## AOAC Official Method 2002.09
## *Listeria* in Foods

### Colorimetric Polyclonal Enzyme Immunoassay Screening Method (TECRA Listeria Visual Immunoassay) Using TECRA Listeria Enrichment Broth
### First Action 2002

*Note*: Method is based upon **995.22** (17.10.06) but with optimized enrichment protocols for additional foods and with omission of the toxic antifungal agent cycloheximide.

[Method is screening procedure for detection of *Listeria* spp. in raw meats; fresh produce/vegetables; processed meats; seafood; dairy foods cultured/noncultured; fruit and fruit juices. Assay is not confirmatory because polyclonal antibodies may cross-react with a small percentage of non-*Listeria* organisms. Enrichments positive by TECRA Listeria Visual Immunoassay (TLVIA) method must be streaked on selective media and confirmed by biochemical and hemolysis tests as described in *Bacteriological Analytical Manual*.]

*Caution:* *See Appendix B*, safety notes on handling microorganisms. *L. monocytogenes* infection can cause fetal death. Pregnant women and persons who are immunocompromised because of illness, medication, or advanced age should avoid handling this organism. Sterilize contaminated equipment and media before disposal or reuse.

*See* Table **2002.09** for the results of the interlaboratory study supporting acceptance of the method.

#### A. Principle

TLVIA detects *Listeria* antigens from enriched foods, food ingredients, and environmental samples by an enzyme-linked immunosorbent assay (ELISA) performed in "sandwich" configuration. If *Listeria* antigens are present, they are captured by specific high affinity polyclonal antibodies adsorbed to wells. All other materials are washed away. "Sandwich" is completed by addition of enzyme-labeled polyclonal antibodies (i.e., conjugate) specific for *Listeria*. Following washing of wells and addition of colorless substrate, development of green color indicates presumptive positive reaction. Determination of positive results by TLVIA can be performed either visually or spectrophotometrically at 414 ± 10 nm, for single wavelength reader, and referenced against 490 ± 10 nm for dual wavelength readers.

#### B. Apparatus

(**a**) *Serological pipets*.—Calibrated to deliver 1 mL, graduated in 0.1 mL units.

(**b**) *Micropipets*.—Accurately dispensing 0.2, 0.05, and 0.02 mL.

(**c**) *Test tubes*.—13 × 100 and 16 × 125 mm, with caps.

(**d**) *Boiling water bath*.—Alternatively, autoclave with flowing steam, set at 100°C may be used.

(**e**) *Incubators*.—Maintaining 35–37 and 28–30°C.

(**f**) *Plastic squeeze bottle*.—500 mL, for dispensing wash solution.

(**g**) *Package insert*

(**h**) *Sample record sheet*

(**i**) *Color card*.—For visual interpretation of positive and negative results.

(**j**) *Enzyme immunoassay reader*.—Optional. Photometer with 414 ± 10 nm screening filter that reads through microtiter wells. Use either single wavelength reader set to zero (blank) while reading through unreactive substrate well or well with water, or dual wavelength reader, with second reference filter set at 490 ± 10 nm set to zero (blank) on an empty cell.

#### C. Media and Reagents

(**a**) *Wash concentrate*.—One vial (25 mL/vial). Contains 1.0 g Tris, 6.0 g NaCl, 0.1 g Tween 20, and 2.0 mg thimerosal in water.

(**b**) *Positive control*.—One vial. Contains lyophilized purified *Listeria* antigen, 0.02 g gelatin, 0.04 g borate buffer, and 0.2 mg thimerosal.

(**c**) *Control diluent*.—One vial (6 mL/vial). Contains 0.01% saline, 0.01 g Tris, 1.0 mg Tween 20, and 1.0 mg thimerosal in water.

(**d**) *Conjugate*.—Two vials. Contain lyophilized anti-*Listeria* antibodies, 0.1 g borate buffer, 0.02 g gelatin, and 0.1 mg thimerosal. Reconstituted conjugate is stable 30 days when stored at 2–8°C.

(**e**) *Conjugate diluent*.—Two vials (13.5 mL/vial). Contain 0.2 g borate buffer and 2.0 mg thimerosal in water.

(**f**) *Substrate*.—One vial. Contains lyophilized 0.01 g 2,2′-azino-di(3-ethylbenzthiazoline sulfonate) and 0.1 g $NaH_2PO_4 \cdot 2H_2O$.

(**g**) *Substrate diluent*.—One vial (26 mL/vial). Contains 0.1 g acetic acid and 0.003 g $H_2O_2$ in water.

(**h**) *Stop solution*.—One vial (6 mL/vial). Contains 0.15 g NaF in water.

