Porting the acceptance of the method.

Presence of the color of chromogen to yellow. Absorbance at 450 nm is measured, horseradish peroxidase with substrate converts chromogen to conjugate solution. Conjugate binds to the fluorescein label present onto solid support. The unbound probe is removed by washing, and facilitates capture of probe:target hybrid nucleic acid molecules hybridization solution. Base pairing between poly dA and poly dT-coated plastic dipstick (solid phase) is then introduced into the hybridization solution. Base pairing between poly dA and poly dT facilitates capture of probe:target hybrid nucleic acid molecules onto solid support. The unbound probe is removed by washing, and dipsticks are incubated in horseradish peroxidase–antifluorescein conjugate solution. Conjugate binds to the fluorescein label present on hybridized detector probe. Unbound conjugate is washed away, and dipsticks are incubated in substrate–chromogen solution. Reaction of horseradish peroxidase with substrate converts chromogen to a blue compound. The reaction is stopped with acid, which changes the color of chromogen to yellow. Absorbance at 450 nm is measured. Absorbance in excess of the threshold value indicates the presence of Listeria in test portions.

B. Apparatus

(a) Photometer.—Capable of measuring absorbance at 450 nm of 1 mL solution in 12 × 75 mm tubes in reference and test portion wells.

(b) Tube racks.—Three plastic, heat-resistant (to 65°C) racks, to hold 50 tubes (12 × 75 mm). Minimum of 5 wells per row with 18 mm spacing between wells (measured between centers of wells).

(c) Dipstick holders.—Plastic device to hold 5 dipsticks in a row with 18 mm spacing between dipsticks (center to center).

(d) Wash basins.—Four metal, or plastic, heat-resistant (to 65°C) basins, 10 × 10 × 9 cm, with covers.

(e) Tubes.—Glass, 12 × 75 mm.

(f) Water baths.—(1) Capable of maintaining 65 ± 1°C, to hold one tube rack, one wash basin, with 5 cm water level. (2) Capable of maintaining 37 ± 1°C, to hold one tube rack with 5 cm water level.

(g) Repeater pipet.—Capable of accurately delivering 0.1, 0.25, and 0.75 mL, with syringe-barrel tips (optional). Alternately, serological pipets may be used.

(h) Sterile capped tubes.—To contain 1 mL volume.

(i) Sterile cotton applicator swabs.

Items (a)–(d) are available from GENE-TRAK Systems (94 South St, Hopkinton, MA 01748, USA). Substitute materials from other sources must be tested for equivalence.

C. Reagents

(Caution: Probe solution and positive control solution contain 0.1% sodium azide. Disposal of this reagent into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent explosion hazards.)

Store pretreatment reagent concentrate, lysis reagent concentrate, probe solution, enzyme conjugate 100× concentrate, substrate–chromogen solution, positive control solution, and negative control solution at 2–8°C. Store all other solutions and dipsticks at room temperature (<30°C).

(a) Pretreatment reagent concentrate.—150 mg Lysozyme and 3000 units mutanolysin in 0.1M potassium phosphate buffer.

(b) Pretreatment reagent buffer.—0.1M Tris pH 7.1–7.4, 0.01M disodium ethylenediamine tetraacetate (EDTA), and 0.0075% bromphenol blue.

(c) Lysis reagent concentrate.—Serine protease derived from Tritirachium album (Protease K is suitable).

(d) Lysis reagent buffer.—5% n-lauroyl sarcosine (Sarkosyl is suitable), 0.005M EDTA, 0.26M Tris pH 7.2–7.6, 1.0M NaCl, and 0.05% brilliant yellow.

(e) Listeria probe solution.—Fluorescein-labeled, Listeria-specific, synthetic oligonucleotide DNA probe and polydeoxyadenylic acid (dA)-tailed, Listeria-specific, synthetic oligonucleotide DNA probe in 0.1M Tris, pH 7.5; 0.001M EDTA; 0.1% bovine serum albumin; 0.01% octyl phenol ethylene condensate, nonionic detergent (NP-40 is suitable); 0.2% cresol red; and 0.1% sodium azide. Probes must exhibit specificity for Listeria and lack of cross-reactivity with bacteria of other genera. Specificity is determined by testing pure cultures of selected bacteria, grown in nonselective media to titer 10^5/mL, in assay. Test panel for specificity should include multiple strains of all Listeria species and strains of other bacteria that may be present in dairy products, seafoods, and meats, especially other Gram-positive bacteria.

