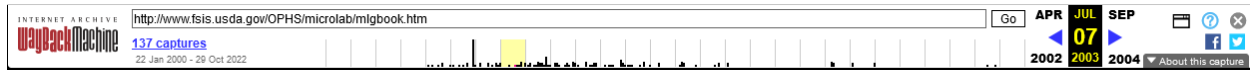


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FOOD SAFETY AND INSPECTION SERVICE
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Microbiology Laboratory Guidebook

The Guidebook contains current protocols for analytical tests required by FSIS regulatory activities on meat, poultry and egg products. Specifically, microbiological methods are presented for sample preparation, isolation and identification of the major foodborne pathogenic microorganisms and their toxins, meat tissue species identification, and the detection of extraneous materials and antimicrobial residues. Media and reagent formulations, and Most Probable Number Tables are contained in an appendix.

*All methods are offered as PDF documents. The documents were created using Adobe Acrobat version 5.0; however, they may not be fully accessible to users of assistive technology. If you require an alternative format, please contact: Microbiology Branch, Laboratory QA/QC Division, USDA/FSIS/OPHS, 950 College Station Road, Athens, GA 30629, Phone (706) 546-3449

*Note: To read and print a PDF file, you must have the [Adobe® Acrobat® Reader](#) installed on your PC. You can download a version suitable for your system, free of charge, from the Adobe Home Page. Adobe also provides tools and information to help make Adobe PDF files accessible to users with visual disabilities at <http://access.adobe.com>.

Chapters Available On This Site

[COVER, FOREWORD, GENERAL CONSIDERATIONS](#)

1. [SAMPLE PREPARATION FOR MEAT, POULTRY AND PASTEURIZED EGG PRODUCTS](#)
2. [PHYSICAL EXAMINATION OF MEAT AND POULTRY PRODUCTS](#)
3. [EXAMINATION OF FRESH, REFRIGERATED AND FROZEN PREPARED MEAT, POULTRY AND PASTEURIZED EGG PRODUCTS](#)

The Wayback Machine - <https://web.archive.org/web/20030707231919/http://www.fsis.usda.gov:80/Ophs/...>



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- CHAPTER NUMBERS 22–31 RESERVED FOR FUTURE USE.
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 - PART A Detection of Light Filth in Prepared Infant Foods Containing Meat and Poultry
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 - PART C Tentative Confirmation of CAST Results for Sulfonamide Residues in Meat and Poultry Tissue
 - PART D Detection of Antimicrobial Residue By Fast Antimicrobial Screen Test (FAST)

PART E Evaluation of Antimicrobial Residues in Meat and Poultry Tissue By A
Modified Fast Antimicrobial Screen Test (M-FAST)

34. BIOASSAY FOR THE DETECTION, IDENTIFICATION AND QUANTITATION OF
ANTIMICROBIAL RESIDUES IN MEAT AND POULTRY TISSUE
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35. [DETECTION OF ANTIBIOTIC AND SULFONAMIDE RESIDUES IN MEAT TISSUE
BY COMMERCIALLY AVAILABLE IMMUNOASSAY KITS](#)
36. [EQUIPMENT CALIBRATION, MAINTENANCE, AND PERFORMANCE
VERIFICATION](#)
(Revision #1; 7/11/00)

APPENDIX: VOLUMES 1.02 & 2.02

[MEDIA AND REAGENTS](#) (46 pp.)

[MOST PROBABLE NUMBER TABLES](#) (9 pp.)

Pen and Ink Changes

September 25, 2000: FSIS has also approved and issued the following changes to specific sections in other chapters of the 3rd Edition, 1998 Microbiology Laboratory Guidebook (MLG). These specific word changes to original referenced sections are as described below:

MLG 3rd edition, 1998, **Chapter 34, Section 34.838 Confirmatory Identification of Tetracycline, page 34-41.** Substitute the following paragraph:

To distinguish between the three tetracyclines, confirmatory identification can be achieved by thin layer chromatographic analysis outlined in **Section 34.9 Identification of Tetracyclines in Tissue by Thin Layer Chromatography** or by chemical confirmation using HPLC following FSIS chemical method "Analysis of Tetracyclines in Beef, Pork, and Poultry Tissues."

October 2, 2000: FSIS has also approved and issued the following changes to specific sections in other chapters of the 3rd Edition, 1998 Microbiology Laboratory Guidebook (MLG). These specific word changes to original referenced sections are as described below:

MLG 3rd edition, 1998, **Chapter 34, Section 34.837 Tentative Identification of Tetracycline in Tissue from the Seven Plate Bioassay System, page 34-41.** Add the following paragraph:

If the zone(s) of inhibition on the BC plate indicate(s) the concentration of tetracycline to be below the cutoff level for that species and tissue, then the test code 2298 (generic tetracycline) is assigned and the sample is considered a non-violation and the analyst does not proceed with further testing (confirmatory identification and quantitation). The cutoff level is the smallest BC zone size, specific for tissue and animal species, that will result in a calculated violative level for any of the three tetracyclines, minus two (2) millimeters. If the zones of inhibition indicate the presence of tetracycline at or above the cutoff level, then confirmatory identification and quantitation analysis is performed as outlined in **Sections 34.838 and 34.839** respectively.

Printed Version

Due to various chapter revisions and pen and ink changes made to the paper version

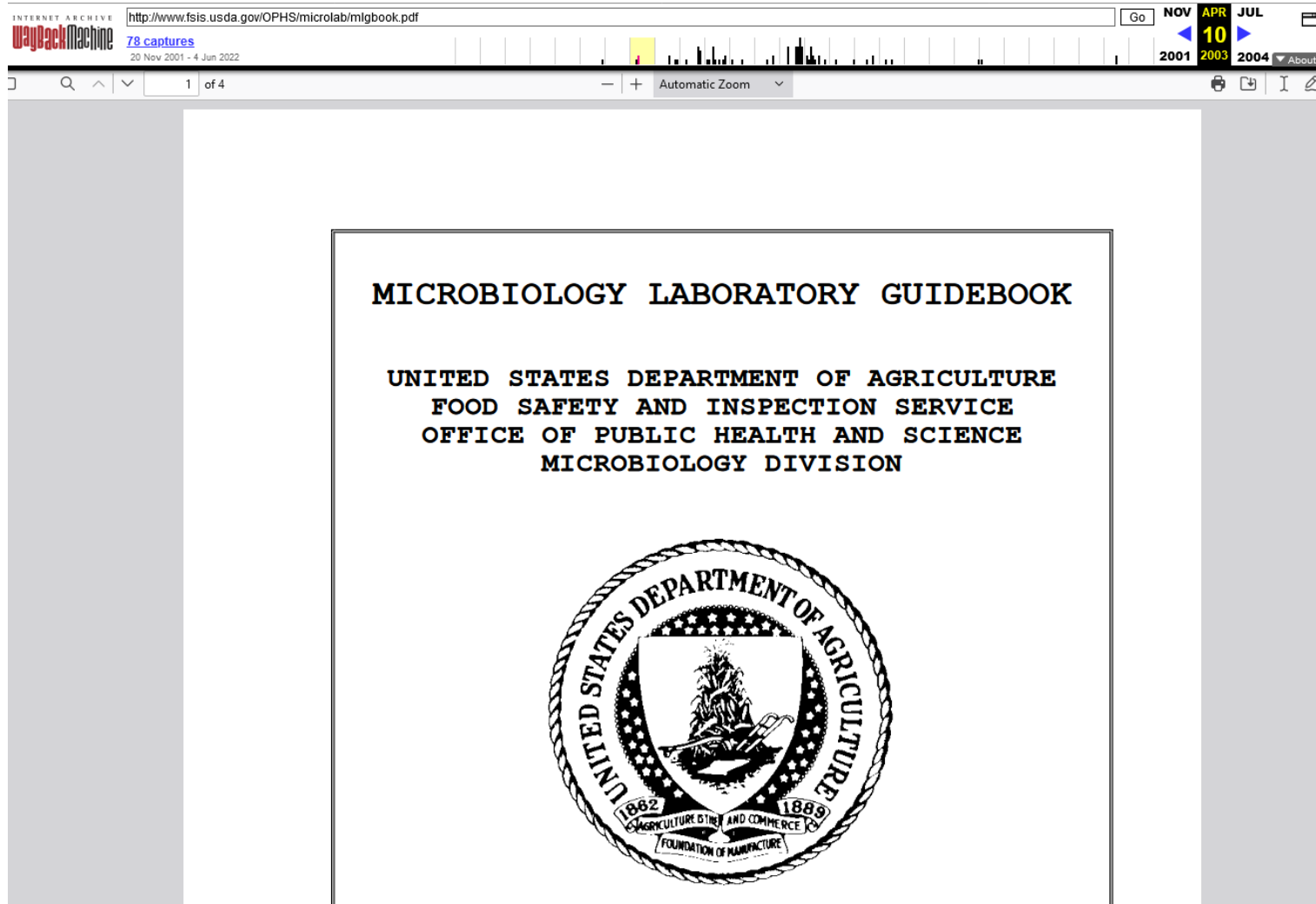
Microbiology Laboratory Guidebook publication, it is not completely up to date and is no longer available for commercial purchase.

