AOAC Official Method 993.12
Listeria monocytogenes in Milk and Dairy Products
Selective Enrichment and Isolation Method
First Action 1993
Final Action 1996

IDF–AOAC Method

[Applicable to isolation of *Listeria monocytogenes* from Camembert, raw milk, skim milk powder, and ice cream at Spearmann-Karber 50% endpoint levels of 1.6 colony-forming units (cfu) per 25 g test portion, and from Limburger at 4.1 cfu/25 g.]

See Table **993.12A** for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle

Test portion is enriched by culturing in selective liquid medium. Enrichment culture is subcultured on diagnostic and selective isolation solid medium. Presumptive *Listeria* colonies are purified by subculture on nonselective solid medium before identification by appropriate morphological, physiological, and biochemical tests.

B. Apparatus

(*Note*: Sterilize all culture media and reagents unless specified otherwise. Sterilize all apparatus that will come into contact with culture media, reagents, and cultures. Sterilize by dry heat ≥1 h at 173°C or by moist heat with saturated steam ≥15 min at 121°C, as appropriate. Filter-sterilize stock chemical solutions as indicated. Commercially available sterile items such as plasticware may be used.)

(**a**) *Autoclave.*—Capable of maintaining 121 ± 1°C.

(**b**) *Oven.*—Capable of maintaining 173 ± 3°C.

(**c**) *Incubators.*—Capable of maintaining 25 ± 1°C, 30 ± 1°C, and 37 ± 1°C.

(**d**) *Water baths.*—Capable of maintaining 37 ± 1°C and 45 ± 1°C.

(**e**) *Blender.*—Rotary (8–45 × 10^3 rev/min), with sterilization-resistant glass or metal containers with fitted lids; or peristaltic-type (Stomacher) blender with peristaltic-type sterile plastic bags as containers. Container volume should be 2× volume of test sample plus medium.

(**f**) *Inoculating loops.*—Pt-Ir or Ni-Cr (if resterilized) or disposable sterile plastic for single use. Loop diameter ca 3 mm.

(**g**) *Inoculating needles.*—Same composition as loops, (**f**).

(**h**) *Inoculating loop or needle sterilizer.*—Bunsen burner, electric incinerator, or equivalent.

(**i**) *pH meter.*—Calibration accuracy of ±0.1 unit at 25 ± 1°C.

(**j**) *Refrigerator.*—Capable of maintaining 2–5°C.

(**k**) *Collimated source of white light.*

(**l**) *Mirror.*—Flat or concave.

(**m**) *Tripod.*—For oblique transmitted (Henry) illumination of microbial colonies on solid culture media in Petri dishes.

(**n**) *Phase-contrast microscope.*—Equipped with oil immersion objective.

(**o**) *Glass or plasticware.*—Petri dishes; culture bottles or flasks; 16 × 125 mm culture tubes with metallic or nonmetallic closures; measuring cylinders; 1, 10, and 25 mL graduated pipets; microscope slides; and Durham tubes.

(**p**) *Filter sterilizing apparatus.*—Disposable sterile filter unit, with 0.45 μm pore size filters, for use with vacuum source.

(**q**) *Centrifuge.*—Capable of processing 10–100 mL at 900 × *g*.

C. Reagents, Culture Media, and Cultures

(*Note*: Use chemical exhaust hood to dispense toxic powders [acriflavin, cycloheximide]. Whenever possible, use dehydrated basic components or complete dehydrated media. Before sterilizing media, adjust pH with 1M HCl or 1M NaOH using pH meter to obtain

Table 993.12A Interlaboratory study results for *Listeria monocytogenes* in milk and dairy products—selective enrichment and isolation method

Product	*L. monocytogenes*, cfu/25 g	Sensitivity rate[a] (error)	Specificity rate[b]	False-positive rate[c]	False-negative rate[d]
Limburger cheese	12	0.675 (0.114)	1.000	0.00	0.245
	120	0.950 (0.024)	1.000	0.00	0.048
Camembert cheese	12	0.975 (0.018)	1.000	0.00	0.024
	120	1.000 (0.000)	1.000	0.00	0.000
Ice cream	12	0.963 (0.028)	1.000	0.00	0.036
	120	1.000 (0.000)	1.000	0.00	0.000
Raw milk	12	0.888 (0.050)	1.000	0.00	0.101
	120	0.975 (0.018)	1.000	0.00	0.024
Skim milk powder	12	0.925 (0.039)	1.000	0.00	0.070
	120	0.938 (0.047)	1.000	0.00	0.059

[a] Sensitivity rate = (sum of number of analyzed positives among "known" positives per laboratory) / (sum of number of "known" positives per laboratory).
[b] Specificity rate = (sum of number of analyzed negatives among "known" negatives per laboratory) / (sum of number of "known" negatives per laboratory).
[c] False-positive rate = (sum of number of analyed postitives among "known" negatives per laboratory) / (sum of number of analyzed positives among both "known" positives and "known" negatives per laboratory).
[d] False-negative rate = (sum of number of analyzed negatives among "known" postives per laboratory) / (sum of number of analyzed negatives among both "known" positives and "known" negatives per laboratory).

