Credit: Leo Gude and Sherre Chambliss).

Streak a MOX plate directly from the primary UVM enrichment. Streak a loopful or a drop approximating 0.1 mL of the UVM over the surface of the plate for isolation. 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 direct MOX at 35 ± 2°C for 26 ± 2 h.

Rapid Screening *Listeria monocytogenes* Test Procedure

Following incubation of MOPS-BLEB, perform the rapid screen on the secondary enrichment using the current 3M™ Molecular Detection System User guide, or equivalent rapid screen technology for preparing reagents, performing the remainder of the assay, and reading the results.

a) Samples that are rapid screen-negative will be reported as negative for *Listeria monocytogenes* if the concurrent 26 ± 2 h direct (UVM-streaked) MOX plate is negative. Cultural analysis will continue for samples that are rapid screen-negative but have typical colonies on the 26 ± 2 h direct MOX plates or have a rapid screen positive or inconclusive result.

b) Alternatively, for samples with a rapid screen-inconclusive or an invalid result, the laboratory is to investigate. Based on the findings, the laboratory shall analyze the inconclusive result samples by:

- repeating the rapid screen analysis from the lysate step or
- preparing new rapid screen lysate tubes and repeating the analysis

If reanalysis of an inconclusive rapid screen sample is unsuccessful, repeat sample preparation from the sample reserve or discard the sample.

c) In analytical runs where the positive control results are NOT positive, all samples are affected, and an investigation shall be performed. Based on the findings the laboratory must:

- repeat the rapid screen analysis from the lysate step
- prepare new rapid screen lysate tubes and repeating the analysis or
- analyze all the samples culturally.

d) If circumstances (e.g., a power outage or equipment failure) do not allow testing using the rapid screen system, the laboratory shall continue cultural analysis by streaking all samples and controls from MOPS-BLEB medium onto MOX plates and then proceed with all isolation and identification procedures.

Examination of Direct MOX and MOX Plating of MOPS-BLEB

- Examine the direct (UVM-streaked) MOX plates for colonies typical of *Listeria* spp. At 26 ± 2 h, suspect colonies are typically small (ca. 1 mm) and are surrounded by a zone of darkening due to esculin hydrolysis.
    - If suspect colonies are present on direct MOX, transfer suspect colonies to HL agar then proceed with the isolation and purification procedure.
    - If there are no typical colonies on 26 ± 2 h incubated direct MOX plate and the rapid screen result is negative, then the direct plating result is negative.
    - If there are no typical colonies on the direct MOX plate but the rapid screen result is positive/inconclusive or no rapid screen was performed, then re-incubate the MOX plate for an additional 26 ± 2 h.
        - After re-incubation, suspect colonies are transferred to HL agar. The direct branch result is negative if there are no typical colonies on MOX after re-incubation.
- After the 18 – 24 h of secondary enrichment in MOPS-BLEB at 35 ± 2°C (from the "Secondary Enrichment in MOPS-BLEB and Direct MOX Plating of UVM" section), rapid screen positive samples are streaked from MOPS-BLEB onto MOX. Inoculate a MOX plate for isolation using a loopful of the MOPS-BLEB, or by streaking a drop approximating 0.1 mL MOPS-BLEB, 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.
    - After inoculating the plate, use a loop to streak for isolation from the swabbed area onto the remainder of the plate. Then, incubate the secondary enrichment MOX plate at 35 ± 2°C for 26 ± 2 h.
        - If typical colonies are present on the secondary enrichment MOX (small colonies surrounded by a zone of darkening due to esculin hydrolysis), transfer suspect colonies to HL agar to proceed with the isolation and purification procedure.
        - If no suspect colonies are evident on secondary enrichment MOX plate, then re-incubate the MOX plate for an additional 26 ± 2 h. After re-incubation, suspect colonies are transferred to HL agar. The secondary enrichment result is negative if there are no typical colonies on MOX after re-incubation.
- If no suspect MOX or HL colonies have been demonstrated on the direct (UVM streaked) MOX plate or the secondary enrichment (MOPS-BLEB streaked) MOX plate, the sample is considered negative for *Listeria monocytogenes*.

Isolating and Confirming *Listeria monocytogenes* and Further Analysis

1) 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. Streak *Listeria innocua* as a negative control on a HL plate. Incubate the streaked HL plates at 35 ± 2°C for 22 ± 4 h.