Current chapters are now posted on this web site.

[Learn more](#) about the Office of Public Health and Science.

URL: <http://www.fsis.usda.gov/ophs/Microlab/mlgbook.htm>

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MICROBIOLOGY LABORATORY GUIDEBOOK

UNITED STATES DEPARTMENT OF AGRICULTURE
FOOD SAFETY AND INSPECTION SERVICE
OFFICE OF PUBLIC HEALTH AND SCIENCE
MICROBIOLOGY DIVISION



B. P. DEY, DVM, MS, MPH, Ph.D., Editor
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Editorial Board

A. M. McNAMARA, Sc.D., R. P. MAGEAU., Ph.D. and
S. S. GREEN., Ph.D.

3RD EDITION, 1998
VOLUMES 1 & 2

FOREWORD

The 1993 *Escherichia coli* O157:H7 outbreak in the Pacific Northwest focused national attention on food safety. Since then, the number of requests for reprints on analytical methods used by the Microbiology Division, Office of Public Health and Science, Food Safety and Inspection Service, United States Department of Agriculture, has increased dramatically. Scientists within the Division have responded to these requests by completely revising and updating our Microbiology Laboratory Guidebook (MLG) for publication.

This MLG is our laboratory guidebook for the microbiological analysis of meat, poultry, and egg products that fall under the jurisdiction of USDA. It contains methods that FSIS prefers to use for the analysis of these foods. Since USDA does not endorse or approve methods for use by the food industry, inclusion of a particular method in the MLG should not be construed in this manner. Similarly, the mention of specific brand or trade names for a product, medium, chemical or reagent associated with methods contained herein does not constitute endorsement or selectivity by the authors or USDA over similar products that might also be suitable.

The use of the MLG comes with several caveats. This guidebook was written for microbiologists, and its interpretation and use should only be undertaken by trained microbiologists. FSIS assumes no responsibility for any economic, personal injury or other damage that may occur to individuals or organizations because of the use of methods contained in this guidebook. Users should note and pay particular attention to the safety caution symbol (†) and written warnings associated with certain hazardous chemicals or dangerous biological materials used in some of the methods. Users must act in a responsible manner at all times to protect themselves and the environment during performance of these methods. This guidebook must be supplemented with quality assurance and quality control programs as well as chemical, biological, and employee safety hazards management programs in order to operate a microbiology laboratory. These programs are beyond the scope of this guidebook and are the sole responsibility of the user to develop and implement.

This guidebook contains protocols for analytical tests that are required by FSIS regulatory activities. Some protocols, such as the Bioassay procedure for antibiotic residue detection and quantitation, may not be of value to commercial laboratories nor do we expect others to try to commercialize them. They are included here primarily as informational material since they are part of our current analytical methods.

The 1998, 3rd edition MLG publication consists of two separate volumes with a newly revised format utilizing a loose-leaf binder.

This format should make the updating of chapters easier by allowing the substitution of a single chapter or page versus reprinting of the entire MLG. Because we anticipate the addition of new materials, the chapter numbers between volumes are not continuous in order to accommodate all changes.

Publishing this new 3rd edition MLG replaces all previous MLG versions and supersedes all Laboratory Communications, which should be discarded.

Finally, to produce a work of this magnitude requires a team of dedicated scientists and support staff. I would like to thank the following people for their efforts: Larry H. Dillard, Joseph Y. Chiu and James G. Eye for coordinating the FSIS Technical Support Laboratory reviews of the manual; Microbiology Division staff members Bhabani P. Dey, Stanley S. Green, Charles P. Lattuada, Bonnie E. Rose, Richard P. Mageau, and Gerri M. Ransom for composing, editing and proofreading many chapters; and Julie M. Hall for providing secretarial support in typing most of the chapters under trying conditions and meeting the demands of a diverse group of scientists.

Ann Marie McNamara, Sc.D.
Director
Microbiology Division
Office of Public Health and Science
Editorial Board, MLG

January 1998

GENERAL CONSIDERATIONS

Before any analyst attempts to perform the microbiological methods contained within this Microbiology Laboratory Guidebook (MLG), it might be helpful to call attention to the following general considerations in the use of this guidebook.

In order to maximize the achievement of successful results when using the various methods in this MLG, it should be clearly understood that all methods and procedures should be performed at all times in a manner as close as possible to the prescribed directions. Particular attention should be paid to all details provided in a given analytical procedure. Changes or shortcuts should not be attempted in a method simply to accommodate factors, for example, such as processing a large number of similar samples through the method at the same time.

All chemicals, media, immunoreagents and commercial test kits should be within current shelf expiration dates and be subjected to quality control and quality assurance procedures to insure their proper performance for their intended purpose and use within the methods presented in this MLG. All instrumentation should be subjected to continuous maintenance and appropriate quality control procedures to insure unquestionably correct performance during use in all methods. The use of positive and negative test controls at all times, as specified for a given procedure, should be implemented. Adequate documentation and record keeping should be employed for all analytical results, test controls, quality assurance and quality control procedures, instrument maintenance programs, and any observed laboratory deviations to the above or in methods performance.

Although all of the methods described in this guidebook have exact numerical values given for performance parameters such as weight and volume measures, pH, time and temperature to achieve optimum results, it should be clearly understood that an acceptable range exists within which optimum results can still be expected to be achieved without compromising the integrity of the method. For any given method, unless otherwise clearly stated within the text of this MLG, the following allowable ranges for the given parameters are considered to be acceptable and are applicable:

Weight and volume measures: $\pm 1\%$
pH: ± 0.2 units
Time: hours ± 1 hour; minutes $\pm 1\%$
Temperature: $\pm 1.0^{\circ}\text{C}$

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Laboratory QA/QC Division
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Microbiology Laboratory Guidebook Notice of Change

Chapter new, revised, or archived: MLG 4.02

Title: Isolation and Identification of *Salmonella* from Meat, Poultry, and Egg Products

Effective Date: 10/25/02

Description and purpose of change(s):

The Microbiology Laboratory Guidebook (MLG) chapters are currently under revision. The formatting is being changed to meet the requirements of the laboratory's document control system. Additional content is being added to meet the requirements of ISO 17025. The chapter has been revised to include a statement of the method detection limits, a section on safety precautions and a revised section on quality control practices. Previous pen-and-ink changes are also incorporated into the revision.