(f) Wash solution, 20× concentrate.—1.0M Tris, pH 7.5; 0.4M EDTA; 3.0M NaCl; and 0.2% Tween-20.

(g) Enzyme conjugate, 100× concentrate.—Horseradish peroxidase–antifluorescein polyclonal antibody conjugate.

(h) Substrate–chromogen solution.—Hydrogen peroxide and tetramethylbenzidine.

(i) Stop solution.—2.0M H₂SO₄.

(j) Dipsticks.—Polystyrene dipsticks, 8 cm (5 cm handle, 3 cm fin). Fin has 5 paddle-like protrusions coated with polydeoxythymidylic acid (dT). Binding capacity of dT-coated dipsticks should exceed 250 ng complementary sequence, dA. Dipsticks should be tested in combination with matrix of other reagents to ensure proper method sensitivity.

(k) Positive control solution.—Listeria-specific oligonucleotide DNA at 20 ng/mL total concentration (sufficient to produce absorbance value = 1.0 when tested in assay) in 0.1M Tris, pH 7.5; 0.001M EDTA; 0.1% bovine serum albumin; 0.01% nonionic detergent (NP-40); and 0.1% sodium azide.

(l) Negative control solution.—Formaldehyde-inactivated Streptococcus faecium in phosphate-buffered saline, (m)(2), in concentrations sufficient to produce absorbance value >0.15 in assay when stringency conditions of assay (hybridization and/or wash temperatures) are not correct. Correctly performed assay should yield absorbance value 0.15 for negative control. Actual cell concentration used may vary depending on strain of organism used and media and conditions used for its preparation. Also contains 0.05% 2-bromo-2-nitropropane-1,3-diol (Bronopol).
(m) **Phosphate-buffered saline (PBS) solutions.**—(1) 10x stock solution.—Dissolve 12.0 g Na$_2$HPO$_4$, 2.2 g NaH$_2$PO$_4$, and 8.5 g NaCl in H$_2$O to 1 L. Autoclave 15 min at 121°C. (2) Working solution.—Dilute stock solution 1:9 with sterile H$_2$O. Mix well. Adjust pH to 7.5 with 0.1M HCl or 0.1M NaOH if necessary.

(n) **Phosphate-buffered Listeria enrichment broth (PEB).**—Combine 30.0 g Trypticase soy broth powder, 6.0 g yeast extract, 1.35 g KH$_2$PO$_4$, 9.6 g Na$_2$HPO$_4$, and 1 L H$_2$O and autoclave 15 min at 121°C. Add 15 mg acriflavin HCl, 40 mg nalidixic acid, and 50 mg cycloheximide to sterile media just before use. Stock solutions, 0.5% (w/v), of acriflavin HCl and nalidixic acid are prepared in H$_2$O and filter sterilized. Stock solution, 1% (w/v), of cycloheximide is prepared in 40% ethanol and filter sterilized.

(o) **UVM-2 broth.**—Dissolve 5.0 g proteose peptone, 5.0 g tryptone, 5.0 g lab Lemco powder, 5.0 g yeast extract, 20.0 g NaCl, 1.35 g KH$_2$PO$_4$, 12.0 g Na$_2$HPO$_4$, 1.0 g esculin, and 1.0 mL nalidixic acid solution (2% in 0.1M NaOH) in 1 L H$_2$O. Autoclave 15 min at 121°C. Do not overheat. Store in refrigerator. Just before use, add 1.0 mL of 2.5% filter-sterilized acriflavin per L of medium.

(p) **Modified UVM-2 broth.**—Add 5.0 g/L LiCl to UVM-2 broth before autoclaving [see (o)].

(q) **Modified LCA agar.**—Combine 52 g brain–heart infusion agar, 10 g LiCl, 10.0 g glycine anhydride, and 1 L H$_2$O and autoclave 15 min at 121°C. Cool to 45–50°C and add 5.0 mL filter-sterilized ceftazidime solution (10 mg/mL). Stir molten agar with magnetic mixing bar and pour 20 mL portions into 100 mm diameter Petri dishes. Refrigerate LCA agar plates in plastic bags. Plates may be stored 1 month at 4°C. Prepare 10 mg/mL ceftazidime solution by dissolving 50 mg ceftazidime in 5.0 mL H$_2$O. Discard any remaining ceftazidime solution.

