# Testing Environmental Sponge Samples for Next-Day *Listeria* Species Results

Jennifer Pelowitz, Wendy Lauer, Joshua Whitworth
Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Dr, Hercules, CA 94547 USA

## Abstract

A global food and agriculture company partnered with Bio-Rad Laboratories, Inc. to evaluate methods to detect high levels of *Listeria* spp. in environmental sponge samples with a target 6–12 hr sample enrichment period to obtain quantitative PCR (qPCR) results in one day. Current methods typically have incubation times of 24 hr. In previous studies, we identified the minimum incubation period required to detect multiple strains of *Listeria* at an inoculum of 30–50 colony forming units (CFU) with the iQ-Check™ *Listeria* spp. PCR Detection Kit method with and without preheating the enrichment media (data not shown). Initial testing showed that with prewarmed media, positive test results for *Listeria* spp. can be obtained following a 4–5 hr incubation period with the iQ-Check Standard II Protocol. To further challenge the method and simulate real-world conditions, environmental sponges were obtained from a manufacturing production plant containing normal flora for subsequent inoculation with *Listeria* in the laboratory. A new highly nutritive enrichment medium, *Listeria* Special Broth II (LSB II), was tested and proved to facilitate the reduced enrichment time.

## Introduction

*Listeria* spp. are gram-positive, rod-shaped bacteria that are motile by flagella. They are hardy and survive many harsh environmental conditions such as low temperature, low pH, freezing, drying, and heating. These environmental organisms are found in soil and water, on vegetation, and on surfaces in food processing facilities. The genus *Listeria* is often used as an indicator of improper sanitation procedures for equipment and possible contamination with pathogenic *Listeria monocytogenes*. *Listeria* ssp. have been found throughout the environment of processing plants on various surfaces including equipment, tools, vents, floors, and food transport racks, making control of the bacteria on environmental surfaces a critical control point. The timely acquisition of *Listeria* environmental results allows for corrective actions to be taken, a pivotal factor in bolstering food safety protocols and protecting the integrity of the food supply chain.

## Methods

Environmental samples collected using polyurethane environmental sponges with HiCap Neutralizing Broth (World Bioproducts, catalog #EZ-10HC-PUR) were received from the production plant and randomly assigned a number, inoculum, and incubation time (Table 1). Samples were directly inoculated with a target 30–50 CFU of either *Listeria monocytogenes*, ATCC 13932 (actual CFU, 43) or *Listeria grayi*, ATCC 25401 (actual CFU, 49), and 100–1,000 CFU of competing organism *Staphylococcus aureus*, ATCC 6538 (actual CFU, 482). Sponges were enriched with 60 ml *Listeria* Special Broth II (LSB II, Bio-Rad Laboratories, Inc., #12017378) prewarmed to 37°C and incubated for 4, 5, or 6 hr. After incubation, samples were processed with the Standard II Protocol (document #10000167777), and the resulting DNA samples were tested in triplicate using the iQ-Check *Listeria* spp. Detection PCR Kit (Bio-Rad, #3578113) and the FAST thermal protocol on a CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad, #3600037).



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**Table 1. Sample log and experimental setup.**

| Sample | Plant Location | Inoculum Target Organism | Inoculum Competing Organism | Enrichment Time, hr |
|---|---|---|---|---|
| 1 | Bottom of belt | *L. grayi* | *S. aureus* | 4 |
| 2 | Hose #1 | *L. grayi* | *S. aureus* | 4 |
| 3 | Rails under belt | *L. grayi* | *S. aureus* | 4 |
| 4 | Ceiling exhaust by dicer | *L. monocytogenes* | *S. aureus* | 4 |
| 5 | Inedible barrel wheel mounts | *L. monocytogenes* | *S. aureus* | 4 |
| 6 | Floor under shaker table | *L. monocytogenes* | *S. aureus* | 4 |
| 7 | Stairs to leveling belt | *L. grayi* | *S. aureus* | 5 |
| 8 | Hose #2 | *L. grayi* | *S. aureus* | 5 |
| 9 | Metal detector control panel | *L. grayi* | *S. aureus* | 5 |
| 10 | Legs of metal detector #2 | *L. monocytogenes* | *S. aureus* | 5 |
| 11 | Dicer framework | *L. monocytogenes* | *S. aureus* | 5 |
| 12 | Blade cart | *L. monocytogenes* | *S. aureus* | 5 |
| 13 | Drain unclogging tool | *L. grayi* | *S. aureus* | 6 |
| 14 | Incline belt to shaker table | *L. grayi* | *S. aureus* | 6 |
| 15 | Legs of metal detector #1 | *L. grayi* | *S. aureus* | 6 |
| 16 | Drain | *L. monocytogenes* | *S. aureus* | 6 |
| 17 | Legs at chiller exit | *L. monocytogenes* | *S. aureus* | 6 |
| 18 | Chiller door | *L. monocytogenes* | *S. aureus* | 6 |
| 19 | Framework of metal detector #2 | *L. grayi* | — | 5 |
| 20 | Crack in floor behind chiller | *L. monocytogenes* | — | 5 |

