

United States Department of Agriculture**Food Safety and Inspection Service****MLG 4.14****Isolation and Identification of *Salmonella* from Meat, Poultry, Pasteurized Egg, Siluriformes (Fish) Products and Carcass and Environmental Sponges**

This method describes the laboratory procedure for performing the isolation and identification of *Salmonella* from meat, poultry, pasteurized egg, Siluriformes (Fish) products and carcass and environmental Sponges.

Notice of Change

This method is revised to update the instructions for processing meat carcass and environmental sponges along with an updated picture. Minor updates also include removal of matrix requirements for control preparation and the addition of *Siluriformes* ready-to-eat (RTE) preparation instructions.

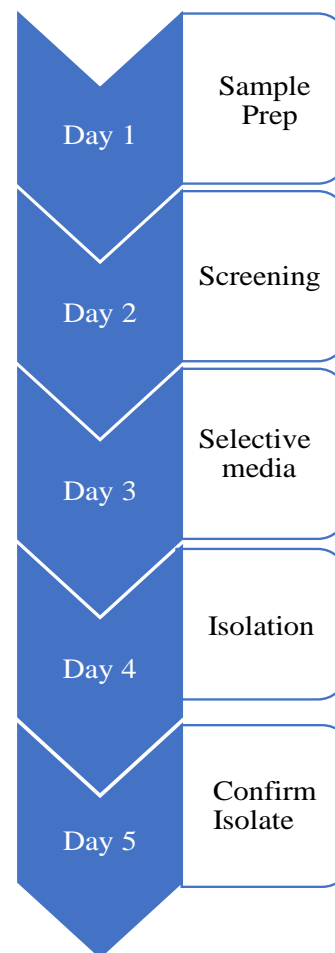
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Introduction

The following methods describe the analysis of various meat, poultry, egg, sponge, & rinse samples, for *Salmonella*. They are not intended for the isolation and identification of *Salmonella typhi*. Successful isolation of *Salmonella* from food can be related to factors including food preparation procedures, the number of organisms present, sample handling after collection, and others. Competitive flora is one of the most important isolation factors with raw products. Each sample has its own unique flora contributing to isolation success.

The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products are available for laboratory use. FSIS utilizes the following performance criteria when evaluating the suitability of an alternative laboratory method or product for a given analyte and sample matrix pair. A criteria value of 90% or greater is required of sensitivity, specificity, accuracy, and positive or negative predictive values. Additionally, performance criteria are relative to the reference cultural method for that analyte and sample matrix as outlined in the corresponding MLG chapter. Method validation is necessary to demonstrate the equivalence of alternative tests as detailed in the document titled “FSIS Guidance for Evaluating Test Kit Performance.”



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

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

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

KEY FACT

Salmonella are gram-negative, rod-shaped bacteria that cause disease in humans. *Salmonella enterica* is often associated with foodborne illness and is subdivided into multiple species and serotypes. Specific serotypes are often linked to foodborne outbreaks. *S. enterica* is widely dispersed in nature and can be found in the intestinal tracts of domestic and wild animals.

Safety Precautions

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

QUALITY CONTROL

Lab Quality Control Procedures

Include at least two method controls in all analyses. These controls must include a positive culture and an un-inoculated medium control. Incubate the controls along with the samples and analyze them in the same manner as the samples. Confirm at least one positive control isolate with each sample set.

Salmonella controls are to include an H₂S+ positive, fluorescent strain of *Salmonella* (*Salmonella enterica* serovar Typhimurium, commercially available) and an un-inoculated medium control.

The positive control cultures will be inoculated into an appropriate enrichment broth at a low-level inoculum level e.g., by preparing a test organism suspension in broth or saline equivalent in turbidity to a 0.5 McFarland standard. Using a 1 μ L loop, inoculate the broth or streak the plates to be tested. Alternatively, commercially prepared bacterial pellets will be used. Once the control cultures are started, incubate the controls along with the samples, and analyze them in the same manner as the samples. Confirm at least one isolate from the H₂S positive control sample. Confirmation of at least one colony from the H₂S negative control is required when confirming H₂S negative samples. In the absence of a positive test sample, control cultures will be terminated at the same point as the sample analyses.