(*Note*: The Spearmann-Karber 50% endpoint detection limit for all products except Limburger was 1.6 cfu/25 g test portion; 95% confidence limits were 1.48–1.68. The corresponding values for Limburger cheese were 4.1 cfu/25 g and 3.2–5.5 cfu/25 g.)

Table 993.12B Diagnostic reactions of *Listeria* species

	Acid production		CAMP reaction[a]	
Species	Rhamnose	Xylose	*S. aureus*	*R. equi*
L. monocytogenes	+	–	+	–[b]
L. innocua	±	–	–	–
L. ivanovii	–	+	–	+
L. seeligeri	–	+	+	–
L. welshimeri	±	+	–	–
L. grayi	–	–	–	–
L. murrayi	±	–	–	–

[a] Only the 3 CAMP-positive species exhibited hemolysis in sheep blood agar stabs.

[b] Weak positive reactions may occur, especially at 48 h incubation. These are insignificant compared to massive positive reaction of *L. ivanovii.*

stipulated value [0.1 unit] after sterilization at 25 ± 1°C. Prepared culture media may be stored at 2–5°C in dark for no more than 1 month.)

(**a**) *HCl.*—1M solution.

(**b**) *NaOH.*—1M solution.

(**c**) *Acriflavin stock solution.*—50 mg acriflavin monohydrochloride in 10 mL H_2O. Filter sterilize.

(**d**) *Nalidixic acid (sodium salt) stock solution.*—50 mg Na nalidixate in 10 mL H_2O. Filter sterilize.

(**e**) *Cycloheximide stock solution.*—100 mg cycloheximide in 10 mL 40% (v/v) aqueous ethanol (4 mL C_2H_5OH + 6 mL H_2O). Filter sterilize.

(**f**) *Selective enrichment medium base.*—Add 30 g tryptone soya broth powder (30 g tryptone soya broth powder contains 17 g pancreatic digest of casein, 3 g papaic digest of soybean meal, 5 g NaCl, 2.5 g dibasic potassium phosphate, and 2.5 g glucose) and 6 g yeast extract powder to 1 L H_2O. Adjust pH to 7.3. Dispense 225 mL portions into 500 mL culture bottles or flasks and sterilize by autoclaving.

(**g**) *Complete selective enrichment medium.*—Aseptically add 0.45 mL acriflavin stock solution, (**c**); 1.8 mL nalidixic acid salt stock solution, (**d**); and 1.15 mL cycloheximide stock solution, (**e**), to 225 mL selective enrichment medium base, (**f**).

(**h**) *Supplement mixture for isolation medium (Oxford agar).*—Dissolve 200 mg cycloheximide, 10 mg colistin sulfate, 2.5 mg acriflavin, 1 mg cefotetan, and 5 mg fosfomycin in 5 mL 50% (v/v) aqueous ethanol (2.5 mL C_2H_5OH + 2.5 mL H_2O). Filter sterilize.

(**i**) *Isolation medium base.*—For 500 mL complete medium, mix 19.5 g Columbia agar base (19.5 g Columbia agar base contains 11.5 g special peptone, 1.0 g starch, 2.5 g NaCl, and 5 g agar; special peptone varies with manufacturer), 0.5 g esculin, 0.25 g ferric ammonium citrate, and 7.5 g lithium chloride.

(**j**) *Complete isolation medium (Oxford agar).*—Suspend 27.75 g isolation medium base, (**i**), in 500 mL H_2O. Slurry should be pH 6.9. Boil to dissolve agar fraction. Autoclave, cool to 50°C, and add 5 mL supplement mixture, (**h**). Dispense 15 mL portions into sterile Petri dishes and let gel.

(**k**) *Nonselective solid culture medium (tryptone soya yeast extract agar, TSYE).*—Weigh and mix 30 g tryptone soya broth powder [formula in (**f**)], 6 g yeast extract powder, and 15 g agar. Add 1 L H_2O, mix, and boil to dissolve. Adjust for pH 7.3 after sterilization. Dispense 6 mL portions into tubes and autoclave. Slant tubes to gel. For agar medium plates, sterilize larger volumes, dispense 15 mL portions into Petri dishes, and let gel.