QD-F-Micro-0004.00

Approved: B. Cottingham, 4/18/02



Microbiology Laboratory Guidebook Notice of Change

Chapter new, **revised**, or archived: MLG 4.02

Title: Isolation and Identification of *Salmonella* from Meat, Poultry, and Egg
Products

Effective Date: 10/25/02

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United States Department of Agriculture
Food Safety And Inspection Service, Office of Public Health and Science

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Title: Isolation And Identification of <i>Salmonella</i> From Meat, Poultry And Egg Products		
Revision:02	Replaces: MLG Chapter 4 Revision 1	Effective: 10/25/02

Procedure Outline

- 4.1 Introduction
 - 4.1.1 General
 - 4.1.2 Limits of Detection
- 4.2 Safety Precautions
- 4.3 Quality Control Procedures
 - 4.3.1 Method Controls
 - 4.3.2 Specific Procedure Controls
- 4.4 Equipment, Reagents, Media and Test Kits
 - 4.4.1 Equipment
 - 4.4.2 Reagents
 - 4.4.3 Media
 - 4.4.4 Cultures
 - 4.4.5 Commercially Available Test Kits (optional)
- 4.5 Isolation Procedures
 - 4.5.1 Sample Pooling
 - 4.5.2 Breeding Mixes, Dehydrated Sauces and Dried Milk
 - 4.5.3 Ready-to-Eat Foods
 - 4.5.4 Fermented Product
 - 4.5.5 Raw Meat
 - 4.5.6 Carcass Sponge Samples
 - 4.5.7 Whole Bird Rinses
 - 4.5.8 Liquid, Frozen, Cooked or Dried Egg Samples
 - 4.5.9 Sanitation Series Food Homogenates
 - 4.5.10 Most Probable Numbers (MPN) Determination
- 4.6 Examination of and Picking Colonies from Plating Media
 - 4.6.1 Picking colonies
 - 4.6.2 Screening Media
- 4.7 Biochemical Procedures
- 4.8 Serological Tests
 - 4.8.1 Somatic (O) Antigen Agglutination Tests
 - 4.8.2 Flagellar (H) Antigen Agglutination Tests
- 4.9 Storage of Cultures
- 4.10 References

Approved by Phyllis Sparling, 10/17/02

United States Department of Agriculture
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4.1 Introduction

4.1.1 General

This method describes the analysis of various meats, meat products, sponge and rinse samples, eggs, and egg products for *Salmonella*. It is not intended for the isolation and identification of *Salmonella* Typhi.

Success in isolating *Salmonella* from any food can be related to a number of factors including food preparation procedures, the number of organisms present, sample handling after collection, etc. With raw meat samples the competitive flora may be the most important factor. It varies from sample to sample and from one kind of meat to another.

Another consideration is whether the examination is for routine monitoring or epidemiological purposes. The analyst may choose to augment the method for epidemiological purposes with additional enrichment procedures and culture media, two temperatures of incubation, intensified picking of colonies from plates, and/or rapid screening methods.

All isolates must be identified as *Salmonella* biochemically and serologically.

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

4.1.2 Limits of Detection

The *Salmonella* detection limit for this method has been determined to be less than 1 colony forming unit (cfu)/g in a 25 g sample.

4.2 Safety Precautions

Salmonella are generally categorized as BioSafety Level 2 pathogens. CDC guidelines for manipulating Biosafety Level 2 pathogens should be followed whenever live cultures of *Salmonella* are used. A Class II laminar flow biosafety cabinet is recommended for procedures in which infectious aerosols or splashes may be created. All available Material Safety Data Sheets (MSDS) must 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 all MSDS sheets.

Approved by Phyllis Sparling, 10/17/02

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4.3 Quality Control Procedures

4.3.1 Method Controls

Include at least three method controls in all analyses. These controls must include a *Salmonella* spp. H₂S-negative culture, a *Salmonella* spp. H₂S-positive culture and an uninoculated media control. To facilitate identification of control isolates, the laboratory may use strains of uncommonly found serogroups. *S. Abaetetuba*, serogroup F, is suggested as a readily available, H₂S-positive culture that is not commonly found in meats or meat products. *Salmonella* serotype Choleraesuis is typically negative for H₂S production. These cultures may be obtained from ATCC. Other serotypes may be found that have aberrant H₂S-negative strains. The inoculum level for the positive controls should approximate 30 to 1000 cfu per sample. A 1 microliter loopful of a suspension of a fresh culture equivalent to a 0.5 McFarland Standard may be used for this purpose. Alternatively, commercially prepared bacterial pellets containing concentrations of 100 to 1000 cfu/pellet may be used according to the manufacturer's instructions. The control cultures should be inoculated into either a meat matrix or the matrix that is being analyzed. Incubate the controls along with the samples, and analyze them in the same manner as the samples. Confirm at least one isolate from each positive control sample. In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses.

4.3.2 Specific Procedure Controls

The biochemical and serological tests used for confirmation of the sample isolates require the use of appropriate controls to verify that the results are valid. *Salmonella* 'O' antisera should be tested with QC control sera before initial use, and with a saline control for each test. Biochemical kit and rapid test manufacturers may specify control cultures for use with their products. If not specified, quality control procedures for biochemical tests and test media should include cultures that will demonstrate pertinent characteristics of the product.

4.4 Equipment, Reagents, Media and Test Kits

All of the materials listed below may not be needed. Media and reagents specific to the biochemical test method that is used will be needed in addition to the materials listed below. See Section 4.7.

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4.4.1 Equipment

- a. Sterile tablespoons, scissors, forceps, knives, glass stirring rods, pipettes, petri dishes, test tubes, bent glass rods ("hockey sticks") as needed
- b. Blending/mixing equipment: Sterile Osterizer-type blender with sterilized cutting assemblies, and blender jars or Mason jars and adapters for use with Mason jars; *or* a Stomacher™ (Tekmar or equivalent) with sterile Stomacher bags
- c. Sterile Stomacher™ 3500 bags, plain, clear polypropylene autoclave bags (ca. 24" x 30 - 36"), or Whirl-Pak™ bags (or equivalent)
- d. Incubator, 35 ± 1°C
- e. Incubator or water bath, 42 ± 0.5°C
- f. Water bath, 48-50°C
- g. Glass slides, glass plate marked off in one-inch squares or agglutination ring slides
- h. Balance, 2000 g capacity, sensitivity of 0.1 g
- i. Inoculating needles and loops
- j. Vortex mixer

4.4.2 Reagents

- a) Crystal violet dye, 1% aqueous solution, steamed
- b) Butterfield's phosphate diluent
- c) Saline, 0.85%
- d) Saline, 0.85% with 0.6% formalin for flagellar antigen tests
- e) Calcium carbonate, sterile
- f) *Salmonella* polyvalent O antiserum
- g) *Salmonella* polyvalent H antiserum
- h) *Salmonella* individual O grouping sera for groups A-I (antisera for further O groups are optional)
- i) (Optional) Oxoid *Salmonella* Latex Test (Unipath Company, Oxoid Division, Ogdensburg, NY) or equivalent
- j) Additional reagents as needed for biochemical tests

4.4.3 Media

- a) Buffered peptone water (BPW)
- b) TT broth (Hajna)

Approved by Phyllis Sparling, 10/17/02

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- c) Modified Rappaport Vassiliadis (mRV) broth, Rappaport-Vassiliadis R10 broth, or Rappaport-Vassiliadis Soya Peptone Broth (RVS)
- d) Brilliant green sulfa agar (BGS; contains 0.1% sodium sulfapyridine)
- e) Xylose lysine Tergitol™ 4 agar (XLT4) or Double modified lysine iron agar (DMLIA)
- f) Triple sugar iron agar (TSI)
- g) Lysine iron agar (LIA)
- h) Trypticase soy broth (TSB) or Tryptose broth
- i) Trypticase soy agar (TSA)
- j) Nutrient agar slants
- k) Nutrient broth, semi-solid
- l) Tryptic soy agar with 5% sheep blood agar
- m) Additional media as needed for biochemical tests

4.4.4 Cultures

At least one H₂S-positive strain of *Salmonella* and one H₂S-negative strain of *Salmonella* are required for method controls.

4.4.5 Commercially Available Test Kits (optional)

Any screening method under consideration for *Salmonella* testing must meet or exceed the following performance characteristics: sensitivity ≥ 97%, specificity ≥ 90%, false-negative rate ≤ 3%, and false-positive rate ≤ 10%.

4.5 Isolation Procedures

4.5.1 Sample Pooling

NOTE: Follow sample pooling instructions in specific program protocols. Otherwise, do not use sample pooling.

When examining products that are expected to be *Salmonella*-free, sample pooling can save valuable time, labor and materials. There are several ways this can be done. Pooling at the non-selective enrichment step is appropriate when the likelihood of finding *Salmonella* is unlikely or when, if a positive is found, it is not important to know which particular sample contained the organism.

Approved by Phyllis Sparling, 10/17/02

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- a. Those samples that are to be examined only for *Salmonella* may be pooled in the blenders. Up to 30 samples may be combined, depending on the capacity of the blenders and culture flasks. The proportions of sample volume to BPW must be maintained at the 1 to 10 ratio (1 part sample to 9 parts BPW). The BPW should be pre-warmed to the temperature of incubation.
- b. When food homogenates (Section 4.5.9) are pooled, the culture flask should be warmed in a water bath to bring the contents up to incubation temperature before placing it in the incubator.
- c. Incubation of large pools should be prolonged to two days if growth is not apparent in one day. Subculture 5 ± 0.5 ml of the incubated non-selective broth pool into 100 ± 1 ml of TT (Hajna), 1 ± 0.1 ml into 100 ± 1 ml of mRV, and proceed as usual.