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

Equipment, Reagents, Media, and Cultures

Table 1: Equipment and supplies for MLG 4

| Equipment | Supplier | Purpose |
|--|--|--|
| Incubators, static 42 ± 1°C, 35 ± 2°C | General lab supplier | Incubation of primary enrichment |
| Refrigerator (2 – 8°C) | General lab supplier | Store media and sample reserves |
| Heating block, 35 ± 2°C, 95 ± 3°C, 99°C | General lab supplier | Prepare sample DNA |
| Balance, sensitivity to at least ± 0.1 g | General lab supplier | Weigh samples |
| Bruker® MALDI Biotyper | Bruker Inc. | Proteomic Confirmation |
| Biochemical test kit and system, GN cards VITEK® 2 system | <i>bioMérieux</i> Vitek, Inc. | Biochemical identification (optional confirmatory technique) |
| Sterile inoculating loops | General lab supplier | Spread/streak plates |
| Pipets and sterile filter tips for the 5uL, 20uL volumes | General lab supplier | Add and mix reagents |
| Transfer pipet (plastic) | General lab supplier | Isolate preparation |
| Stomacher bags | General lab supplier | Primary bag for preparation and enrichment |
| Blending/mixing equipment: Paddle blender or equivalent | General lab supplier | Mix samples |
| Cloth sampling Device | Fremonta, Cat. # MT-S100 or General supplier | Raw Beef Trim Sample Collection Device |
| 3M Molecular Detection System | 3M, Model # MDS100 | Screen primary enrichment |
| Cooling block (20 – 25°C) | General lab supplier | Prepare sample DNA |
| Biomérieux GENE-UP® PCR instrument | <i>bioMérieux</i> GENE_UP | Salmonella Quantification |

Table 2: Kits and Reagents for MLG 4

| Kits and Reagents | Supplier | Purpose |
|--|---------------------------------|--|
| Bruker® MALDI Biotyper reagents | Bruker Inc. or General supplier | Proteomic Confirmation |
| 3M Molecular Detection Assay 2 – Salmonella | 3M, Catalog #MDA2SAL96 | Screen primary enrichment for <i>Salmonella</i> analyses |
| Crystal violet dye, 1% aqueous solution | General lab supplier | Sample Preparation |
| Calcium carbonate, sterile | General lab supplier | Sample Preparation |
| GENE-UP® QUANT <i>Salmonella</i> Test Kit | <i>bioMérieux</i> GENE_UP | Salmonella Quantification |

Media

- Buffered peptone water (BPW)
- Trypticase Soy Agar with 5% Sheep Blood (SBA)
- Tetrathionate Broth (TT) (Hajna)
- Nutrient agar slants
- Rappaport-Vassiliadis Soya Peptone Broth (RV), Modified Rappaport Vassiliadis (mRV) broth, or Rappaport-Vassiliadis R10 broth
- Brilliant Green Sulfa Agar (BGS; contains 0.1% sodium sulfapyridine)
- Double Modified Lysine Iron Agar (DMLIA)
- Saline (0.45%)
- Modified Tryptone Soy Broth (mTSB)