(**l**) *Nonselective liquid culture medium [tryptone soya yeast extract (TSYE) broth].*—Weigh and mix 30 g tryptone soy broth powder [formula in (**f**)] and 6 g yeast extract powder. Add 1 L H_2O, mix, and boil to dissolve. Adjust for pH 7.3 after sterilization. Dispense 6 mL portions into tubes; autoclave.

(**m**) *Stock carbohydrate solutions.*—Weigh 5 g each rhamnose and xylose; dissolve each separately in 100 mL H_2O. Filter sterilize.

(**n**) *Carbohydrate utilization broth base.*—Combine 10 g proteose peptone, 1 g beef extract, 5 g NaCl, and 0.02 g bromocresol purple. Add 1 L H_2O, mix, and boil to dissolve. Dispense 10 mL portions into tubes containing Durham tubes. Autoclave.

(**o**) *Complete carbohydrate utilization broths.*—(*1*) Aseptically add 1 mL rhamnose stock carbohydrate solution, (**m**), to 9 mL carbohydrate utilization broth base, (**n**). (*2*) Similarly, prepare broth using xylose stock carbohydrate solution, (**m**).

(**p**) *Motility agar medium.*—Combine 20 g casein peptone, 6.1 g meat peptone, and 3.5 g agar. Add 1 L H_2O. Mix and adjust to pH 7.3 after sterilization. Dispense 6 mL portions into tubes and autoclave.

(**q**) *Sheep blood agar basal medium.*—Weigh 40 g blood agar base No. 2 powder [39.5 g blood agar base No. 2 powder contains 15 g proteose peptone, 2.5 g liver digest, 5 g yeast extract, 5 g NaCl, and 12 g agar; in 1 L of basal medium, 40 g is used to allow for added volume of the sheep red blood cell suspension, (**s**)], add 1 L H_2O, mix, and adjust for pH 7.0 after sterilization. Dissolve by boiling and autoclave in ≤500 mL portions. Cool to 45°C and use promptly.

(**r**) *Washed sheep red blood cells.*—Centrifuge defibrinated sheep blood 30 min at 900 × *g*. Aseptically aspirate and discard supernate. Gently resuspend pellet in sterile saline, (**v**), to original volume. (*Note*: Horse red blood cells may be substituted for sheep red blood cells.)

(**s**) *CAMP (Christie, Atkins, and Munch-Peterson) test overlay medium.*—Prepare 100 mL sterilized molten sheep blood agar basal medium, (**q**). Temper to 45°C and add 7 mL washed sheep red blood cell suspension, (**r**). Hold at 45°C. Use promptly.

(**t**) *CAMP test basal medium.*—Pour 10 mL portions blood agar basal medium, (**q**), into sterile Petri dishes and let gel.

(**u**) *Complete CAMP test medium.*—Pour 3 mL CAMP test overlay medium, (**s**), onto CAMP test basal medium dishes, (**t**) (prewarmed to 37°C), as thin layer and let gel. Dry medium surface at 37°C before use.

(**v**) *Saline solution.*—0.8% (w/v) NaCl solution. Dissolve 8 g NaCl in 1 L H_2O. Dispense in 100 mL portions and sterilize.

(**w**) *CAMP test stock cultures.*—Weakly β-hemolytic strain of *Staphylococcus aureus* (NCTC 1803 is suitable); *Rhodococcus equi* (NCTC 1621 is suitable); and standard *Listeria* spp. strains (*L. innocua*, *L. ivanovii*, and *L. monocytogenes* are suitable). Establish stock cultures by inoculating TSYE agar slants, (**l**), and incubating 24–48 h at 37°C. Store stock cultures at 4°C. Maintain viable stock cultures by subculturing on fresh slants at 37°C at least monthly.

(**x**) *Catalase test substrates.*—Prepare 3% (v/v) H_2O_2 by diluting 30% (v/v) H_2O_2.

(**y**) *Gram stain solutions.*—(*1*) *Hucker's ammonium oxalate–crystal violet solution.*—Dissolve 2 g crystal violet (90% [w/v] dye content) in 20 mL ethanol; dissolve 0.8 g $(NH_4)_2C_2O_4 \cdot H_2O$ in 80 mL H_2O; mix these solutions and filter through paper into staining bottle. Age 24 h before use. (*2*) *Gram's iodine solution.*—Dissolve 1 g I_2 and 2 g KI in 300 mL H_2O. To facilitate dissolution, grind solids with mortar and pestle before and during addition of H_2O in

several mL portions. Store in amber bottle. (*3*) *Safranin*.—Dissolve 2.5 g safranin in 100 mL ethanol. For use, add 10 mL safranin solution to 100 mL H_2O. (*4*) *Acetone–alcohol*.—Mix equal volumes of acetone and ethanol.