(r) **Diagnostic reagents.**—Necessary for culture confirmation of positive DNA hybridization assays (refer to current edition of Bacteriological Analytical Manual [BAM], Gaithersburg, MD, USA).

Items (a)–(l) are available as Colorimetric GENE-TRAK™ Listeria Assay (DNA Hybridization Test for Detection of Listeria) from GENE-TRAK Systems.

### D. General Instructions

Include one positive control and one negative control with each group of test portions.

Do not touch fin portion of dipstick with fingers; hold by handle only. Do not reuse dipsticks or wash solution.

Use separate pipets or tips for each test portion and kit reagent to avoid cross-contamination. Exercise care not to contaminate substrate–chromogen solution with enzyme conjugate.

Return reagents requiring refrigeration to 2–8°C immediately after use. Refer to storage requirements on individual reagent bottle labels.

Treat all materials in contact with bacterial cultures as biohazardous materials and decontaminate by appropriate methods.

### E. Test Portion Preparation

(a) **Primary enrichment.**—Proceed according to product type as follows: (1) **Dairy products.**—For solid and semi-solid products, aseptically weigh 25 g test portion into sterile high-speed blender jar. Add 225 mL sterile PEB, C(n), prewarmed to 35°C, and blend 2 min

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Table 993.09  Interlaboratory study results for detection of *Listeria* in dairy products, seafoods, and meats by colorimetric deoxyribonucleic acid hybridization

<table>
<thead>
<tr>
<th>Food</th>
<th>Listeria level</th>
<th>Method agreement, %</th>
<th>Incidence (%) of false negatives among total positive test portions</th>
<th>Incidence (%) of false positives among method negative test portions</th>
<th>Sensitivity rate</th>
<th>Specificity rate</th>
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<tbody>
<tr>
<td></td>
<td>DNA Culture</td>
<td>DNA Culture</td>
<td>DNA Culture</td>
<td>DNA Culture</td>
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<tr>
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<td>15.6 4.7</td>
<td>84.4 95.3</td>
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<tr>
<td></td>
<td>Low</td>
<td>76.9</td>
<td>16.4 8.2</td>
<td>83.6 91.8</td>
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<td>—</td>
<td>—</td>
<td>1.6 0.0</td>
<td>98.4 100.0</td>
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<td>1.7 1.7</td>
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<td>3.4 10.2</td>
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<td></td>
<td>Control</td>
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<td>—</td>
<td>—</td>
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<tr>
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<td>16.9 8.5</td>
<td>83.1 91.5</td>
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</tr>
</tbody>
</table>

* Undefined.
at 10 000–12 000 rpm. For liquid products, add 225 mL sterile PEB, prewarmed to 35°C, and shake gently. Incubate 24 ± 4 h at 35°C. (2) Red meats and poultry.—Aseptically weigh 25 g test portion into sterile high-speed blender jar. Add 225 mL sterile UVM-2 broth, C(o), prewarmed to 35°C, and blend 2 min at 10 000–12 000 rpm. Incubate 24 ± 4 h at 35°C. (3) Seafoods.—Aseptically weigh 25 g test portion into sterile high-speed blender jar. Add 225 mL sterile Modified UVM-2 broth, C(p), prewarmed to 35°C, and blend 2 min at 10 000–12 000 rpm. Incubate 24 ± 4 h at 35°C.

(b) Secondary enrichment (all sample types).—Mix incubated primary enrichment culture well, and dip sterile cotton swab into culture. Swab onto entire surface of modified LCA agar plate, C(g), expressing as much liquid from swab as possible. Incubate LCA plate 24 ± 2 h at 35°C. Harvest growth from LCA plate with sterile swab and resuspend by swirling swab vigorously 5 s in 1 mL PBS, C(m), in sterile, capped tube. Express as much liquid from swab as possible before discarding swab.

F. DNA Hybridization Assay

(a) Fill water baths ca 5 cm and adjust to 37 ± 1°C and 65 ± 1°C.

(b) Prepare pretreatment reagent by adding 12 mL pretreatment reagent buffer, C(b), to pretreatment reagent concentrate, C(a), and mix by gentle swirling. Place on ice.

(c) Prepare lysis reagent by adding 6 mL lysis reagent buffer, C(d), to lysis reagent concentrate, C(e), and mix by gentle swirling. Place on ice. (Note: Reconstituted pretreatment reagent and lysis reagent are stable 60 days stored at −20°C. To thaw, place bottles in 37°C water bath 10 min, then place on ice.)