In cases in which it is important to identify particular samples that may contain *Salmonellae*, it is still possible to take advantage of labor-saving by pooling. In such cases, the samples may be started in the usual way in non-selective broth. After incubation, up to ten of these cultures may be pooled in selective enrichment broth. Maintain the 0.5 to 10 ratio for inoculation of TT broth and the 0.1 to 10 ratio for inoculation of mRV broth. The remaining non-selective broths (or portions of them) are refrigerated. The total volume of selective enrichment broth used will be the same, but the number of plates to be streaked is reduced. If a positive pool is found, all the pooled samples are started individually in selective enrichment broth by going back to the refrigerated non-selective broths.

4.5.2 Breeding Mixes, Dehydrated Sauces and Dried Milk

For dehydrated sauces, dried milk, and breeding mixes add BPW as described for powdered egg in Section 4.5.8.

4.5.3 Ready-to-Eat Foods

Follow program requirements for preparing sample and sub-sample composites. Outbreak investigation requirements may differ, in which case, follow the client specifications for those samples.

- a. Weigh 325 g of the composite sample into a Stomacher bag (or sterile blender jar if required by the client or sample type).

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- b. Add approximately one third to half of 2925 ml of ambient temperature sterile buffered peptone water. Blend or stomach approximately 2 minutes, and then add the remainder of the 2925 ml of BPW.
- c. Incubate at $35 \pm 1^{\circ}\text{C}$ for 20-24 h.
- d. Transfer 0.5 ± 0.05 ml of incubated broth into 10 ml TT and 0.1 ± 0.02 ml into 10 ml of mRV broth.
- e. Incubate the enrichment broths at $42 \pm 0.5^{\circ}\text{C}$ for 22-24 h, or in a water bath at $42 \pm 0.5^{\circ}\text{C}$ for 18-24 h.
- f. Streak above enrichments on BGS and either DMLIA or XLT4 agar plates. Use one 10-microliter loopful for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.
- g. Incubate at $35 \pm 1^{\circ}\text{C}$.
- h. Examine in 18-24 h. Select colonies. Refer to Section 4.6 et seq.
- i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies as above. Reserve, under refrigeration, all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

4.5.4 Fermented Products

Follow the procedure for ready-to-eat foods (Section 4.5.3) except:

- a. Blend/stomach the sample with 10 g of sterilized calcium carbonate.
- b. Use buffered peptone water that contains 1 ml of a 1% aqueous solution of crystal violet per liter.

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4.5.5 Raw Meat

If the sample is not already ground, in some cases it may be best to mince it with scissors or leave it whole (e.g. chicken wings) to avoid jamming blender blades with skin or connective tissue. Whirl-Pak™ bags can be used in culturing these samples.

- a. Weigh 25 ± 0.5 g of meat into a sterile blender jar, other sterile jar or a Whirl-Pak™ or Stomacher™ bag. HACCP program samples collected using a sampling ring are allowed a weight range of 25 ± 2.5 g.
- b. Add 225 ml of BPW. Stomach or blend, as required, for approximately two minutes or shake thoroughly.
- c. Incubate at $35 \pm 1^{\circ}\text{C}$ for 20-24 h.
- d. Transfer 0.5 ± 0.05 ml into 10 ml TT broth and 0.1 ± 0.02 ml into 10 ml mRV broth.
- e. Incubate at $42 \pm 0.5^{\circ}\text{C}$ for 22-24 h.
- f. Streak on DMLIA or XLT4 and BGS agar plates. Use one loopful of inoculum for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.
- g. Incubate at $35 \pm 1^{\circ}\text{C}$.
- h. Examine in 18-24 h. Select colonies. See Section 4.6 et seq.
- i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies as above. Reserve, under refrigeration, all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

4.5.6 Carcass Sponge Samples

- a. Add 50 ml of BPW to the sample bag containing the moistened sponge to bring the total volume to 60 ml. Mix well.

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- b. Incubate at $35 \pm 1^{\circ}\text{C}$ for 20-24 h.
- c. Follow the procedures in Section 4.5.5, d-i.

4.5.7 Whole Bird Rinses

Due to differences between sample types/sizes (e.g. chicken vs. turkey carcasses), follow instructions given in the specific program protocol. For chicken carcasses, aseptically drain excess fluid from the carcass and transfer the carcass to a sterile Stomacher™ 3500 bag, a plain, clear polypropylene bag (ca. 24" x 30-36"), or equivalent. Pour 400 ml (or other volume specified in program protocol) of Buffered Peptone Water (BPW) into the cavity of the carcass contained in the bag. Rinse the bird inside and out with a rocking motion for one minute (ca. 35 RPM). This is done by grasping the broiler carcass in the bag with one hand and the closed top of the bag with the other. Rock with a reciprocal motion in about an 18-24 inch arc, assuring that all surfaces (interior and exterior of the carcass) are rinsed. Transfer the sample rinse fluid to a sterile container.

Use 30 ml of the sample rinse fluid obtained above for *Salmonella* analysis. Add 30 ml of sterile BPW, and mix well. Incubate at $35 \pm 1^{\circ}\text{C}$ for 20-24 h, and then proceed according to 4.5.5 (d-i).

NOTE: If analyses other than *Salmonella* are to be performed, the carcass may be rinsed in Butterfield's Phosphate Diluent instead of BPW. In this case, add 30 ml of 2X BPW to 30 ml of carcass-rinse fluid, mix well, and continue as above.

4.5.8 Liquid, Frozen, Cooked or Dried Egg Samples

- a. Mix the sample with a sterile spoon, spatula, or by shaking.
- b. Aseptically weigh a minimum of 100 g of egg sample into a sterile blender jar, other sterile jar, or a Whirl-Pak™ or Stomacher™ bag containing 900 ml of sterile BPW. If a special sample or specification requires a sample size other than 100 g, the ratio of egg sample to BPW is to be maintained at 1:10.
- c. Mix the inoculated BPW well by shaking, stomaching, or blending.

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- d. With dried egg samples, gradually add BPW to the sample. Add a small portion of sterile BPW and mix to obtain a homogeneous suspension. Add the remainder of the BPW. Mix until a lump-free suspension is obtained.
- e. Incubate at $35 \pm 1^{\circ}\text{C}$ for 20-24 h, and then proceed according to 4.5.5 (d-i).

4.5.9 Sanitation Series Food Homogenates (optional)

To isolate salmonellae from food samples homogenized as outlined in MLG 3 Section 3.3.1, as part of a sanitation test series, use the 10^{-1} food homogenate dilution (See also this chapter, Section 4.5.1 Sample Pooling).

- a. Weigh 250 g of food homogenate into a sterile jar (this contains 25 g of product).
- b. Add 25 ml of 10x BPW (broth made to ten time's normal strength).
- c. Incubate 24-26 h at $35 \pm 1^{\circ}\text{C}$.
- d. Transfer 0.5 ± 0.05 ml into 10 ml of TT broth and 0.1 ± 0.02 ml into 10 ml of mRV broth.
- e. Incubate at $42 \pm 0.5^{\circ}\text{C}$ for 22-24 h.
- f. Streak on DMLIA or XLT4 and BGS agar plates. Use one loopful for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.
- g. Incubate 18-24 h at $35 \pm 1^{\circ}\text{C}$.
- h. Select colonies. See Section 4.6 et seq.
- i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies per Section 4.6. Reserve all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

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4.5.10 Most Probable Numbers (MPN) Determination

Due to differences between sample types (e.g. whole chicken rinse vs. ground beef) and sample sizes (e.g. 25 g vs. 325 g) follow MPN instructions given in the specific program protocol. See also MLG Appendix 2, Most Probable Number Tables.