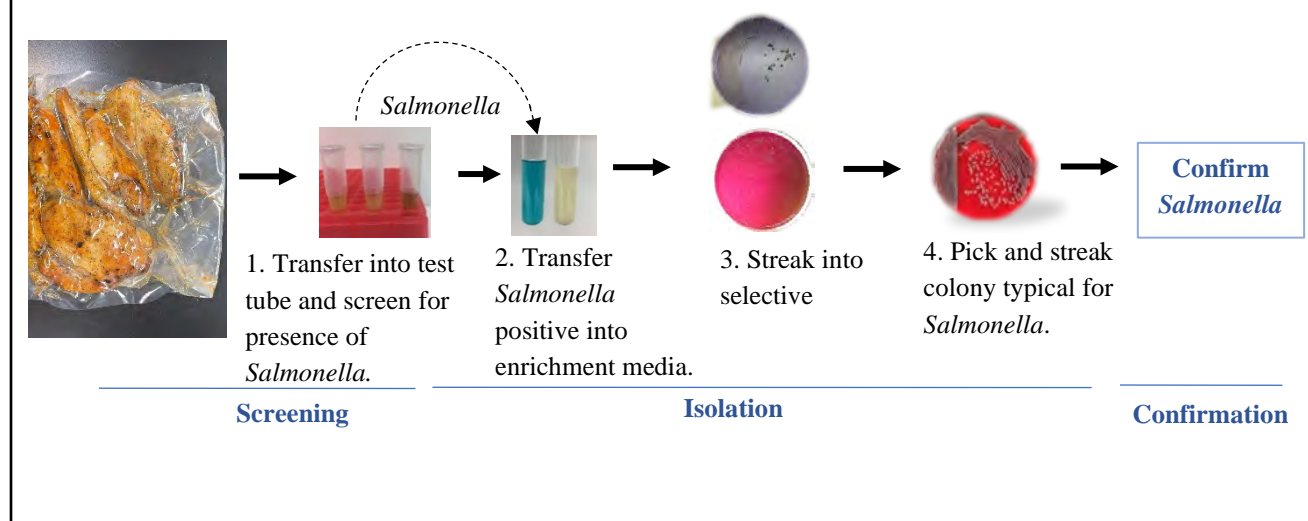
Cultures and Controls

Analyze a positive *Salmonella* control and an un-inoculated media control with each sample set. Confirm at least one isolate from the positive control sample. In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analysis. Fresh bacterial cultures on or in media yielding healthy growth following MLG protocols. For more information, refer to the section entitled “Lab Quality Control Procedure”.

Preparing Samples

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

Figure 1: Overview of the steps for isolating *Salmonella* from meat, poultry, egg, sponge, and rinse samples.



Intact retail packages must be disinfected at the incision sites immediately prior to incision for sampling using an appropriate disinfectant, e.g., 3% hydrogen peroxide, ca. 70% ethanol or ca. 70% isopropanol. If the package does not appear to be clean, scrub gently using soapy water and rinse thoroughly prior to disinfection. A sterile scalpel will be used for cutting the packaging. Aseptically pull the packaging away to expose the product for sampling.

For ready-to-eat (RTE) sausages in casing, the shell/casing is an integral part of the sample and must be free of pathogens and toxins. The casing is not to be disinfected since some casings are permeable and the disinfectant can be introduced into the core of the product. Consumers often slice through an inedible casing and then remove it thus any contamination on the surface of the casing could be transferred to the edible core of the product. Sample preparation and enrichment incubation times can vary by matrix and program. Refer to Table 1 and the following sample preparation sections.

Table 3. Sample Preparation and Enrichment Guide

| Product | Sample Preparation | | Incubation |
|--|--|---|-----------------------------|
| | Portion Size | Enrichment Amount determined by volume or weight | Cultural or rapid screen |
| Ready-to-Eat Meat, Poultry and Siluriformes Products | 325 ± 6.5 g | 975 ± 19.5 mL BPW | 35 ± 2°C for 18 – 24 hr. |
| Raw Poultry Products | 325 ± 32.5 g | 1625 ± 32.5 mL BPW | 35 ± 2°C for 20 – 24 hr. |
| Raw Meat and Raw Beef Mixed Products | 325 ± 32.5 g | 975 ± 19.5 mL mTSB | 42 ± 1°C for 15 – 24 hr. |
| Poultry Carcass and Environmental Sponges | 1 sponge pre- moistened with 10 mL buffer | 50 ± 1 mL BPW to bring total volume to 60 mL* | 35 ± 2°C for 20 – 24 hr. |
| Raw Beef Product Cloth Samples | 1 cloth pre- moistened with 25 mL buffer | Bring total volume to 200 ± 4 mL mTSB | 42 ± 1°C for 15 – 24 hr. |
| Meat Carcass and Environmental Sponges | 1 sponge pre- moistened with 10 mL buffer | 50 ± 1 mL mTSB to bring total volume to 60 mL* | 42 ± 1°C for 15 – 24 hr. |
| Whole Bird and Parts Rinses | 30 ± 0.6 mL sample rinse fluid | 30 ± 0.6 mL BPW | 35 ± 2°C for 20 – 24 hr. |
| Pasteurized Liquid, Frozen or Dried Egg Products | 100 ± 2 g | 900 ± 18 mL BPW | 35 ± 2°C for 18 – 24 hr. |
| Fermented Products | 325 ± 6.5 g + 10 g of sterilized calcium carbonate | 2925 ± 58.5 mL BPW with 1 mL of a 1% aqueous solution of crystal violet per liter | 35 ± 2°C for 18 – 24 hr. |
| Dried Products (Breeding Mix, Dehydrated Sauce, Soup Mix, Dried Milk) | 325 ± 6.5 g | 2925 ± 58.5 mL BPW | 35 ± 2°C for 18 – 24 hr. |