(**i**) *Sample additive*.—One vial (6 mL/vial). Contains 1.0 g Tris, 0.1 g Tween, and 1.0 mg thimerosal in water.

(**j**) *Test (Removawell) strips (polyclonal antibodies to Listeria) and holder for securing wells or strips*

(**k**) *TECRA Listeria Enrichment Broth (complete medium) (TLEB)*.—Prepare according to manufacturer's instructions.

(**l**) *TLEB base*.—Prepare according to manufacturer's instructions.

(**m**) *TECRA Listeria enrichment supplement*.—Use according to manufacturer's instructions.

(**n**) *Fraser broth*.—5.0 g Protease peptone, 5.0 g tryptone, 5.0 g Lab Lemco powder (meat extract), 5.0 g yeast extract, 20.0 g NaCl, 1.35 g $KH_2PO_4$, 12.0 g $Na_2HPO_4$, 1.0 g esculin, 3.0 g LiCl, and 20 mg nalidixic acid. Suspend ingredients in 1.0 L water. Dispense 10 mL portions into 16 × 125 mm test tubes. Cap test tubes and autoclave 15 min at 121°C on slow exhaust and cool to 20–25°C. Just before use add the following filter-sterilized reagent additives: 0.1 mL (2.5 mg/mL) acriflavine hydrochloride and 0.1 mL (5% in distilled water) ferric ammonium citrate. Use of commercially available Fraser broth is also acceptable if its formulation is the same as that described.

(**o**) *Diagnostic reagents*.—Necessary for culture confirmation of presumptive positive TLVIA tests.

Items **B**(**g**)–(**j**) and **C**(**k**)–(**m**) are available from TECRA International (13 Rodborough Rd, French's Forest, NSW 2086, Australia). Items **C**(**a**)–(**j**) are provided in the kit. Shelf life of test kit is 13 months from date of manufacture when held at 2–8°C.

#### D. General Instructions

Refrigerate all components in TLVIA kit at 2–8°C when not in use. Bring components to room temperature before use. Kit components are intended for use as integral unit. Conjugate, positive control, and antibody coated strips are matched and should not be interchanged with components from other kits. Discard all unused materials when new kit is opened. Do not use kit after expiration date.

**Table 2002.09. Statistical analyses of interlaboratory study results comparing new enrichment methods for TECRA Listeria Visual Immunoassay with cultural reference methods**

| Food (No. of labs) | MPN[a] (CFU/g) | Method agreement, %[b] | Total No. of test portions[c] | Test portions positive: TLVIA Pres. | TLVIA Conf. | Culture Pres. | Culture Conf. | $\chi^{2d}$ | Incidence of false negatives among total positive test portions, %[e]: TLVIA | Culture | Sensitivity rate, %[e]: TLVIA | Culture | Incidence of false positives among total negative test portions, %[f]: TLVIA | Culture | Specificity rate, %[f]: TLVIA | Culture |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fish fillets (15) | <0.03 U | 100 | 90 | 0 | 0 | 0 | 0 | | | | | | 0 | 0 | 100 | 100 |
| | 0.23 L | 96.7 | 90 | 77 | 79 | 80 | 80 | 1.33 | 3.8 | 0 | 96.3 | 100 | | | | |
| | 2.4 H | 98.9 | 90 | 89 | 90 | 90 | 90 | 0 | 1.1 | 0 | 98.9 | 100 | | | | |
| Turkey (10) | <0.03 U | 100 | 60 | 0 | 0 | 0 | 0 | | | | | | 0 | 0 | 100 | 100 |
| | 0.16 L | 95 | 60 | 55 | 56 | 52 | 52 | 0.35 | 8.3[g] | 13.3[g] | 91.7[g] | 86.7[g] | | | | |
| | 0.59 H | 98.3 | 60 | 59 | 60 | 60 | 60 | 0 | 1.7[g] | 0[g] | 98.3[g] | 100[g] | | | | |
| Raw ground beef (12) | 0.074 U | 56.9 | 72 | 66 | 67 | 43 | 43 | 14.7[d] | 5.7 | 38.6 | 94.3 | 61.4 | 0 | 0 | 100 | 100 |
| | 0.43 L | 86.1 | 72 | 68 | 68 | 63 | 63 | 0.9 | 5.6 | 12.5 | 94.4 | 87.5 | | | | |
| | >11[h] H | 98.6 | 72 | 72 | 71 | 72 | 72 | 0 | 1.4 | 0 | 98.6 | 100 | | | | |
| Ice cream (10) | <0.03 U | 100 | 60 | 8 | 0 | 0 | 0 | | | | | | 13.3 | 0 | 86.7 | 100 |
| | 0.092 L | 80 | 60 | 55 | 52 | 48 | 48 | 0.75 | 7.1 | 14.3 | 92.9 | 85.7 | | | | |
| | 0.43 H | 98.3 | 60 | 60 | 60 | 59 | 59 | 0 | 0 | 1.7 | 100 | 98.3 | | | | |
| Lettuce (10) | <0.03 U | 100 | 60 | 2 | 0 | 1 | 0 | 0 | | | | | 3.3 | 1.6 | 66.7 | 98.4 |
| | <0.03 L | 83.3 | 60 | 19 | 12 | 7 | 5 | 2.5 | 14.3 | 50 | 85.7 | 50 | | | | |
| | 0.15 H | 75 | 60 | 38 | 37 | 42 | 41 | 1.1 | 21.8 | 10.9 | 78.2 | 89.1 | | | | |