4.6 Examination of and Picking Colonies from Plating Media

4.6.1 Picking Colonies

- a. After the recommended incubation interval, examine the selective-differential agar plates for the presence of colonies meeting the description for suspect *Salmonella* colonies. Pick well-isolated colonies.
 - BGS. Select colonies that are pink and opaque with a smooth appearance and entire edge surrounded by a red color in the medium. On very crowded plates, look for colonies that give a tan appearance against a green background.
 - XLT4. Select black colonies or red colonies with or without black centers. The rim of the colony may still be yellow in 24 h; later it should turn red.
 - DMLIA. Select purple colonies with or without black centers. Since salmonellae typically decarboxylate lysine and ferment neither lactose nor sucrose, the color of the medium reverts to purple.
- b. Pick up to three colonies from each plate, if available. (NOTE: Before any sample is reported as *Salmonella*-negative, a total of three typical colonies, if available, from each selective agar plate must be examined). Pick only from the surface and center of the colony. Avoid touching the agar because these highly selective media suppress growth of many organisms that may be viable.

If there are typical colonies on a plate, that are not well isolated, pick from the typical colonies and re-streak directly to selective agar plates. Alternatively, place a loopful of growth into a tube of TT or mRV broth and incubate overnight, then re-streak to selective agars.

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4.6.2 Screening Media

- a. Inoculate TSI and LIA slants in tandem with a single pick from a colony by stabbing the butts and streaking the slants in one operation. If screw cap tubes are used, the caps must be loosened. Incubate at $35 \pm 1^{\circ}\text{C}$ for 24 ± 2 h.
- b. Examine TSI and LIA slants as sets. Note the colors of butts and slants, blackening of the media and presence of gas as indicated by gas pockets or cracking of the agar. Note also the appearance of the growth on the slants along the line of streak. Discard, or re-streak for isolation, any sets that show "swarming" from the original site of inoculation. Discard sets that show a reddish slant in lysine iron agar. Isolates giving typical *Salmonella* spp. reactions and isolates which are suggestive, but not typical of *Salmonella* spp. should be confirmed by a combination of biochemical and serological procedures. Refer to Table 1 for a summary of TSI-LIA reactions. The motility testing in the last column of the table is optional.
- c. (Optional: for some biochemical test kits) Streak a TSA + 5% sheep blood agar plate from either the TSI or LIA slant. Incubate 18-24 h at $35 \pm 1^{\circ}\text{C}$.

4.7 Biochemical Procedures

Commercially available biochemical test kits, including automated systems may be used for biochemical identification. If the VITEK[®] test kit is used, the cytochrome oxidase and gram stain tests are optional. Alternatively, use traditional methods of biochemical identification. Refer to AOAC Official Method 967.27 or "Edwards and Ewing's Identification of Enterobacteriaceae", 4th Edition, for biochemical reactions of *Enterobacteriaceae* and for fermentation media and test procedures.

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Table 1.

Triple Sugar Iron Agar			Lysine Iron Agar		Polyvalent Sera		Disposal
Butt	Slant	H ₂ S	Butt	H ₂ S	O	H	
Y	R	+	P	+	+	+	B. & M. T.
Y	R	+	P	+	+	-	B. & M. T.
Y	R	-	P	-			B. & M. T.
Y	R	-	Y	-	+	+	* B. & M. T.
Y	R	-	Y	-	-	-	Discard
Y	R	+	Y	+/-			B. & M. T.
Y	Y	-	Y or P	-			Discard
Y	Y	+	P	+			** B. & M. T.
NC	NC						Discard

Y = Yellow; R = Red; P = Purple; B. & M. T. = Biochemical and motility tests; NC = No change in color from uninoculated medium.

* *Salmonella* Typhisuis (found seldom in swine in U.S.)

** *Salmonella enterica* subsp. *arizonae* or *S. enterica* subsp. *diarizonae*

4.8 Serological Tests

4.8.1 Somatic (O) Antigen Agglutination Tests

At a minimum, isolates should be tested with polyvalent O antiserum reactive with serogroups A through I. Following a positive reaction with polyvalent O antiserum, it is necessary to type the isolate using individual *Salmonella* antisera for O groups A through I.

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Testing for O groups A through I should encompass the majority of the *Salmonella* serotypes commonly recovered from meat and poultry products. Occasionally, however, an isolate will be recovered which is typical of *Salmonella* biochemically and is serologically poly H-positive, but is non-reactive with antisera to groups A through I. Such an isolate should be reported as "*Salmonella* non A-I" or "*Salmonella* O group beyond I".

Use growth from either the TSI or LIA slant. Test first with polyvalent O antiserum. Include a saline control with each isolate. If there is agglutination with the saline control alone (autoagglutination), identify such a culture by biochemical reactions only. If the saline control does not agglutinate and the polyvalent serum does, test the culture with *Salmonella* O grouping antisera. Record positive results and proceed to H agglutination tests.

4.8.2 Flagellar (H) Antigen Agglutination Tests

Inoculate trypticase soy broth or tryptose broth. Incubate at $35 \pm 1^{\circ}\text{C}$ overnight or until growth has an approximate density of three on the McFarland scale. Add an equal amount of saline containing 0.6% formalin and let sit one hour. Remove one ml to each of two 13 x 100 mm test tubes. To one of the tubes, add *Salmonella* polyvalent H serum in an amount indicated by the serum titer or according to the manufacturer's instructions. The other tube serves as an autoagglutination control. Incubate both tubes at $48-50^{\circ}\text{C}$ in a water bath for up to 1 h. Record presence or absence of agglutination.

If desired, use Spicer-Edwards pooled serum or H typing serum. Find details in "Edwards and Ewing's Identification of Enterobacteriaceae" (Ewing, 1986).

The Oxoid *Salmonella* Latex Test, or equivalent, may be used as an optional method for H antigen agglutination testing. Follow the manufacturer's instructions. If a suspect *Salmonella* isolate is negative by the latex test, perform the poly H tube agglutination test described above.

4.9 Storage of Cultures

Do not store cultures on TSI agar because this tends to cause roughness of O antigens. For short-term (2-3 months) storage, inoculate a nutrient agar slant, incubate at $35 \pm 1^{\circ}\text{C}$ overnight and then store at $4-8^{\circ}\text{C}$.

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Store "working" *Salmonella* stock cultures on nutrient agar slants. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at $35 \pm 1^{\circ}\text{C}$, and then store them at $4-8^{\circ}\text{C}$. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the opportunity for contamination. Cultures may be subcultured up to 5 times. After this period the culture must be re-confirmed biochemically or a new culture initiated.

For long term storage freeze cultures using cryo-beads, i.e. Cryostor™ or equivalent, lyophilize or use the procedure that follows. Subculture *Salmonella* isolates by picking a colony with an inoculating needle and stabbing it into semi-solid nutrient broth (0.75% agar). Incubate at $35 \pm 1^{\circ}\text{C}$ overnight, and then seal with hot, paraffin-soaked corks. Household wax is better than embedding paraffin because it stays relatively soft at room temperature making the corks easy to remove. Store the cultures in the dark at room temperature. Such cultures will remain viable for several years.

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Exhibit 1058
Mullins Decl. Appendix N-4
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Microbiology Laboratory Guidebook Notice of Change

Chapter new, revised, or archived: MLG 5.03

Title: Detection, Isolation, and Identification of *Escherichia coli* O157:H7 and O157:NM (Nonmotile) from Meat Products

Effective Date: 10/25/02

Description and purpose of change(s):

The Microbiology Laboratory Guidebook method chapters are currently under revision. The formatting is being changed to meet the requirements of the laboratory's document control system. Additional content is being added, i.e. Section 5.1.2. Limits of Detection, to meet the requirements of ISO 17025. Safety Precautions are also included in the revised chapters.



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Procedure Outline

- 5.1 Introduction
 - 5.1.1 General
 - 5.1.2 Limits of Detection
- 5.2 Safety
- 5.3 Quality Control Practices
- 5.4 Equipment, Materials, Media, Reagents and Test Kits
 - 5.4.1 Equipment
 - 5.4.2 Media, Reagents and Cultures
 - 5.4.3 Test Kits
- 5.5 Detection Procedure
- 5.6 Isolation Procedure
- 5.7 Identification and Confirmation
- 5.8 Storage of Cultures
- 5.9 Selected References

5.1 Introduction

5.1.1 General

The following method is used for the analysis of raw and ready-to-eat meat products for *Escherichia coli* O157:H7 and O157:NM (O157:H7/NM). The method is based on enrichment in a selective broth medium, application of a rapid screening test, immunomagnetic separation (IMS) in paramagnetic columns, and plating on a highly selective medium.