(d) For each 25 tests performed, dilute 65 mL 20× wash solution concentrate, C(f), to 1235 mL H2O; prepare one wash basin with 300 mL 1× wash solution, cover, and place in 65°C water bath; and prepare 3 wash basins with 300 mL 1× wash solution at room temperature and cover basins until needed.

(e) Label 12 × 75 mm tubes for test portions, plus one positive control, and one negative control. Place tubes in rack in rows of 5.

(f) Vortex or otherwise mix each PBS growth resuspension, E(b), positive control solution, C(k), and negative control solution, C(l). Add 0.5 mL to tubes as labeled. Return controls to 2–8°C.

(g) Add 0.10 mL reconstituted pretreatment reagent, (b), to each tube. Shake rack of tubes by hand 5 s. Incubate tubes 15 min in 37°C water bath. (Note: If, after addition of pretreatment reagent, resulting solutions are not purple, recheck that pretreatment reagent has been added.)

(h) Without removing rack from water bath, add 0.10 mL reconstituted lysis reagent, (e), to each tube. Briefly remove rack from water bath and shake rack by hand 5 s. Incubate tubes 15 min in 37°C water bath. (Note: If, after addition of lysis reagent, resulting solutions are not green, recheck that lysis reagent has been added.)

(i) Place one dipstick for each tube into dipstick holders. Rinse dipsticks 2–3 min in 1× wash solution at room temperature. Remove excess solution by blotting to absorbent paper (touch tip of dipstick fin to paper).

(j) Add 0.10 mL probe solution, C(e), to each tube. Place dipsticks into test portion tubes. Mix contents in tubes by raising and lowering dipsticks 5× (Note: If, after addition of probe solution, resulting solutions are not red, recheck that probe solution has been added.)

(k) Move rack of tubes to 65°C water bath and incubate 1 h.

(l) Set up and label second rack of 12 × 75 mm tubes. Prepare sufficient 1× enzyme conjugate by mixing 100× enzyme conjugate concentrate, C(g), and 1× wash solution 1:100. Dispense 0.75 mL 1× enzyme conjugate into each empty tube.

(m) Remove dipsticks from tubes in 65°C water bath. Wash dipsticks sequentially, with gentle shaking 1 min each, first in 65°C wash solution (do not remove wash basin from 65°C water bath), then in room-temperature wash solution. Blot dipsticks on absorbent paper. Place dipsticks into second set of tubes containing enzyme conjugate. Incubate 20 min at room temperature.

(n) Set up and label third rack of 12 × 75 mm tubes. Add one tube for blank. Dispense 0.75 mL substrate–chromogen solution, C(h), into each empty tube.

(o) Remove dipsticks from enzyme conjugate tubes. Wash dipsticks sequentially with gentle shaking 1 min each in remaining 2 basins containing room-temperature 1× wash solution. Blot dipsticks on absorbent paper. Place dipsticks into tubes containing substrate–chromogen solution. Incubate 30 min at room temperature. Remove dipsticks from tubes and discard.

(p) Add 0.25 mL stop solution, C(i), to each tube containing substrate–chromogen solution, including blank. Shake rack by hand to mix tube contents.

(q) To measure absorbance value, A (at 450 nm), wait for reading to stabilize before recording result for each tube. Determine negative control absorbance by placing tube labeled “Blank” in reference well and negative control tube in test portion well. Determine positive control absorbance by placing tube labeled “Blank” in reference well and positive control tube in test portion well. Determine test portion absorbance by placing negative control tube in reference well and test portion tube in test portion well.

G. Data Analysis

For negative control, A should be ≤0.15; for positive control, A should be 1.00. If these results are not obtained, assay should be repeated.

Negative criterion.—Test portion is considered negative (nonreactive for presence of Listeria) if A is ≤0.10.

Positive criterion.—Test portion is considered positive (reactive for presence of Listeria) if A is >0.10.

H. Confirmation of Positive DNA Hybridization Results

Test portions found positive by DNA hybridization assay must be confirmed by standard culture methods. For confirmation, streak PBS growth resuspension on a Listeria selective plating medium and continue with biochemical identification of presumptive Listeria isolates according to standard methods in current edition of BAM.


Revised: June 2000