* Or maintain a 1:6 ratio for different project buffer volumes, e.g., 25 mL buffer + 125 mL enrichment.

KEY FACT

Sample temperature of $\leq 15^{\circ}\text{C}$ is required upon receipt for raw poultry and raw beef products.

Disinfect the surface of intact sample package(s) prior to opening.

FSIS Regulatory programs require *Salmonella* testing in concurrence with STEC for raw beef products. mTSB serves as a universal media for culturing STEC and *Salmonella* from a single sample.

RTE Meat, Poultry, Siluriformes Products and Pasteurized Egg Products

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

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

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

- If the meat, or poultry component is separate and distinct from other non-meat ingredients, analyze only the representative meat/poultry portion of the RTE product. Examples include products with the meat/poultry portion separate from any vegetable/dessert component, or fajita kits with meat/poultry, onions/peppers, and tortillas in three separate internal packages/bags within an outer package.
- When meat, or poultry, is combined with other ingredients to form the product (e.g., beef stew containing vegetables, potatoes, etc.), analyze representative meat/poultry portions in combination with other ingredients.
- Weigh the composite sample into a large sterile bag.
- Add ambient temperature sterile BPW. Stomach approximately two minutes.
- Incubate at $35 \pm 2^{\circ}\text{C}$ for 18 – 24 hr.
- Proceed to the section entitled “Rapid Screening *Salmonella* Test Procedure” for use of the rapid screen or refer to the section entitled “Selective Enrichment and Plating” to continue the cultural analysis.

Raw Poultry Products



If the sample is not already ground, it is best to mince it with sterile scissors or leave it whole (e.g., chicken wings). Add BPW. Stomach, or hand massage until clumps are dispersed.

Incubate at $35 \pm 2^{\circ}\text{C}$ for 20 – 24 hr.

Proceed to the section entitled “Rapid Screening *Salmonella* Test Procedure” for use of the rapid screen or refer to section entitled “Selective Enrichment and Plating” to continue the cultural analysis.

Raw Meat and Raw Beef Mixed Products



Add the mTSB at a 1:4 dilution, e.g., 325 ± 32.5 g sample with 975 ± 19.5 mL mTSB broth. Stomach, or hand massage until clumps are dispersed.

Incubate at $42 \pm 1^{\circ}\text{C}$ for 15 – 24 hr.

Proceed to the section entitled “Rapid Screening *Salmonella* Test Procedure” for use of the rapid screen or refer to section entitled “Selective Enrichment and Plating” to continue the cultural analysis.

Raw Beef Product Cloth Samples

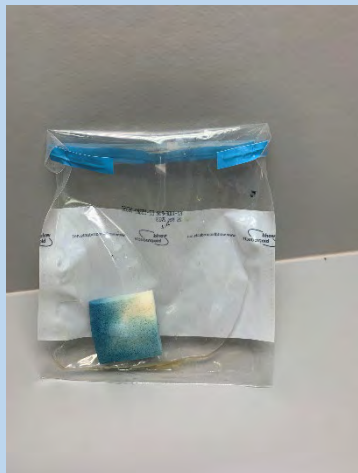


For raw beef cloth samples that are premoistened with 25 mL of buffer, tare the balance with a dry cloth then bring total volume of sample up to 200 mL \pm 4 mL with mTSB for enrichment. Aseptically manipulate the cloth to ensure it is submerged into the mTSB before stomaching and incubation. Mix well.