The following definitions are used for reporting purposes. A potential positive sample causes a positive reaction on the screen test kit. A presumptive positive sample has typical colonies, observed on Rainbow Agar, and reacts specifically with O157 antiserum. A sample is a confirmed positive sample for *E.coli* O157:H7 or *E. coli* O157:NM when the isolate is confirmed biochemically and serologically, and the presence of Shiga toxin(s) or Shiga toxin gene(s) is demonstrated.

Unless otherwise stated all measurements cited in this method have a tolerance of $\pm 2\%$.

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5.1.2 Limits of Detection

This test has been shown to consistently detect less than 1 colony forming unit (cfu)/g in a 65 g sample.

5.2 Safety

E. coli O157:H7/NM is a human pathogen with a low infectious dose. (Ingestion of 100 cells can cause disease.) The use of gloves and eye protection is mandatory and all work surfaces must be disinfected prior to and immediately after use. Laboratory personnel must abide by CDC guidelines for manipulating Biosafety Class II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. All available Material Safety Data Sheets (MSDS) should be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the materials should read all MSDS sheets.

5.3 Quality Control Practices

- a. Rainbow[®] Agar plates have a shelf life of 2 weeks.
- b. All media and E-Buffer must be pre-warmed to 18-35°C prior to use.
- c. The recommended fluorescent strain of *E. coli* O157:H7 must be used in this procedure to monitor for cross contamination. The protocol for the use of fluorescent strains of *E. coli* O157:H7 as positive controls follows:

Wild-type strains of *E. coli* O157:H7 transformed with pGFP produce a green fluorescent protein. As a result of this transformation, fluorescent strains of *E. coli* O157:H7 possess the unique property of expressing bright green fluorescence visible in the dark when illuminated by long-wave UV light. This property, which sets them apart from typical *E. coli* O157:H7, makes them useful positive controls for analyses of meat samples for *E. coli* O157:H7/NM. At different steps in the procedure, both test samples and (fluorescent) positive controls can be tested for the bright green fluorescence as a Quality Control measure to make sure that positive sample isolates actually came from the test sample and not from accidental contamination by the positive control cultures.

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Results of studies done at the FSIS Beltsville Microbial Pathogens Laboratory showed that these fluorescent cultures can be subjected to *E. coli* O157:H7/NM isolation and identification procedures without losing their fluorescent properties. These strains retain their fluorescent properties when grown in SOB media with added ampicillin (SOB + A). These cultures must be transferred every 7 days to fresh SOB + A media, according to the protocol outlined below. The fluorescent colonies are ready to be used as positive controls on day 3 of the following protocol, and for the next 6 consecutive days without losing their fluorescent properties. If these cultures are not needed on a continuous basis, they can be stored at refrigeration temperatures on SOB + A agar plates in zip-lock bags or sealed with parafilm® for 1 month and then transferred, or started up again 2 days before needed. Strict adherence to the protocol described below is essential, in order to ensure that the fluorescent strains do not lose their ability to express green fluorescence.

- i. Test the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain) on SOB + A agar plate for fluorescence by illuminating colonies under long-wave UV light in the dark.
- ii. Select only fluorescing colonies and inoculate into 10 ml of SOB + A broth in a tube. Incubate at $35 \pm 2^{\circ}\text{C}$ overnight.
- iii. Streak the culture from the SOB + A broth onto a SOB + A agar plate. Incubate at $35 \pm 2^{\circ}\text{C}$ overnight.
- iv. Examine colonies on the plate for fluorescence. The fluorescent colonies are ready to be inoculated into modified EC broth + novobiocin (mEC+n) at this stage. These cultures on SOB + A agar plates can be stored refrigerated and be used as positive controls for 6 more days. Incubate the inoculated mEC+n positive control culture at $35 \pm 2^{\circ}\text{C}$ overnight, along with the test samples.
- v. Continue analysis per Sections 5.5-5.7 and test the Blood Agar Plates of the fluorescent positive controls and any positive sample cultures for fluorescence.

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5.4 Equipment, Materials, Media, Reagents and Test Kits

5.4.1 Equipment

- a. Balance, sensitivity of 0.1 g
- b. Stomacher™ 400 or 3500 with appropriate sizes of sterile Stomacher™ bags, with or without mesh. (Tekmar Co., Cincinnati, Ohio), or equivalent
- c. Incubator, static $35 \pm 2^{\circ}\text{C}$
- d. Micropipettors to deliver 15-1000 μl with sterile disposable filtered micropipet tips
- e. Mechanical Pipettor with 1.0 ml, 5.0 ml, 10.0 ml sterile pipettes
- f. Inoculating loops, “hockey sticks” or spreaders, and needles
- g. UV light (long-wave, e.g. VWR # 36553-124, or equivalent)
- h. Filter unit, 0.2 μm , nylon, sterile
- i. Infrared thermometer
- j. LabQuake® Agitator (or equivalent) with clips to hold microcentrifuge tubes
- k. Sterile disposable 12 x 75 mm polypropylene tubes (e.g. Fisher # 14-956-1B, or equivalent)
- l. Microcentrifuge and sterile 1.5 ml microcentrifuge tubes
- m. Sterile 50 ml conical tubes (e.g. Falcon® # 2070, or equivalent) or sterile bottles
- n. Sterile 40 μm Cell Strainer (Falcon® # 2340, or equivalent)
- o. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02, or equivalent)
- p. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09, or equivalent)
- q. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03, or equivalent)
- r. Tray, autoclavable, approximately 130 mm x 83 mm (e.g. VWR # 62663-222, or equivalent) for use with the OctoMACS®

5.4.2 Media, Reagents and Cultures

- a. Modified EC broth with novobiocin (mEC+n) (or equivalent)
- b. Rainbow® Agar O157 (Biolog Inc., Hayward California, 94545) containing 10 mg/L novobiocin plus 0.8 mg/L potassium tellurite, or equivalent selective medium
- c. Tryptic soy agar with 5% sheep blood

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- d. SOB + A Medium
- e. E Buffer, approximately 7 ml per sample [Buffered Peptone Water, Bovine Albumin Sigma # 7906 (or equivalent) , and Tween-20[®], or equivalent]
- f. Disinfectant (Lysol[®] I. C., 2.0%, or equivalent)
- g. Dynal[®] # 710.04 anti-*E. coli* O157 antibody-coated paramagnetic beads (Dynal Inc., Lake Success, NY 11042), or equivalent
- h. *E. coli* O157:H7 strain 465-97 (positive control used throughout method)
- i. *E. coli* ATCC strain 25922 (negative control for bead capture and screen tests)

5.4.3 Test Kits

- a. The screening test for the detection of *E. coli* O157:H7/NM should meet or exceed the following performance characteristics:

Sensitivity	≥98%
Specificity	≥90%
False Negative Rate	≤ 2%
False Positive Rate	≤10%

- b. *E. coli* O157:H7 latex agglutination test kit (RIM[®] *E. coli* O157:H7 Latex Test Kit, REMEL, 12076 Santa Fe Drive, Lenexa, KS 66215, or equivalent)
- c. Biochemical test kits and systems [Vitek[®] GNI and GNI Plus cards (bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395), or equivalent]
- d. Shiga Toxin test kit [Premier[®] EHEC, cat. # 608096 (Meridian Diagnostics, Inc., 3471 River Hills Dr., Cincinnati, OH, 45244), or equivalent]

5.5 Detection Procedure

- a. Sample Preparation
 - i. Raw ground beef microbiological testing programs.
Randomly collect five 65 ± 2 g sub-samples (total of 325 ± 10 g) that are representative of the entire sample. Place each 65 ± 2 g sub-sample in a

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sterile Strainer Stomacher™ bag. Add 585 ml mEC+n broth and pummel for two minutes in a Stomacher™.

