United States Department of Agriculture

Food Safety and Inspection Service

Office of Public Health Science

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# Laboratory Guidebook
# Notice of Change

Chapter new, **revised**, or archived: MLG 8.13

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

Effective Date: 10/01/2021

Description and purpose of change(s):

Changes made to provide procedural clarifications including:

- Added section about cross-sectioning during sample prep.
- Added reference to methodology variation in event of lab closure.
- Added detailed section on the preparation of environmental aqueous chilling and surface rinse solutions.

# United States Department of Agriculture
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| MLG 8.13 | |  |
|---|---|---|
| Title: Isolation and Identification of *Listeria monocytogenes* from Red Meat, Poultry and Egg Products, Ready-To-Eat, Siluriformes (Fish) and Environmental Samples | | |
| Revision: .13 | Replaces: .12 | Effective: 10/01/2021 |

**Procedure Outline**


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## 8.1 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 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 method for that analyte and sample matrix as outlined in the corresponding MLG chapter. Method validation is necessary to demonstrate the equivalence of alternative tests as detailed in the document titled "FSIS Guidance for Evaluating Test Kit Performance."

*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 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 0.5 McFarland 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.

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, Test Systems and Cultures

### 8.4.1 Equipment

a. Electronic top-loading balance capable of weighing a minimum of 25 ± 0.1 g (500 g capability recommended)
b. Paddle blender or blade type blender with sterilized blender cutting assemblies and jars
c. Incubator, 30 ± 2°C
d. Incubator, 35 ± 2°C
e. Vortex mixer
f. UV lamp, blue light excitation 475-495 nm light
g. 3M™ Molecular Detection System
    - 3M™ Molecular Detection Heat Block Insert MDSHBIN

- 3M™ Molecular Detection Speed Loader Tray MDSSLT
- 3M™ Molecular Detection Cap/ Decap Tool-Lysis
- 3M™ Molecular Detection Chill Block Insert MDSCBIN held at 20-25°C
- VWR Digital 2 Block Heater or equivalent set at 100 ± 1°C
- Pipettor to deliver 20 µL and sterile disposable filter tips
- Eight channel Pipettor to deliver 20 µL and sterile disposable filter tips

h. Bruker® MALDI Biotyper, see manufacturer's instructions

### 8.4.2 Supplies

a. Supplies for all samples:

i. Sterile tablespoons, scissors, forceps, knives, glass stirring rods, petri dishes, test tubes, bent glass rods ("hockey sticks") as needed
ii. Sterile, filter or non-filter bags
iii. Non-filter plastic bags for double-bagging sample homogenates (optional)
iv. Pipettor and sterile disposable tips for dispensing 100 µl and 1 ml
v. Disposable plastic and/or platinum inoculating needles and loops
vi. Sterile cotton-tipped applicators (i.e., swabs)

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)
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)

### 8.4.4 Reagents and Test Systems

a. Biochemical test panel (VITEK® 2 Compact System or equivalent)
b. 3M™ Molecular Detection Assay 2 – *Listeria monocytogenes*
c. Bruker® MALDI Biotyper reagents, see manufacturer's instructions

### 8.4.5 Cultures

a. At least one *Listeria monocytogenes* positive control strain is required. 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.

b. *Listeria innocua* negative control strain is required for HL plates.

## 8.5 Detection and Isolation Procedures

### 8.5.1 Sample preparation

a. Meat, poultry, and RTE Siluriformes products: A 25 ± 1 g portion is analyzed for meat, poultry, and Siluriformes testing or a 125 ± 5 g portion for programs allowing compositing of five product subsamples. A 25 ± 1 g portion is used for pasteurized egg products testing. Refer to Table 1.

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. 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.

*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.

Therefore, it is important for analysts to maximize surface area when preparing RTE samples for *Listeria monocytogenes* enrichment. In particular, for intact RTE samples (examples: whole prosciutto, whole deli products, hotdogs), cut thin slices length-wise from the surface of the product to maximize surface area sampled. Do not cut discs since this does not maximize surface area for these types of RTE products

Analysts are to prepare the *Listeria monocytogenes* enrichment sample first maximizing available surface area and then move to

the *Salmonella* enrichment sample preparation from RTE samples. *Salmonella* enrichment is primarily used to verify whether a product was under processed so a preparation including at least some interior product is advantageous.

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 ≤ -10°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.

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 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 per sponge | 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 approximately 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.

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

b. For environmental sponge samples:

Add 225 ± 5 ml (or 225 ± 5 g) of UVM broth to each bagged single sponge sample or 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. Stomach for 2 ± 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 ± 2°C for 20 -26 h. Proceed to Section 8.5.3.

For environmental aqueous chilling solutions and surface rinse solutions such as water, brine and propylene glycol solutions, see Section 8.9 for detailed procedure. Briefly:

i. 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.

ii. Add 225 ± 5 ml (or 225 ± 5 g) of UVM broth. Stomach 2 ± 0.2 minutes. Ensure that the filters are submerged. Incubate the homogenate at 30 ± 2°C for 20 - 24 h. Proceed to Section 8.5.3.