Helpful Tip: *L. innocua* analysis begins and ends with HL plates. It is not required to proceed with *L. innocua* to biochemical testing, although the analyst may choose to do so if atypical *L. monocytogenes* strains are found to be present in the samples.

2) After incubation, examine the HL plate(s) against backlight for translucent colonies surrounded by a small zone of β-hemolysis.

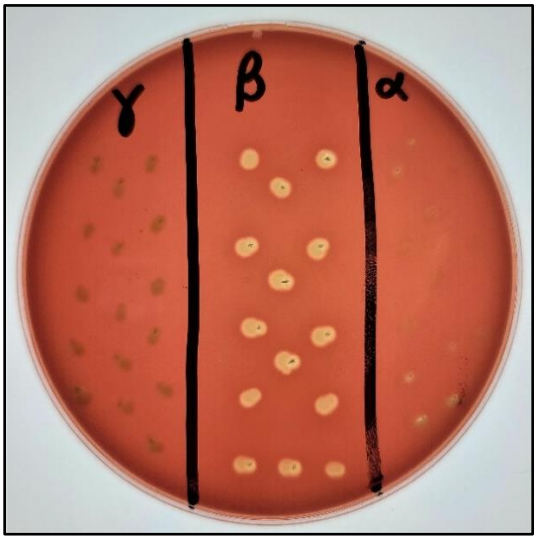


Figure 11: Example of an HL plate streaked with α, β, and γ hemolytic *Listeria monocytogenes* (Photo Credit: Leo Gude and Sherre Chambliss).

a. If at least one suspect colony is clearly isolated, proceed to confirmatory testing. Hold all HL plates containing suspect colonies (room temperature or refrigeration) until confirmatory testing is complete.
b. 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 at 35 ± 2°C for 22 ± 4 h.
c. If a suspect isolate is an atypical strain, re-test the isolate using the approved FSIS validated rapid screening technology. To prepare the sample for testing, add a portion of the atypical isolate to 50 µL of Molecular Grade PCR Water and mix. Then follow the rapid screening technology's instructions to analyze the sample.

KEY FACT

The definition of an atypical *Listeria monocytogenes* isolate is a rapid screening positive result, typical colonies on the MOX plate, and non-β-hemolytic result on the HL plate.

d. If no suspect isolates are present on HL, pursue follow-up of MOX or HL isolates from other branches of analysis (e.g. Rapid screen follow-up vs. UVM Primary Enrichment 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*. Samples are considered presumptive positive when they produce suspect β-hemolytic colonies on HL plates.

Helpful Tip: Removal of a few colonies may assist the analyst in observing medium clearing for weakly hemolytic strains. Samples are considered presumptive positive when they produce suspect β-hemolytic colonies on HL plates.

3) Pick typical presumptive positive isolated colony(s) from HL and streak onto a purity HL plate and an SBA plate or equivalent for confirmation testing. Incubate plates at 35 ± 2°C for 16 – 24 h.
   a. 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.

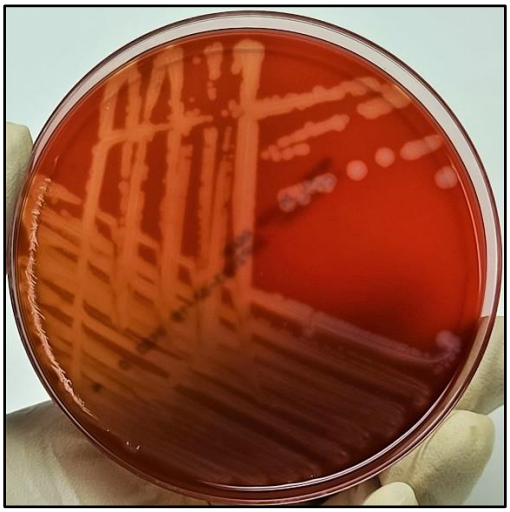


Figure 12: Example of an SBA plate streaked with *Listeria monocytogenes* (Photo Credit: Leo Gude and Sherre Chambliss).

4) Perform confirmatory tests using a single isolated colony. If using UV positive controls, perform a fluorescence check on the SBA plate for the positive control and presumptive positive sample following incubation. Use long wave UV light to examine plates for purity and evidence of cross contamination with the positive control. Only the positive control culture should fluoresce. If the presumptive positive sample SBA plates are pure and uncontaminated, perform the proteomic confirmation method.
   b. Commercially available test systems such as Bruker® MALDI Biotyper or validated equivalent systems are to be employed. Refer to the manufacturer's instructions for the use of the instrument, preparation of reagents, and troubleshooting guidance.