- ii. Cooked meat patties and semi-dry and dry fermented sausages. Randomly prepare five 65 ± 2 g sub-samples (total of 325 ± 10 g) that are representative of the entire sample. When appropriate, sample representative portions from both the outer surface (shell) and inner section (core) of RTE products, especially semi-dry and dry fermented sausages. Place each 65 ± 2 g sub-sample in a sterile Strainer Stomacher™ bag. Add 585 ml mEC+n broth and pummel for two minutes in a Stomacher™.
- iii. Outbreak-related samples. Randomly collect thirteen 25 ± 1 g sub-samples (total of 325 ± 13 g) that are representative of the entire sample. Place each 25 ± 1 g sub-sample in a sterile Strainer Stomacher™ bag and add 225 ml of mEC+n broth. Pummel for 2 minutes in a Stomacher™.
- b. Incubate all bags (static) with their contents for 20 to 24 h at $35 \pm 2^\circ\text{C}$. Include a positive, negative, and uninoculated medium control for each group of samples tested. Use the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97) as a positive control and *E. coli* ATCC strain 25922 as the negative control.
- c. From the enrichment cultures in the Stomacher™ bags, perform the screening test for *E. coli* O157:H7/NM following the manufacturer's instructions. The enrichment culture may be analyzed immediately upon removal from the incubator without waiting for tempering to room temperature. To prevent clogging the pipette tip, be sure to collect the appropriate size sample from the enrichment culture outside the inner strainer bag.
- d. Samples negative by the screening test can be reported as negative for *E. coli* O157:H7/NM and discarded.
- e. Samples positive by the screening test should be reported as potential positives. Begin isolation procedures from the enrichment culture in the Stomacher™ bag.

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5.6 Isolation Procedure

Note: Steps a.-l. may be performed in a sequence that is convenient to the laboratory personnel.

- a. Prepare E Buffer by mixing 0.5 g Bovine Albumin and 50 µl Tween-20[®] into 100 ml Buffered Peptone Water (BPW). Filter sterilize (0.2 µm) and store at 2-8°C.
- b. Remove Rainbow[®] Agar plates from 2-8°C storage, allowing 3 plates for each screen-positive culture and each control. Be sure that plates have no visible surface moisture at the time of use. If necessary, dry plates (e.g. for up to 30 minutes in a laminar flow hood with the lids removed) prior to use. Dried plates that are not used should be labeled "dried", placed in bags and returned to 2-8°C.
- c. Remove a bottle of E Buffer from 2-8°C storage. Decant 7 ml of E Buffer for each culture and each control into a sterile tube or bottle and allow it to warm to at least 18°C. (Return the stock E Buffer to 2-8°C.)
- d. For each positive control, negative control and screen-positive culture to be analyzed, order and label 50 ml conical centrifuge tubes so that the positive control is first, followed by the negative control, then all cultures. Maintain this order for subsequent steps.
- e. For each positive control, negative control, and screen-positive culture, label two sterile 1.5 ml microcentrifuge tubes (for step g and step s), one 50 ml conical centrifuge tube (for step h.) and two 12 x 75 mm capped tubes (one for step p.). For each pair of 12 x 75 mm tubes, label one tube and add 0.9 ml E Buffer (for step q.).
- f. Prepare the Dynal #710.04 *E. coli* O157:H7 immunomagnetic bead suspension by following Table 1 below. Be sure to include the positive and negative controls in the total number of cultures. Use the bead suspension immediately (step g), or hold at 2-8°C. Return the stock vial of Dynal #710.04 *E. coli* O157:H7 immunomagnetic beads to 2-8°C.
- g. Vortex the bead solution briefly (2-3 seconds), then add 50 µl to a labeled

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microcentrifuge tube (from step e), one for each control and screen-positive culture. Use immediately or hold these tubes at 2-8°C.

- h. Place a 40 µm Cell Strainer on a labeled 50-ml conical centrifuge tube (from step e.). Pipet 5 ± 1 ml of each control and enrichment culture into the respective Cell Strainer and collect at least 1.0 ml of filtrate.
- i. Do not proceed with more than the number of tubes that the OctoMacs[®] magnet(s) will hold. Transfer 1.0 ml of a filtrate (step h.) to the corresponding microcentrifuge tube containing the immunomagnetic bead suspension (step g.) and place in the clips of the LabQuake[®] tube agitator. Rotate the tubes for 10-15 min at 18-30°C.
- j. Attach the OctoMACS[®] Magnet to the Multistand.
- k. Position a tray on the base of the Multistand so that it will collect the filtrate passing through the columns. Add approximately 300 ml of 2% Lysol[®] I. C. (or equivalent) disinfectant to cover the bottom of the tray.
- l. Label and place the appropriate number of Large Cell Separation columns on the OctoMACS[®] Magnet. Insert columns from the front making sure the column tips do not touch any surfaces. Leave the plungers in the bags at this time to maintain sterility.
- m. Transfer at least 0.5ml E Buffer to the top of each column and let the buffer run through.
- n. Resuspend, then transfer each culture and control from step i. to its corresponding column.
- o. After a culture or control has drained through, wash the column by applying 1.0 ml of E Buffer to each column and allow to drain. Repeat 3 more times for a total of 4 washes.
- p. After the last wash has drained, remove the column from the OctoMACS[®] Magnet and insert the tip into an empty labeled 12 x 75 mm tube (from step e.).

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Apply 1.0 ml of E Buffer to the column, and using the plunger supplied with the column, *immediately* flush out the beads into the tube. Use a smooth, steady motion to avoid splattering. Cap the tubes. Repeat this for each column. If the OctoMACS[®] magnet is to be used for a second set of cultures, it must be decontaminated as described in step u, below. Repeat steps j.-s. for the additional cultures.

- q. Vortex the tubes from step p. briefly to resuspend the beads. Make a 1:10 dilution of each treated bead suspension by adding 0.1 ml of the bead suspension to a 12 x 75 mm labeled tube containing 0.9 ml E Buffer (from step e.).
- r. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (from step p. and step q.) onto a labeled Rainbow[®] Agar plate. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate.
- s. Vortex the tubes containing undiluted beads (from step p.) and transfer to a labeled microfuge tube (from step e.) and centrifuge at least one minute using a bench-top microcentrifuge to concentrate the beads. Withdraw and discard the supernatant without disturbing the beads. Add 0.1 ml of E Buffer to the beads, resuspend the beads and transfer the beads to a labeled Rainbow[®] Agar plate. Spread plate the beads as described in step r.
- t. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 24-26 h at 35 ± 2°C.
- u. Decontaminate the OctoMACS[®] Magnet by applying 2% Lysol[®] I. C. (or equivalent) disinfectant directly to the surface. After approximately ten minutes, rinse with deionized or tap water. Allow the unit to air-dry or use absorbent paper towels to dry the unit.

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Table 1.

# of Cultures	ul of Beads*	ul of E-Buffer	# of Cultures	ul of Beads*	ul of E-Buffer
1	15	135	26	145	1305
2	20	180	27	150	1350
3	25	225	28	155	1395
4	30	270	29	160	1440
5	35	315	30	165	1485
6	40	360	31	175	1575
7	45	405	32	180	1620
8	50	450	33	185	1665
9	55	495	34	190	1710
10	60	540	35	195	1755
11	65	585	36	200	1800
12	70	630	37	205	1845
13	75	675	38	210	1890
14	80	720	39	215	1935
15	85	765	40	220	1980
16	90	810	41	230	2070
17	95	855	42	235	2115
18	100	900	43	240	2160
19	105	945	44	245	2205
20	110	990	45	250	2250
21	120	1080	46	255	2295
22	125	1125	47	260	2340
23	130	1170	48	265	2385
24	135	1215	49	270	2430
25	140	1260	50	275	2475
* Dynal® anti- <i>E. coli</i> O157:H7 antibody-coated paramagnetic beads (vortex briefly before use)					

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5.7 Identification and Confirmation

- a. After incubation, *E. coli* O157:H7 colonies have black or gray coloration on Rainbow[®] Agar. When *E. coli* O157:H7 colonies are surrounded by pink or magenta colonies, they may have a bluish hue. Mark colonies typical of *E. coli* O157:H7 and perform latex agglutination assays for O157, following manufacturer's instructions. Streak all latex positive colonies, up to a total of five from each sample (one per sub-sample, if possible) onto Blood Agar plates. Incubate Blood Agar plates for 16-24 h at 35 ± 2°C.

Note: If no typical colonies are present, hold the original Rainbow[®] plates at 20-24°C for an additional 6-24 h then re-examine for typical colonies.