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

a. 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. Proceed to Section 8.5.4 for use of the rapid screen assay on MOPS-BLEB tubes or refer to Section 8.5.5.b to continue the cultural analysis.

b. 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. Proceed to Section 8.5.5.a for examination of direct MOX plates.

### 8.5.4 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 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 hour direct (UVM-streaked) MOX plate is negative. Cultural analysis will continue on samples that are rapid screen-negative but have typical colonies on the 26-hour 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 may investigate. Based on the findings, the laboratory may 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

c. In analytical runs where the positive control results do not show as positive, all samples are affected and an investigation shall be performed. Based on the findings the laboratory may analyze the samples by:
- repeating the rapid screen analysis from the lysate step
- preparing new rapid screen lysate tubes and repeating the analysis or
- analyzing all of the samples culturally.

d. If reanalysis of an inconclusive rapid screen sample is unsuccessful, repeat sample preparation from the sample reserve or discard the sample. If circumstances (e.g. a power outage or equipment failure) do not allow testing using the rapid screen system, the laboratory shall complete the

cultural method by streaking all samples and controls from MOPS-BLEB medium onto MOX plates per MLG 8 Section 8.5.5.b then proceed with all isolation and purification procedures.

### 8.5.5 Examination of Direct MOX and MOX Plating of MOPS-BLEB

a. Examine the direct (UVM-streaked) MOX 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.

   i. If suspect colonies are present on direct MOX, transfer suspect colonies to HL agar as described in Section 8.5.6 to proceed with isolation and purification procedure.

   ii. If there are no typical colonies on 26 ± 2 hour incubated direct MOX plate and the rapid screen result is negative, then the direct branch result is negative.

   iii. 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 hour.

   After re-incubation, suspect colonies are transferred to HL agar as described in Section 8.5.6. The direct branch result is negative if there are no typical colonies on MOX after re-incubation.

b. After 18-24 h of secondary enrichment in MOPS-BLEB at 35 ± 2°C, 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 hours.

i. 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 as described in Section 8.5.6 to proceed with isolation and purification procedure.

ii. If no suspect colonies are evident on secondary enrichment MOX plate, then re-incubate the MOX plate for an additional 26 ± 2 hour. After re-incubation, suspect colonies are transferred to HL agar as described in Section 8.5.6. The secondary enrichment result is negative if there are no typical colonies on MOX after re-incubation.

c. If no suspect MOX and/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*.

### 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. Streak *Listeria innocua* as a negative control on a HL plate. Incubate the streaked HL plates at 35 ± 2°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.

If using UV positive controls, perform a fluorescence check on the HL 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.

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 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. 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*.

Note: 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.

## 8.6 Isolate Confirmation Procedure

Pick typical presumptive positive isolated colony(s) from HL and streak onto SBA or equivalent for confirmation testing. Incubate SBA plates at 35 ± 2°C for 16-24 hr.

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.

Bruker® MALDI Biotyper or other commercially available, validated, and equivalent test systems are to be employed.

Refer to manufacturer's instructions for the use of the instrument, preparation of reagents, and troubleshooting guidance. 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.

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.

If an isolate or any presumptive positive colony picks from SBA are **inconclusive**, then the isolates are submitted to Whole Genome Sequencing.

## 8.7 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 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.

## 8.8 Appendix: Alternative Method(s)

### 8.8.1 Methodology Variation

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

### 8.8.2 Biochemical confirmation

FSIS laboratories may elect to use biochemical confirmation methods (VITEK® 2) for reasons including: Bruker® MALDI Biotyper is unavailable, interruption in reagent supply chain, or results comparison.

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.

To use the VITEK® 2 Compact System for biochemical testing:

a. 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 biochemical test.

b. Follow the instructions provided by the manufacturer for inoculation and loading the VITEK® 2 Compact System.

c. SBA or HL agar may be used for preparing the inoculum suspension. All growth on the agar of choice must represent the same clone.

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

## 8.9 Appendix: Preparation of Environmental Aqueous Chilling and Surface Rinse Solutions

### 8.9.1 Additional Supplies

a. 2- 2 L filtering flasks (autoclaved)
b. 2- rubber stoppers (autoclaved)
c. 1- filter funnel adapter- sterile

d. 1- filter funnel with 0.45µm hydrophobic grid membrane filter - sterile
e. 3- approx. 2-3ft. of tubing
f. 1- vacushield vent device
g. 1- 10 mL graduated pipette
h. glass microfiber filters-diameter 47mm (autoclaved)
i. Millipore Sigma vacuum-pressure pump or equivalent
j. 1- 500 mL glass bottle (autoclaved)
k. sterile forceps

**8.9.2 Filtration Configuration**

The overall configuration is shown in Figure 1.