Incubate at 42 \pm 1°C for 15 – 24 hr.

Proceed to the section entitled “Rapid Screening *Salmonella* Test Procedure” for use of the rapid screen or refer to the section entitled “Selective Enrichment and Plating” to continue the cultural analysis.

Carcass Sponges and Environmental Sponges



For poultry carcass or environmental sponges, add 50 mL BPW to the sample bag containing a sponge moistened with 10 mL. Mix well.

Incubate at 35 \pm 2°C for 20 – 24 hr.

For meat carcass or environmental sponges, add 50 mL of mTSB to the sample bag containing a sponge moistened with 10 mL. Mix well.

Incubate at 42 \pm 1°C for 15 – 24 hr.

Proceed to the section entitled “Rapid Screening *Salmonella* Test Procedure” for use of the rapid screen or refer to section entitled “Selective Enrichment and Plating” to continue the cultural analysis.

Whole Bird and Parts Rinses



For whole bird and parts rinses:

Use 30 ± 0.6 mL of the sample rinse fluid obtained above for *Salmonella* analysis. Add 30 ± 0.6 mL of sterile BPW and mix well.

Incubate at $35 \pm 2^\circ\text{C}$ for 20 – 24 hr.

Proceed to the section entitled “Rapid Screening *Salmonella* Test Procedure” for use of the rapid screen or refer to section entitled “Selective Enrichment and Plating” to continue the cultural analysis.

Pasteurized Liquid, Frozen, or Dried Egg Products



For Pasteurized liquid, Frozen, or Dried Egg product:

Mix the liquid sample with a sterile spoon, spatula, or by shaking. Weigh the liquid egg product into a sterile polypropylene bag.

Add sterile BPW and mix well by shaking or stomaching.

With dried egg samples, gradually add BPW to the sample. Add a small portion of the sterile BPW and mix to obtain a homogeneous suspension. Add the remainder of the BPW. Mix until a lump-free suspension is obtained.

Incubate at $35 \pm 2^\circ\text{C}$ for 18 – 24 hr.

Proceed to the section entitled “Rapid Screening *Salmonella* Test Procedure” for use of the rapid screen or refer to section entitled “Selective Enrichment and Plating” to continue the cultural analysis.

Fermented Products



For fermented products, follow the procedure for RTE foods except:

Blend by hand massaging or stomach the sample with 10 ± 0.2 g of sterilized calcium carbonate.

Use BPW that contains 1 mL of a 1% aqueous solution of crystal violet per liter.

Proceed to the section entitled “Rapid Screening *Salmonella* Test Procedure” for use of the rapid screen or refer to section entitled “Selective Enrichment and Plating” to continue the cultural analysis.

KEY FACT

Fermented products can be distinguished based on ingredients list with typical identifications such as: starter cultures, cheese culture, milk starter culture, live culture, and lactic acid bacteria.

Dried Products (Breeding Mix, Dehydrated Sauce, Dried Soup Mix)

Weigh the product into a sterile polypropylene bag.

Add a small portion of the ambient temperature sterile BPW and mix to obtain a homogeneous suspension. Add the remainder of the BPW. Mix until a lump-free suspension is obtained.

Incubate at $35 \pm 2^{\circ}\text{C}$ for 18 – 24 hr.

Proceed to the section entitled “Rapid Screening *Salmonella* Test Procedure” for use of the rapid screen or refer to the section entitled “Selective Enrichment and Plating” to continue the cultural analysis.

KEY FACT

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

Rapid Screening *Salmonella* Test Procedure

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

Samples that are rapid screen-negative will be reported as negative. All other samples will continue to cultural analysis.

For samples with rapid screen results that are considered inconclusive, the laboratory will investigate. Based on the findings, the laboratory must:

- repeat the rapid screen analysis from the lysate step or
- prepare new rapid screen lysate tubes and repeat the analysis.