- b. After incubation, examine the Blood Agar plates for purity under visible light, and evidence of cross contamination with the positive control by using long wave UV light. Only the positive control culture, *E. coli* O157:H7 strain 465-97, should fluoresce. If the Blood Agar plates appear pure and uncontaminated, perform the following confirmatory tests:
- i. Biochemical confirmation.
Inoculate Vitek-GNI or GNI Plus cards or use an equivalent biochemical identification testing system. The cytochrome oxidase and gram stain tests are optional.
- ii. Serological confirmation.
To confirm the absence or presence of O157 and H7 antigens, use an *E. coli* O157:H7 latex test agglutination kit (RIM[®] *E. coli* O157:H7 Latex Test Kit, or equivalent). Use growth from the Blood Agar plate (from step b).
- iii. Shiga toxin/toxin genes confirmation.
The presence of Shiga toxin(s) in a culture isolate should be confirmed by the use of a toxin assay, e.g., Meridian Premier[®] EHEC Kit, or equivalent. When Shiga toxin(s) is (are) not demonstrated, detection of one or more toxin genes by PCR should be used for confirmation. The positive control culture, *E. coli* O157:H7, is toxin-negative.

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- c. If the isolate confirms as an *E. coli* O157:H7, or *E. coli* O157:NM (or H-indeterminate) and the Shiga toxin(s) and/or one or more toxin genes are present, the sample will be treated as positive for *E. coli* O157:H7, and regulatory action will be taken. The cultures will also be tested by pulsed-field gel electrophoresis (PFGE) for potential epidemiological association.

5.8 Storage of Cultures

For storage requirements of the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain), refer to Section 5.3.c. of this chapter.

Store other "working" *E. coli* stock cultures on nutrient agar slants. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at $35 \pm 1^\circ\text{C}$, and then store them at $4-8^\circ\text{C}$. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the opportunity for contamination. Cultures may be subcultured up to 5 times. After this period the culture must be re-confirmed biochemically or a new culture initiated.

For long term storage freeze cultures using cryo-beads i.e. Cryostor™ or lyophilize.

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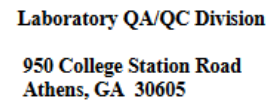
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Exhibit 1058
Mullins Decl. Appendix N-5
(MLG 2003 Chapter 8.03 from Wayback Machine)



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Microbiology Laboratory Guidebook Notice of Change

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

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

Effective Date: 4/29/02

Description and purpose of change(s):

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

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

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Procedure Outline

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- 8.2 Safety Precautions
- 8.3 Quality Control Procedures
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 - 8.4.1 Equipment
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 - 8.4.4 Reagents and Test Systems
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 - 8.5.3 Secondary Enrichment in FB and Direct Plating of UVM
 - 8.5.4 Examination of UVM-streaked MOX and interpretation/plating of 26 h FB
 - 8.5.5 Examination of MOX Plates and Interpretation/Plating of 48 h FB
 - 8.5.6 Isolation and Purification Procedures
- 8.6 Confirmation and Other Identification Procedures
 - 8.6.1 Preliminary Confirmation Tests for *Listeria* spp.
 - 8.6.2 Biochemical Tests
 - 8.6.3 CAMP/CAMP Factor Test
 - 8.6.4 Genetic Identification Tests
- 8.7 Enumeration: Most Probable Number Technique
- 8.8 Culture Storage and Shipment
- 8.9 References

8.1 Introduction

8.1.1 General

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

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

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

8.1.2 Method Detection Limits

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

8.2 Safety Precautions

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

8.3 Quality Control Procedures

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

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

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

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

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

8.4.1 Equipment

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

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8.4.2 Supplies

- a. Supplies for all samples:
 - i. Sterile sample preparation supplies: scalpels, chisels, knives, scissors, spatulas, forceps, disposable or reusable dishes, pans or trays
 - ii. Stomacher® Strainer 400™ sterile filter bags or non-filtered bags or equivalent
 - iii. Non-filter plastic bags for double-bagging sample homogenates (optional)
 - iv. Pipets (e.g. 1 ml)
 - v. Pipettor and sterile disposable tips for dispensing 100 µl
 - vi. Microscope slides, cover slips and immersion oil
 - vii. Disposable plastic and/or platinum inoculating needles and loops
 - viii. Wax or waterproof ink marker
 - ix. Sterile cotton-tipped applicators (i.e. swabs)
- b. Additional supplies necessary for environmental samples:
 - i. 0.45 µm hydrophobic grid membrane filter system (e.g. SELeCT 56 mm/100-ml 0.45-µm Monitor, cat# BPM9261, BioPath Inc., 2611 Mercer Ave., W. Palm Beach, FL 33401, 800-645-2302, or equivalent)
 - ii. Vacuum flask/hose and vacuum source
 - iii. Sterile disposable scalpels
 - iv. Sterile forceps
 - v. Non-bactericidal sampling sponges (e.g. Nasco cat # B01299WA, or equivalent)

8.4.3 Media

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

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

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- i. Modified University of Vermont broth (UVM, also known as UVM1)
 - ii. Fraser broth (FB)
 - iii. Modified Oxford agar (MOX)
 - iv. Horse blood overlay agar (HL, also known as HBO)
 - v. Trypticase soy agar with 5% sheep blood (TS-SBA, also known as CAMP test agar)
 - vi. Brain heart infusion broth (BHI broth)
- b. Additional media for environmental samples

Dey-Engley (D/E) neutralizing broth (e.g. Nasco cat# B01256WA, or equivalent)
- c. Optional media

Trypticase Soy Agar-Yeast Extract (TSA-YE)

8.4.4 Reagents and Test Systems

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

8.4.5 Commercially Available Screening Tests

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

8.4.6 Cultures

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

8.5 Detection and Isolation Procedures

8.5.1 Sample preparation

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

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

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

8.5.2 Primary Enrichment in UVM broth

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

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

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

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

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

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

8.5.3 Secondary Enrichment in FB and Direct Plating of UVM

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

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

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

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

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

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

8.5.6 Isolation and Purification Procedures

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

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

8.6 Confirmation and Other Identification Procedures

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

8.6.1 Preliminary Confirmation Tests for *Listeria* spp.

- a. Inoculation of preliminary confirmation test media

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

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

Incubate the HL purity streak plate at $35 \pm 2^\circ\text{C}$ for 22 ± 4 h. Proceed to Section 8.6.2.

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b. Purity streak test (optional)

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

c. Tumbling motility test

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

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

8.6.2 Biochemical Tests

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

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

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

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

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

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system as described in Section 8.6.4.a.

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

8.6.3 CAMP/CAMP Factor Test

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

- a. β -lysin CAMP factor test

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

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

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

b. Traditional CAMP test

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

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

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

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

8.6.4 Genetic Identification Tests

- a. Ribosomal RNA-based tests

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

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

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

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

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

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

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

8.7 Enumeration: Most Probable Number (MPN)

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

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

- ii As with the qualitative procedure, at least one colony must be confirmed from three available suspect isolates on the HL plate(s) representing each positive FB in the MPN series, including that from the undiluted UVM/sample homogenate. A ribosomal RNA-based test (Section 8.6.4) may be employed to supplant biochemical/CAMP testing and expedite confirmation of most isolates. However, both biochemical/CAMP testing and ribosomal RNA-based testing must be used in tandem to characterize at least one suspect isolate representing the highest dilution found to contain suspects.
- g. Record the number of tubes per dilution that were ultimately found to be *L. monocytogenes* positive. Use a 3-tube MPN table (e.g. MLG MPN Appendix) to determine the estimated number of *L. monocytogenes* per gram of product. If all tubes are negative but the undiluted UVM/sample homogenate (i.e. the qualitative test) is positive, report the sample as positive at an enumerated level less than the lowest value on the table.

8.8 Culture Storage and Shipment

- a. BHI or TSA-YE slants may be used for short-term storage of *Listeria* spp. The culture should be stabbed into the agar using an inoculating needle. Tubes should be sealed with Parafilm® or equivalent to prevent desiccation and stored at 2-8°C. Under these conditions, *Listeria* spp. can remain viable for many months.
- b. For long-term storage (i.e. for more than one year) or to assure that the genetic character of the strain does not change over time (e.g. lose plasmids or other unstable genetic elements), cultures should be lyophilized and/or frozen

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

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