D. Medium Quality Control

Check newly prepared batches of enrichment and isolation media for ability to support *L. monocytogenes* growth. Add 10–100 cells to control flask containing enrichment medium, **C**(**g**), and after 24 h incubation at 30°C, confirm enrichment by streaking culture onto isolation, **C**(**j**), and purification, **C**(**k**), agars. Similarly, check new batches of other culture media.

E. Test Portion Selection

Using sterile implements and aseptic technique, remove 25 g or 25 mL representative portions [*see* (**a**)–(**f**)] of milk or dairy product into sterile container.

(**a**) *Liquid milk.*—Rapidly invert container 25×to redistribute any sedimented microbes. Avoid causing excessive foaming. Let any foam disperse, but remove test portion within 3 min after mixing.

(**b**) *Dried milk and other dry milk products.*—Mix contents of closed container by repeatedly shaking and inverting. Allowing sufficient container head space (ca 50% v/v) is crucial for proper mixing.

(**c**) *Butter.*—Melt butter in sterile container in 45°C water bath with intermittent agitation. Remove container from bath as soon as butter is melted.

(**d**) *Cheese.*—Test portion should proportionately represent rind and interior.

(**e**) *Ice cream and other edible ices.*—Proceed as for butter, (**c**), but use water bath at 37°C.

(**f**) *Fermented milks, yogurt, custards, and desserts.*—Mix contents of closed container by shaking as for milk, or mix by stirring aseptically with sterile spatula or spoon.

F. Determination

(*Note*: Incubate cultures for stated time ±2 h.)

(**a**) *Selective enrichment.*—As described below, aseptically mix test portion with selective enrichment medium, **C**(**g**), and incubate 48 h at 30°C.

For liquid products (liquid milk, melted butter, ice cream, or other frozen milk products), aseptically pipet (prewarm pipet if necessary) 25 mL to 500 mL flask or equivalent vessel containing 225 mL selective enrichment medium. Mix by swirling.

For dried milk, dried milk products (except casein), fermented milks, yogurt, custards, and desserts, aseptically weigh 25 g into flask containing 225 mL selective enrichment medium. Stopper flask and shake until dissolved or dispersed. Sterile glass beads may be added to assist dispersal of insoluble fractions.

For cheese or casein, aseptically weigh 25 g into sterile blender containing 225 mL selective enrichment medium at 45°C. Blend 1–3 min to disperse thoroughly.

If acidic dairy product test samples lower pH of selective enrichment medium and food mixture below 7.0, aseptically adjust pH to 7.3 at 25 ± 0.1°C, using 1M HCl or NaOH solutions.

(**b**) *Selective isolation of Listeria.*—Streak loopful (ca 10 µL) onto surface of selective solid isolation medium (Oxford), **C**(**j**), and restreak with fresh loops 2–3 times, so well-isolated colonies can develop. Incubate inverted plate 48 h at 37°C.

(**c**) *Presumptive identification and choice of colonies for confirmation of Listeria.*—Examine Oxford agar plate for typical *Listeria* colonies which are small, black, and surrounded by black halos. Choose 5 typical or suspect colonies, or if there are <5 such colonies, choose all to confirm *Listeria*.

(**d**) *Purification of isolates for confirmation of presumptive Listeria.*—Streak chosen colonies individually onto surfaces of separate TSYE plates to obtain well-separated pure colonies. Incubate 24 h at 37°C or until well-developed colonies are obtained.

(**e**) *Examination of purified isolates.*—Examine purification plates, (**d**), using strong beamed white light incident on bottom of plate at 45°C (Henry illumination). For diagram of optical system, *see Bacteriological Analytical Manual* (8th Ed.), p. 147. When culture plate is viewed from above at perpendicular angle, *Listeria* colonies appear bluish and have granular surface. If not well-isolated, presumptive typical colonies should be repurified.

