AOAC Official Method 996.14
Detection of Listeria monocytogenes and Related Listeria Species in Selected Foods and from Environmental Surfaces
Assurance Polyclonal Enzyme Immunoassay (EIA)
First Action 1996
Revised First Action 1999
Final Action 2001
Revised 2001

(Applicable to detection of Listeria monocytogenes and related Listeria species in dairy foods, red meats, pork, poultry and poultry products, seafood, fruits, vegetables, nutmeats, pasta, chocolate, eggs, bone meal, and from environmental surfaces.)

Caution: L. monocytogenes infections can cause fetal death. It is recommended that pregnant women avoid handling this organism. Sterilize contaminated equipment and media before disposal or reuse.

See Table 996.14 for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Proprietary antibodies with a high specificity to L. monocytogenes and related Listeria species antigens are bound to microwell plates. Enriched test portions and positive controls are added to the plate. Any antigens that are present bind to the microwells, forming antibody-antigen complexes. Nonreactive material is washed away. Listeria-specific antibody is added, and the incubation and wash procedures are repeated. Alkaline phosphatase conjugate is then added to bind enzyme to Listeria antigens. After incubation, unbound conjugate is washed away. The substrate, p-nitrophenylphosphate, is added, and the absorbance of the resulting colored product is read spectrophotometrically at 405–410 nm. Readings above cutoff value indicate a presumptive positive; confirm by culture method.

B. Media and Reagents

(a) Wash solution concentrate.—2% Polyoxyethylene 20 sorbitan monolaurate (Tween 20) in water.
(b) Liquid substrate.—p-Nitrophenylphosphate solution, 4.33 mM; 1.6 mg disodium salt/mL.
(c) Positive control.—Stabilized, inactivated Listeria antigen.
(d) Antibody solution.—Highly purified antibodies reactive with Listeria antigen.
(e) Conjugate solution.—Alkaline phosphatase-antibody conjugate.
(f) Stop solution.—20% Ethylenediaminetetraacetic acid (EDTA) in water.
(g) Antibody-coated microwells.—Microwell strips, each well coated with Listeria antibody, 96-well holder, and plastic cover.
(h) Modified Fraser broth (mFB) with lithium chloride (mFB + LiCl).—Suspend 55 g commercial Fraser broth base in 1 L water. Stir until completely dissolved. If necessary, warm to dissolve powder but do not overheat. Only after powder has completely dissolved, add 4 g LiCl and stir until completely dissolved. Autoclave at 121°C for 15 min. Do not overheat. Do not add ferric ammonium citrate additive to broth. Alternatively, prepare a 45% (w/v) LiCl solution by dissolving 45 g LiCl in enough water for final volume of 100 mL. Filter sterilize the solution through a 0.2 μm filter. Add 2 mL sterile LiCl stock to 225 mL presterilized mFB. If using a commercially prepared 8M LiCl stock solution (Sigma), add 2.65 mL per 225 mL sterilized mFB.
(i) Buffered Listeria enrichment broth (BLEB).—Suspend 36.1 g commercial Listeria enrichment broth in 1 L water. Add 8.5 g 3-(N-morpholino)propanesulfonic acid (MOPS) and 13.7 g MOPS–Na salt. Mix well and heat to dissolve if necessary. Dispense in 9 mL aliquots. Sterilize by autoclaving at 121°C for 15 min.

Items (a)–(g) are available as Assurance Listeria Enzyme Immunoassay (EIA) test kit from BioControl Systems, Inc. (12822 SE 32nd St, Bellevue WA 98005 USA).

C. Apparatus

(a) Incubators.—Maintaining 30–32°C and 35–37°C.
(b) Water bath.—Maintaining 100°C. Alternatively, flowing steam autoclave set at 100°C or a dry heat block may be used.
(c) Microplate washer or plastic squeeze bottle.—For washing microwell strips.
(d) Microplate reader.—Photometer with 405–410 nm filter, reading microwell plates. May include optional printer.
(e) Micropipets.—Accurately dispensing 0.1 and 1.0 mL.
(f) Vortex mixer.—For mixing test portions.
(g) Top loading balance.—For weighing test portions, measuring up to 1000 g, sensitivity of ±0.1 g.
(h) Blender or stomacher.—For homogenizing test portions.