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

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

If circumstances (e.g., a power outage or equipment failure) do not allow testing using the rapid screen system, the laboratory shall continue cultural analysis of all samples by proceeding with isolation and purification steps as per MLG 4, section entitled “Selective Enrichment and Plating Media”.

Selective Enrichment and Plating Media

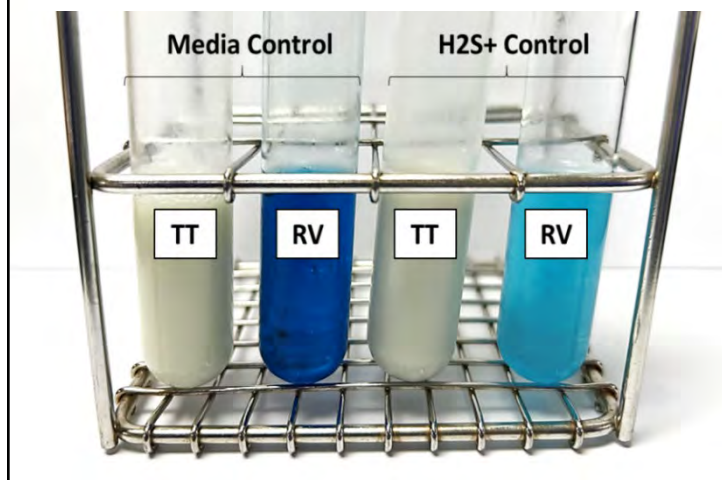
Transfer 0.5 ± 0.05 mL of sample into 10 mL TT broth (Hajna) broth and 0.1 ± 0.02 mL into 10 mL RV broth.

Incubate at $42 \pm 0.5^\circ\text{C}$ for 22 – 24 hr. or in a water bath at $42 \pm 0.5^\circ\text{C}$ for 18 – 24 hr.

Carefully mix contents of tube by vortexing or equivalent means. Streak to BGS and DMLIA agar plates using a $10 \mu\text{L}$ loopful of inoculum for each plate. Streak the entire agar plate with a single sample enrichment.

Incubate at $35 \pm 2^\circ\text{C}$ for 18 – 24 h.

Figure 2: Examples of TT (Hajna) and RV broths inoculated with media control are shown on the left and broths inoculated with *Salmonella* are shown on the right.

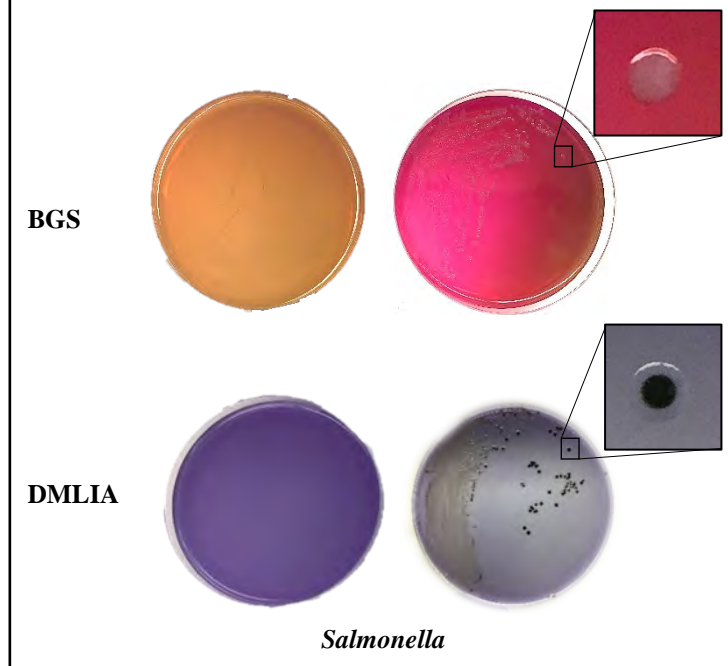


Examination of and Picking Colonies from Plating Media

Examine the BGS and DMLIA plates for the presence of colonies meeting the description of suspect *Salmonella* 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.
- **DMLIA.** Select purple colonies with (H_2S positive) or without (H_2S negative) black centers. Since *Salmonella* typically decarboxylate lysine and ferment neither lactose nor sucrose, the color of the medium reverts to purple.