(**f**) *Gram-stain reaction and cell morphology.*—Suspend cells from suspect colony in 1 drop H_2O on slide to form light emulsion. Smear mixture over slide and let air-dry. Heat-fix dried smear by passing slide through flame. Flood smear with ammonium oxalate–crystal violet solution, **C**(**y**)(*1*), and stain 1 min. Rinse slide gently with tap water. Drain excess water and apply Gram's iodine solution, **C**(**y**)(*2*), to smear 1 min. Rinse stained slide in tap water. Decolorize slanted slide for ca 15–20 s with acetone–alcohol, **C**(**y**)(*4*), stream, stopping as soon as solvent flows colorlessly from slide. Do not over-decolorize. Counterstain with safranin, **C**(**y**)(*3*), 15 s, rinse with tap water, and blot dry with absorbent paper or air-dry. Add 1 drop immersion oil to stained smear and examine microscopically, using high-power oil immersion lens. Look for blue Gram-positive stain reaction and slim, short, rod-shaped morphology of *Listeria* cells. Gram-negative cells stain red. Gram-positive and -negative control cultures should be stained at same time, ideally as separate smears on test slide. Results are acceptable only when controls have stained properly. (*Note*: Other Gram staining techniques may also be used.)

(**g**) *Catalase reaction.*—Suspend one typical colony in 1 drop 3% hydrogen peroxide, **C**(**x**). Observe for any effervescence of oxygen gas bubbles.

(**h**) *Wet mount tumbling motility and cell morphology.*—Inoculate one typical colony on TSYE agar, (**d**), into TSYE broth medium, **C**(**l**), and incubate overnight at 25°C or until grown. Prepare wet mount on slide using 1 drop of culture. Add coverslip, followed by 1 drop of immersion oil. Examine for cell shape and characteristic tumbling motility, using phase-contrast microscope with high-power oil immersion phase-contrast objective.

(**i**) *Hemolysis reaction.*—Draw 5 ×5 cm grid, 25 spaces, on bottom of sheep red blood cell agar plate, **C**(**u**). Dry agar surface at 37°C if visibly wet. With sterile inoculating needle, stab typical colonies from TSYE agar plate, (**h**), into blood agar, one colony/space. Prepare, on same plate, control stabs of CAMP control *Listeria* strains. Incubate plates 48 h at 37°C. Examine test and control strains for hemolysis, holding plates up to bright light to compare test cultures with controls. *L. monocytogenes* shows narrow clear zones of β-hemolysis, zones surrounding *L. ivanovii* are wide and distinct, and *L. innocua* is nonhemolytic. If single-layer commercial red blood cell plates are used, ensure that stab goes from top to bottom of agar.

(**j**) *Inoculum for further biochemical confirmation of identity.*—Inoculate one typical colony from TSYE agar plate, (**d**), into corresponding TSYE broth, **C**(**l**). Incubate 24 h at 37°C. Use as inoculum for following tests.

(**k**) *Carbohydrate utilization test.*—Inoculate each kind of carbohydrate fermentation broth, **C**(**o**), with one loopful of TSYE broth inoculum, (**j**). Incubate 7 days at 37°C. Observe daily for acid-induced color change and gas formation (*see* Table **993.12B**). (*Note*: Sugars ferment without gas production.)

(**l**) *Motility agar test.*—Stab-inoculate motility agar, **C**(**p**), using TSYE broth inoculum, (**j**). Incubate 2–7 days at 25°C. Examine for development of diffuse growth out from stab and for subsurface region of enhanced growth with umbrella-like appearance.

(**m**) *CAMP test.*—Streak *S. aureus* and *R. equi* CAMP cultures, **C**(**w**), on complete CAMP test medium, **C**(**u**), as 2 thin and uniform parallel lines diametrically opposite each other, using inoculating loop or needle [for diagram and further detail, *see Bacteriological Analytical Manual* (8th Ed.), p. 144]. Streak several test broth cultures, (**j**), and control *Listeria* strains, **C**(**w**), similarly, between parallel streaks of CAMP cultures and at 90° to them. Test and control streaks, and CAMP culture streaks should not touch but should be as close as 1–2 mm. Incubate plates 18–24 h at 37°C. Positive reactions are indicated by enhanced zone of β-hemolysis at intersectional approach of test strain with either one or other, but generally not both, of CAMP cultures (*see* Table **993.12B**).

G. Interpretation of Test Results

All *Listeria* spp. are Gram-positive rods (but are more coccoidal in cultures older than 24 h) that move by tumbling in wet mounts and exhibit diffuse growth in motility agar. They are catalase-positive.

For differentiating species, apply combined results of hemolytic and carbohydrate utilization tests to Table **993.12B**. Of 3 hemolytic species, only *L. monocytogenes* fails to use xylose while being rhamnose positive.

After interpretating the results, report presence or absence of *L. monocytogenes* in the test portion, specifying quantity (g or mL) of the test portion tested.

Reference: *J. AOAC Int.* **77,** 395(1994).

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