The sponges were returned to the incubator for an additional 16–18 hr, after which they were streaked to Modified Oxford Medium (MOX, Hardy Diagnostics, #G46) and RAPID'*L.mono* Agar Plates (RLM, Bio-Rad, #3563694) and incubated at 35 ± 2°C for 24–28 hr. Typical colonies were confirmed with qPCR. Simultaneously, 0.1 ml of the primary enrichment was added to 10 ml 4-morpholinepropanesulfonic acid–buffered *Listeria* enrichment broth (MOPS-BLEB) prepared from MOPS, Free Acid, Molecular Biology Grade (MilliporeSigma, #475922-100GM), MOPS-Na ≥ 99%, High Purity (VWR International, LLC., #E413-250G), and Criterion Listeria Enrichment Broth Base, Dehydrated Culture Media (Hardy Diagnostics, #C9301) and incubated at 35 ± 2°C for 18–24 hr. A secondary qPCR analysis was performed with the MOPS-BLEB culture. To remove PCR inhibition, a 1:10 dilution was needed for qPCR on MOPS-BLEB enrichments.

Primary enrichments for sponges that did not show growth were tested with Mquant Quaternary Ammonium Compounds Test Strips (MilliporeSigma, #1.17920.0001).

## Results

### 4-Hr Enrichments

The combination of prewarmed LSB II and DNA sample preparation using the Standard II Protocol following incubation for 4 hr gave inconsistent results. Some samples that tested negative after 4 hr had positive results with a secondary enrichment (Table 2). Samples that were fractionally positive at 4 hr and negative after secondary enrichment were assumed to be the result of the inoculum dying due to quaternary ammonia on the sponges. These unconfirmed positive results could potentially be removed by using iQ-Check Free DNA Removal Solution (FDRS, Bio-Rad, #3594970) if the cause was free DNA in the sample.

**Table 2. Results following a 4-hr enrichment.** Replicates show fractional recovery and inconsistencies with secondary enrichment results.

| | | | Same-Day qPCR Results | | | | | | | Secondary Enrichment qPCR Results | | | | | | | Confirmation Results | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time Point | Sample | Organisms | Cq 1 | Cq 2 | Cq 3 | Avg | Rep 1 | Rep 2 | Rep 3 | Cq 1 | Cq 2 | Cq 3 | Avg | Rep 1 | Rep 2 | Rep 3 | RLM Agar | MOX Agar | PCR Colony Confirm | Quats, mg/L |
| 4 hr | 1 | *L. grayi, S. aureus* | 37.84 | 35.96 | 37.39 | 37.06 | + | + | + | 25.82 | 26.29 | 25.84 | 25.98 | +* | + | + | + | + | + | NA |
| | 2 | | 34.88 | 35.00 | 35.40 | 35.09 | + | + | + | 21.47 | 21.68 | 21.82 | 21.66 | +* | + | + | + | + | + | NA |
| | 3 | | 40.84 | ND | ND | 40.84 | + | – | – | 27.58 | 27.46 | 27.22 | 27.42 | + | + | + | – | – | + | –30 |
| | 4 | *L. monocytogenes, S. aureus* | 35.81 | ND | 38.10 | 36.96 | + | – | + | 18.66 | 18.73 | 19.04 | 18.81 | + | + | + | + | + | + | NA |
| | 5 | | 36.85 | 40.01 | 36.38 | 37.75 | + | + | + | 19.06 | 19.00 | 18.97 | 19.01 | + | + | + | + | + | + | NA |
| | 6 | | 39.39 | 40.53 | ND | 39.96 | + | + | – | ND | ND | ND | ND | – | – | – | – | – | – | –30 |

* RLM positive for *Listeria* ssp. other than *L. monocytogenes*.

Cq, quantification cycle; MOX, Modified Oxford Medium; NA, not tested; ND, not detected; Quats, quaternary ammonium compounds; Rep, replicate samples; RLM, RAPID'*L. mono* Agar Plates.