a. Obtain a 10 mL graduated pipette (G) and insert it into a rubber stopper (B1). Use this to plug the top of a 2 L filtering flask (A1). Alternatively, this flask can be omitted or substituted with a flask with two tube ports.

b. Obtain a second 2 L filtering flask (A2) and plug with a rubber stopper (B2) insert a filter funnel adapter (C) into the stopper (B2).

c. Obtain a sterile filter funnel (D) and unscrew the bottom. Carefully insert an autoclaved glass microfiber filter (H) with sterile forceps and screw the bottom back on. Place the filter funnel (D) into the adapter (C).

d. Insert approx. 2-3ft. of tubing (E1) into the vacuum trap of A2 and connect to the graduated pipette (G) atop A1. If only one flask is being used, A2 is connected directly to E2 tube described below.

e. Insert approx. 2-3ft. of tubing (E2) into the vacuum trap of A1 and insert into the front of a vacushield vent device (F) labeled “fluid side.” Insert one end of tubing (E3) into the back of the vacushield and the other end into the vacuum (I). The apparatus set up is now complete.

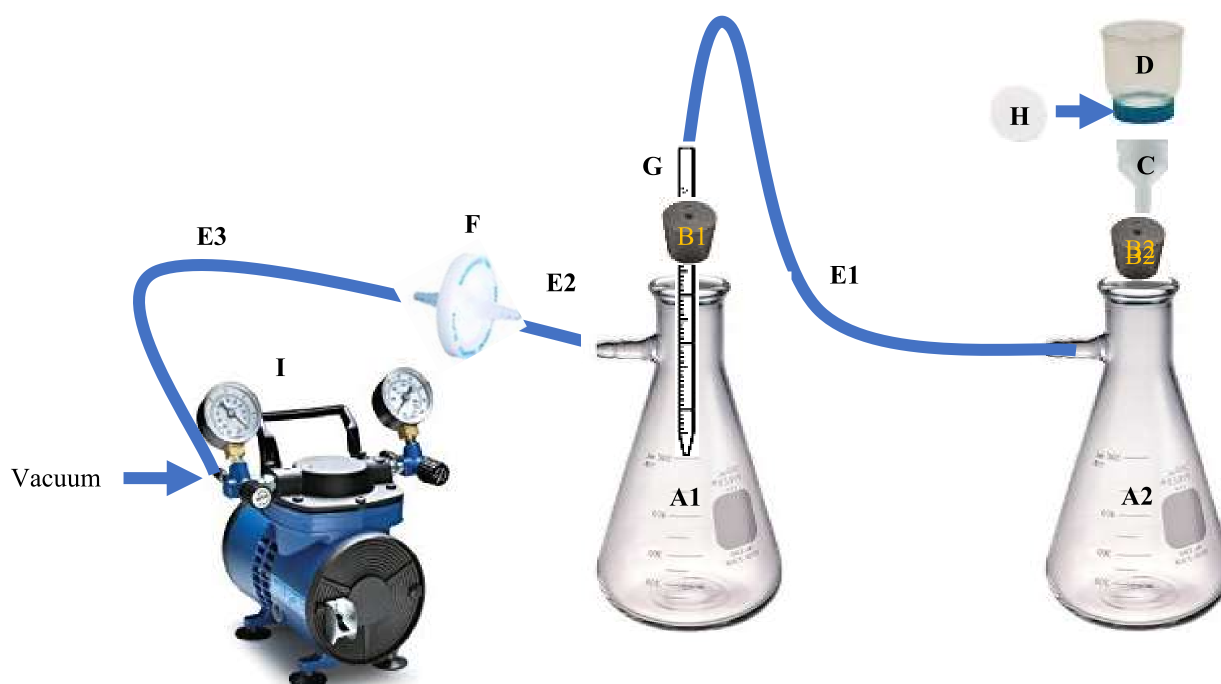


Figure 1. Vacuum filtration configuration for sample preparation of environmental aqueous chilling and surface rinse solutions.

**8.9.3 Sample Preparation**

a. Pour 500±2 ml of the brine sample, environmental aqueous chilling solution or surface rinse 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 the vacuum filter system by following the steps below.

b. Remove the top cap to the filter funnel (D) and switch the pump on. Slowly pour out the premeasured sample into the filter funnel. Once filtration is complete, remove the used glass fiber filter and hydrophobic membrane filter and add both to the original filter bag from step a.

i. If the filters become clogged during this procedure, it might prevent a steady flow of liquid. Switch the vacuum off once the liquid filters through.

ii. Collect clogged filter(s) and replace either the glass fiber filter (H) or the entire filtration system (D + H) and continue pouring the remaining liquid. Do this as many times as needed. All filters should be placed in the enrichment bag as they are removed.

c. Add 225 ± 5 mL of UVM enrichment broth to bag containing the filters and stomach for 2 ± 0.2 minutes. Ensure that the filters are submerged. Incubate at 30 ± 2ºC for 20-24h.

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