Figure 3: Example of BGS and DMLIA. Control plates without *Salmonella* are shown on left. Plates streaked with *Salmonella* are shown on right. Magnification shows isolated colony.



Select well-isolated colonies that meet the description for *Salmonella* spp. from a BGS or DMLIA plate and streak onto SBA. Before any sample is reported as *Salmonella* negative, pick at least three total typical colonies, if available. A representative of each typical colony type must be picked from each plate type before reporting the sample as *Salmonella* negative. 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 are viable.

If there are typical colonies on a plate that are not well isolated, pick from the typical colonies and streak directly to a new set of selective agar plates. Alternatively, transfer typical colonies into a tube of TT broth (Hajna) or RVS broth and incubate overnight, then streak to selective agars.

Incubate BGS and DMLIA plates for an additional 18 – 24 h at $35 \pm 2^\circ\text{C}$. Incubate SBA plate for 16 – 24 h at $35 \pm 2^\circ\text{C}$.

Reexamine initially negative plates and pick colonies as above. After 48-hr incubation, plates with no typical colonies are discarded as negative. Plates with colonies undergoing confirmation testing must be stored at $2 - 8^\circ\text{C}$ until testing is complete. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, select colonies again for confirmation following the section entitled “Examination of and Picking Colonies from Plating Media”.

KEY DEFINITIONS

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

Confirming *Salmonella* and Further Analysis

Perform confirmatory tests using a single isolated colony. Commercially available test systems such as Bruker[®] MALDI Biotyper or validated equivalent systems are to be employed. Refer to the manufacturer’s instructions for the use of the instrument, preparation of reagents, and troubleshooting guidance.

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

The *Salmonella* isolate will be further characterized using Antimicrobial Susceptibility Testing (AST), and Whole Genome Sequencing (WGS).

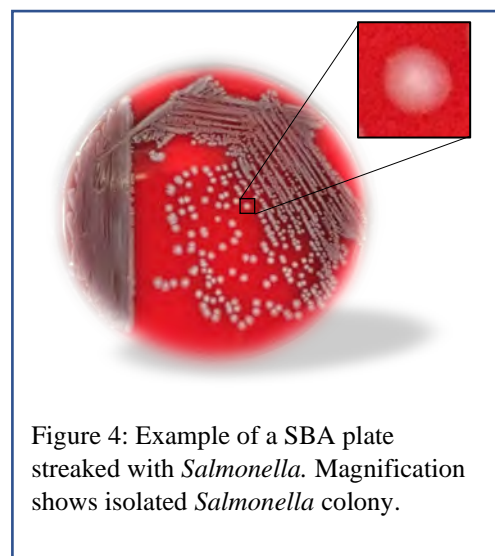


Figure 4: Example of a SBA plate streaked with *Salmonella*. Magnification shows isolated *Salmonella* colony.

Confirmation Criteria

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

Sample *Salmonella* Enumeration Procedure (Poultry Rinsates and Raw Poultry)

Salmonella enumeration is performed on confirmed *Salmonella* positive samples using the bioMérieux GENE-UP® PCR instrument with the GENE-UP® QUANT *Salmonella* Test Kit. Perform *Salmonella* enumeration within 72 hours of sample collection. DNA extractions are stored at $\leq -20^{\circ}\text{C}$ until final isolate confirmation. Upon confirmation, DNA extraction is used to determine the concentration of *Salmonella* spp. in the original, un-enriched poultry rinsate or raw poultry sample. Refer to manufacturer's instructions for use of the instrument, preparation of reagents, and troubleshooting guidance.

Appendix: Alternative Methods

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

To biochemically confirm isolates, inoculate appropriate VITEK® 2 cards (if using VITEK® 2 Compact) or equivalent.

References

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3M® Molecular Detection System User Guide

Bruker® MALDI Biotyper Users Guide. Proteomic isolate confirmation testing.

VITEK® 2 Compact User Guide. Biochemical isolate confirmation testing.

Contact Information and Inquiries

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

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

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