D. General Instructions

Store all reagents at 2–8°C when not in use. Let reagents equilibrate to room temperature before use. Include duplicate positive and one blank test wells with each run of test portions. Use separate pipets for each test suspension and reagent to avoid cross-contamination. Use reagents and components as an integrated unit and do not mix with components from other manufacturing batches or sources. Use dedicated trough or glassware for each reagent to avoid cross-contamination. Do not use reagents beyond stated expiration date. Do not reuse microwells. Store unused microwells in sealed foil pouch.

E. Preparation of Test Suspensions

(a) Primary enrichment.—Food products.—Aseptically weigh 25 g test portion or pipet a 25 mL aliquot into 225 mL mFB + LiCl, B(h). Blend or stomach food to homogenize test portion.

Environmental monitoring.—For environmental sponges, ensure that the sponge is in a horizontal position in the bag and add 60 mL mFB + LiCl, B(h), to sponge bag. If using a swab, add swab to 10 mL mFB + LiCl, B(h).

Mix well via stomacher or vortex mixer. Incubate 28 ± 2 h at 30°C.

(b) Secondary enrichment.—Transfer 1 mL incubated mFB + LiCl to 9 mL BLEB, B(i). For powdered milk products, transfer 0.1 mL incubated mFB + LiCl into 10 mL BLEB, B(i). Vortex tubes and incubate at 30°C for 24 ± 2 h.

(c) Inactivation.—Vortex mix incubated BLEB tubes and transfer 1.0 mL to a clean tube. Inactivate microorganisms at 100°C for 15 min. Cool tubes to 25–37°C before testing. Inactivated broths can be stored at 2–8°C up to 4 days prior to testing. Store remaining BLEB enrichment broths at 2–8°C to confirm presumptive positives. Note: Test tubes that have been stored must be mixed thoroughly before addition of test suspension to microwell.

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Table 996.14  Interlaboratory study results for detection of *Listeria monocytogenes* and related *Listeria* species from environmental surfaces (EIA)

<table>
<thead>
<tr>
<th>Surface/collection device</th>
<th>No. of labs</th>
<th>Total No. test samples</th>
<th>Test samples positive</th>
<th>Sensitivity rate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Incidence of false negatives among total positive test samples, %&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Specificity rate&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Incidence of false positives among total negative test samples, %&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Agreement between EIA and USDA/FSIS methods, %&lt;sup&gt;g&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Steel/swab</td>
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<td>95</td>
<td>28</td>
<td>28</td>
<td>29</td>
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<td></td>
<td></td>
<td>0.0</td>
<td>97</td>
<td>100</td>
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<tr>
<td>Rubber/sponge</td>
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<td>100</td>
<td>74</td>
<td>74</td>
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<td>3.8</td>
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<tr>
<td>Uninoculated</td>
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<td>100</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>96</td>
<td>100</td>
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<tr>
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<tr>
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<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>100</td>
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</tbody>
</table>

<sup>a</sup> Pres. = presumptive positive data; Conf. = culturally confirmed data.

<sup>b</sup> $\chi^2$ as defined by McNemar is $\frac{(|a - b| - 1)^2}{(a + b)}$ where $a =$ test samples positive by EIA and negative by USDA/FSIS, and $b =$ test samples negative by EIA and positive by USDA/FSIS. A $\chi^2$ value $>$ 3.84 indicates significance at $p < 0.05$.

<sup>c</sup> Sensitivity rate is defined as total number of analyzed positive test portions among “known” positive test portions/laboratory divided by total number of “known” positive test portions/laboratory, where “known” positive is defined as test samples confirmed positive by the reference method.

<sup>d</sup> Incidence of false negatives is 100 – sensitivity rate.

<sup>e</sup> Specificity rate is defined as total number of analyzed negative test portions among “known” negative test portions/laboratory divided by total number of “known” negative test portions/laboratory, where “known” negative is defined as samples confirmed negative by the reference method and negative controls.

<sup>f</sup> Incidence of false positives is 100 – specificity rate.

<sup>g</sup> Rate reflects number of confirmed determinations that were equivalent between EIA and USDA/FSIS.

<sup>h</sup> Statistical analysis not applicable. Methods gave equivalent results.
F. Enzyme Immunoassay Procedure

(1) Prepare wash solutions by adding 1.0 mL wash solution concentrate, B(a), to 100 mL water (sufficient to wash 48 wells). Label container. Wash solution is stable for 30 days stored at 2–8°C.