**Table 3. Results following 5- and 6-hr enrichements.** Replicates show consistent results compared to secondary enrichments.

| | | | Same-Day qPCR Results | | | | | | | Secondary Enrichment qPCR Results | | | | | | | Confirmation Results | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time Point | Sample | Organisms | Cq 1 | Cq 2 | Cq 3 | Avg | Rep 1 | Rep 2 | Rep 3 | Cq 1 | Cq 2 | Cq 3 | Avg | Rep 1 | Rep 2 | Rep 3 | RLM Agar | MOX Agar | PCR Colony Confirm | Quats, mg/L |
| 5 hr | 7 | *L. grayi, S.aureus* | 34.79 | 35.46 | 34.71 | 34.99 | + | + | + | 22.36 | 22.19 | 22.41 | 22.32 | + | + | + | +* | + | + | <30 |
| | 8 | | 34.15 | 34.76 | 35.39 | 34.77 | + | + | + | 21.89 | 21.64 | 22.13 | 21.89 | + | + | + | +* | + | + | NA |
| | 9 | | 36.52 | 34.75 | 35.53 | 35.60 | + | + | + | 21.74 | 21.76 | 21.54 | 21.68 | + | + | + | +* | + | + | NA |
| | 19 | *L. grayi* | 34.13 | 36.17 | 37.92 | 36.07 | + | + | + | 19.46 | 19.66 | 19.90 | 19.67 | + | + | + | +* | + | + | NA |
| | 10 | *L. monocytogenes, S. aureus* | 35.85 | 35.03 | 35.69 | 35.52 | + | + | + | 19.13 | 19.14 | 19.60 | 19.29 | + | + | + | + | + | + | NA |
| | 11 | | 37.78 | 36.91 | 36.87 | 37.19 | + | + | + | 19.62 | 19.37 | 19.44 | 19.48 | + | + | + | + | + | + | NA |
| | 12 | | 36.28 | 37.08 | 34.90 | 36.09 | + | + | + | 19.11 | 19.23 | 19.12 | 19.15 | + | + | + | + | + | + | <30 |
| | 20 | *L. monocytogenes* | ND | 39.21 | ND | 39.21 | – | + | – | ND | ND | ND | ND | – | – | – | – | – | – | ~100 |
| 6 hr | 13 | *L. grayi, S. aureus* | 34.59 | 34.86 | 34.02 | 34.49 | + | + | + | 21.30 | 21.48 | 21.39 | 21.39 | + | + | + | + | + | + | NA |
| | 14 | | 37.68 | 35.87 | 35.72 | 36.42 | + | + | + | 22.46 | 22.42 | 22.53 | 22.47 | + | + | + | + | + | + | NA |
| | 15 | | 34.20 | 33.79 | 35.05 | 34.35 | + | + | + | 22.81 | 22.80 | 22.92 | 22.84 | + | + | + | + | + | + | NA |
| | 16 | *L. monocytogenes, S. aureus* | ND | ND | 38.78 | 38.78 | – | – | + | ND | ND | ND | ND | – | – | – | – | – | – | ~75 |
| | 17 | | 35.20 | 35.05 | 34.62 | 34.96 | + | + | + | 19.46 | 19.15 | 19.56 | 19.39 | + | + | + | + | + | + | NA |
| | 18 | | 33.92 | 33.61 | 33.76 | 33.76 | + | + | + | 19.03 | 19.92 | 19.49 | 19.48 | + | + | + | + | + | + | NA |

* RLM positive for *Listeria* ssp. other than *L. monocytogenes*.
Cq, quantification cycle; MOX, Modified Oxford Medium; NA, not tested; ND, not detected; Quats, quaternary ammonium compounds; Rep, replicate samples; RLM, RAPID'*L. mono* Agar Plates.

#### 5- and 6-Hr Enrichments

In contrast to the time point at 4 hr, enrichments ≥5 hr with prewarmed LSB II followed by DNA sample preparation using the Standard II Protocol gave consistent results when compared to secondary enrichment (Table 3). Samples that were fractionally positive and negative after secondary enrichment were assumed to be the result of the inoculum dying due to quaternary ammonia on the sponges.

### Unconfirmed Positive and Negative Results

Samples that produced negative and unconfirmed positive qPCR results after inoculation (20 and 16) were tested with Mquant Quaternary Ammonium Test Strips to determine whether a bactericide cleaner was picked up and not neutralized when swabbing with the environmental sponges. In all cases, sponge enrichments tested positive for 30–100 mg/L of quaternary ammonia. For comparison, two random samples (7 and 12) that tested positive after both primary and secondary enrichments were also analyzed, producing negative results for quaternary ammonia.

### Conclusions

A 4-hr incubation period for the inoculated sponges gave inconsistent results; however, a minimum incubation time of 5 hr gave consistent positive results except in the presence of quaternary ammonia. Additional work can be done to treat unconfirmed positive samples with FDRS and retest to see if the result was due to free DNA in the sample. LSB II was designed to be a highly nutritive enrichment media, particularly for injured *Listeria* cells, allowing for a decreased sample enrichment time. Obtaining *Listeria* spp. environmental results 20 hours earlier than currently available methods is paramount to ensure food safety. Rapid identification of potential sources of contamination enables timely corrective actions, preventing the possible spread of *Listeria* in the food processing environment. This proactive approach safeguards consumers from the risk of consuming contaminated products and minimizes economic and reputational consequences for food manufacturers.

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