KEY DEFINITION

Bruker® Matrix-Assisted Laser Desorption Ionization (MALDI) Biotyper (MBT): A method of ionization for mass spectrometry, commonly applied to the analysis and identification of biomolecules.

c. This method allows for the use of each available preparation method (direct, extended direct, and tube extraction) as needed to identify organisms. Refer to the manufacturer's documentation for a full list of organism coverage and thresholds.

4) If an isolate or any presumptive positive colony picks from SBA are **inconclusive**, then the isolates are submitted to Whole Genome Sequencing. Any **confirmed** *Listeria monocytogenes* isolates will be further characterized using Antimicrobial Susceptibility Testing (AST), and Whole Genome Sequencing (WGS).

Confirmation Criteria

A genus-level identification requires a minimum score of 1.70, and a species-level identification requires a minimum score of 2.00 when using the Bruker® MALDI Biotyper.

Culture Storage and Shipment:

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

Biosafety Chart

SAFETY INFORMATION AND PRECAUTIONS

1. Required protective equipment: Nitrile or latex gloves, lab coat, safety glasses
2. Hazards

Procedure Step	*Hazard*	*Recommended Safety Procedures*
Preparing samples with Primary Enrichment- Disinfecting intact retail packages with ethanol	Ethanol is acutely toxic by oral, dermal, or inhalation means. Highly flammable.	Wear gloves when using ethanol and avoid breathing the fumes directly. Avoid handling ethanol around heat or open flame.
Preparing samples with Primary Enrichment- Spraying down work area with bleach	Bleach is corrosive to the skin with prolonged exposure and may cause skin irritation with short-term exposure. Serious eye damage or eye irritation can occur if accidentally sprayed near eyes.	Wear gloves, safety glasses, and a lab coat when using bleach to prevent dermal or eye exposure.
Isolating and Confirming *Listeria monocytogenes*- Using an Ultraviolet (UV) Lamp	Using UV lamps can cause eye irritation and/or damage if eyes are directly exposed. The cornea of the eye can become inflamed or burned. Prolonged UV lamp exposure can also cause skin irritation and/or damage such as burns.	Refrain from staring directly at the UV lamp when in use. Use gloves when fluorescing samples under the UV lamp. Turn off the UV lamp when not in use.
Rapid Screening, Isolation, and Confirmation of *Listeria Monocytogenes*- Risk to Pregnant and/or Immunocompromised Individuals	Pregnant women are more susceptible to listeriosis than other healthy adults. Listeriosis may cause miscarriage, premature labor, birth defects, or infant death. Listeriosis can also lead to severe illness or death of the pregnant individual. Listeriosis can cause severe illness or death in immunocompromised individuals.	Follow CDC guidelines for manipulating Biosafety Level 2 (BSL-2) pathogens. Pregnant women and immunocompromised individuals should avoid working with *Listeria monocytogenes* at all. Laboratories should inform pregnant women and immunocompromised individuals of the risks of working with *Listeria monocytogenes*. Reasonable accommodations should be made to ensure the safety of individuals in these health groups.

Appendix A: Alternative Methods

1) Methodology Variation

- In some rare instances, (hazardous weather or other unanticipated lab closures) it may be necessary to incubate MOX plates for 48 h without recording the 24 h results. However, the initial transfer of 0.1 mL of enriched UVM into MOPS-BLEB **MUST BE** completed at the specified time interval of 22 ± 2 h.

2) Biochemical Confirmation

- FSIS laboratories can elect to use biochemical confirmation methods (VITEK® 2) for reasons including: Bruker® MALDI MBT is unavailable, interruption in reagent supply chain, or results comparison. To biochemically confirm isolates, inoculate appropriate VITEK® 2 cards (if using VITEK® 2 Compact) or equivalent.
    - Using a pure culture, perform confirmatory biochemical tests. Commercially available test systems (e.g. VITEK® 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 submitted to Whole Genome Sequencing.

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VITEK® 2 Compact User Guide

Contact Information and Inquiries

Inquiries about methods can be submitted through the USDA website via the "Ask USDA" portal at https://ask.usda.gov or please contact:

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This method has been validated, reviewed, approved, and deemed suitable and fit for purpose for use in the USDA FSIS Field Service Laboratories.

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