(2) Install a 405–410 nm filter in microwell plate reader.

(3) Fit required number of microwells, B(g), into holder. Reseal unused microwells. In addition to test wells, allow 3 extra wells for 2 positive controls and 1 blank. Carefully record positions of positive controls, blank, and tests in holder.

(4) Mix inactivated test suspensions, E(c), and positive control, B(c), separately on vortex mixer before pipetting. Use a new pipet tip for each test portion. Pipet 100 μL aliquot of test suspension into each well. Also pipet 100 μL aliquot of positive control into each positive control well. Leave blank well empty.

(5) Cover microplate with plastic cover provided and incubate 30 min at 35–37°C. Do not stack anything on top of microwell holder during incubation. Do not agitate plate during any incubation step.

(6) Following incubation, wash each well 3 times using either (a) or (b) as follows: (a) **Washing procedure.**—Completely remove contents of well with a microwell washer. Immediately fill wells completely with 250 μL of wash solution, F(I). **Note:** Effective washing is critical to obtain accurate data. Avoid overfilling wells to prevent antigen carryover to adjacent nonreactive wells. Avoid underfilling wells to prevent ineffective washing. **(b) Alternative washing procedure.**—Remove contents of well by inverting and vigorously tapping plate. Completely fill each well with wash solution, F(I), using a clean wash bottle. Repeat twice for a total of 3 aspiration/wash cycles per step.

(7) Immediately following removal of the last wash, mix antibody solution, B(d), by gently inverting the bottle several times. Add 100 μL antibody solution to each well, including the control and blank wells. Cover plate with plastic cover and incubate 30 min at 35–37°C.

(8) Following incubation, wash each well 3 times. Refer to washing procedure instructions, F(6).

(9) Immediately following removal of the last wash, mix conjugate solution, B(e), by gently inverting the bottle several times. Add 100 μL conjugate solution to each well, including control and blank wells. Cover plate with plastic cover and incubate 30 min at 35–37°C.

(10) Following incubation, wash each well 3 times. Refer to washing procedure instructions, F(6).

(11) Immediately following removal of the last wash, add 100 μL substrate, B(b), to each well, including control and blank wells. Cover plate with plastic cover and incubate 30 min at 35–37°C. After incubation do not wash wells. Proceed directly to G. If reading will be delayed, add 50 μL stop solution, B(f), to each well. Read within 1 h.

G. Reading

Read control and test well absorbencies (A) at 405–410 nm. To obtain valid results, calibrate the microwell plate reader against the blank well before reading test and control wells. Standardize reader by reading the blank well and adjusting absorbance to zero. Read the absorbance of each well, starting with the 2 positive controls, with a microwell plate reader at 405 or 410 nm. **Note:** When reader is standardized to blank well, certain test wells may read less than zero. This is not uncommon and indicates a negative result.

H. Interpretation of Test Results

**Positive control value.**—The positive control absorbance readings should be >0.8 A units. Absorbance readings below this value may indicate problems with the washing procedure. Contact BioControl Technical Services for more information.

**Cutoff value.**—Calculate the average value of the 2 positive control absorbance readings (in A units) and multiply by 0.25 to establish the cutoff value:

\[
\frac{\text{PC1} \ + \ \text{PC2}}{2} \times 0.25 \quad \text{cutoff value}
\]

where PC = positive control absorbance reading (in A units). Repeat positive controls for each test run. **Note:** Microwell plate reader linear range is variable depending on manufacturer’s specifications. If PC is reported as “over” or a numerical value over 2.5, use 2.5 A for calculation purposes.

**Negative results.**—Test wells with A readings less than the cutoff value are negative.

I. Confirmation of Positive EIA Test Portions

A reading greater than or equal to the cutoff value indicates a presumptive positive test result. See chapter on *Listeria* confirmation in the current edition of *Bacteriological Analytical Manual*, AOAC INTERNATIONAL, Gaithersburg, MD 20877, USA, or *Microbiology Laboratory Guidebook*, U.S. Department of Agriculture-Food Safety Inspection Services, Athens, GA 30604, USA. Isolate from previously enriched BLEB tubes.

References: *J. AOAC Int.* 80, 775(1997); (future issue).