[a] MPN = Most probable number of colony forming units per gram of food. U = uninoculated, L = low, H = high.

[b] Percentage agreement is defined as 100 times the number of test portions for which the confirmed results were in agreement divided by the total number of test portions. Test portions were paired except for turkey.

[c] Different enrichment media and different subtest portions were used for each method. However, efforts were made to pair test portions, and pairing of test portions was assumed in order to calculate performance parameters for all foods, except turkey. For turkey, test portions were not paired on mailing and it was assumed that all inoculated test portions contained *Listeria*.

[d] $\chi^2$ value >3.84 indicates significance at $p \geq 0.05$.

[e] Incidence of false negatives is 100 – sensitivity rate. Sensitivity rate is defined as 100 times the total number of analyzed positive test portions among known positive test portions divided by total number of known test portions; where known positive is defined as test portions confirmed positive by the reference method.

[f] Incidence of false positives is 100 – specificity rate. Specificity rate is defined as the total number of analyzed negative test portions among known negative test portions/laboratory divided by total number of known negative test portions per laboratory; where known negative is defined as test portions confirmed negative by the reference method and negative controls.

[g] For turkey, data were unpaired and Chi square test with Yates correction was used. Sensitivity was calculated using total number of confirmed positive test portions divided by total number of inoculated test portions. Specificity was calculated using total number of presumptive negative test portions divided by number of uninoculated control portions at uninoculated level.

[h] Target MPN was 1–2 CFU/g.

Reconstituted reagents have 2 month shelf life, except for conjugate, which has 1 month shelf life after reconstitution.

Positive and negative controls provided with kit must be run with each set of samples. If results from controls are not within acceptable range, test is invalid.

Do not reuse antibody-coated wells. It is not necessary to perform TLVIA under sterile conditions; however, separate pipet tips must be used for each test portion and kit reagents to avoid cross-contamination.

### *E. Preparation of Test Sample*

(**a**) *Dairy foods.*—Aseptically add 25 mL liquid or 25 g solid test portion to 225 mL TLEB and blend 2 min at high speed in food blender, or stomach 2 min. Incubate 24 h at 35–37°C. Transfer 1.0 mL TLEB culture to 9.0 mL fresh TLEB. Incubate 24 h at 30°C. Transfer 1.0 mL of second TLEB broth culture into 13 × 100 mm clean test tube and perform enzyme immunoassay, **F**.

(**b**) *Raw meat poultry and seafood.*—Aseptically add 25 g test portion to 225 mL TLEB and blend 2 min at high speed in food blender, or stomach 2 min. Incubate 24 h at 35–37°C. Transfer 0.1 mL TLEB culture to 9.9 mL Fraser broth. Incubate 24 h at 30°C. Transfer 1.0 mL Fraser broth culture into 13 × 100 mm clean test tube.

(**c**) *Processed meat, poultry, and seafood*.—Aseptically add 25 g test portion to 475 mL TLEB base and blend 2 min at high speed in food blender, or stomach 2 min. Incubate 4 h at 35–37°C. Add 1 vial (2 mL) TECRA Listeria Selective supplement. Incubate 20 h at 35–37°C. Transfer 1.0 mL TLEB culture to 9 mL fresh TLEB (complete medium). Incubate 24 h at 30°C. Transfer 1.0 mL of second TLEB broth culture into 13 × 100 mm clean test tube.

(**d**) *Other foods.*—Aseptically add 25 mL liquid or 25 g solid test portion to 475 mL TLEB and blend 2 min at high speed in food blender, or stomach 2 min. Incubate 24 h at 35–37°C. Transfer 0.1 mL TLEB culture to 9.9 mL Fraser broth and incubate 24 h at 30°C. Transfer 1.0 mL Fraser broth culture into 13 × 100 mm clean test tube.

### *F. Enzyme Immunoassay*

(*1*) Prepare the following reagents before beginning assay.

Prepare wash solution by diluting wash concentrate, **C**(**a**), to 2 L with water in plastic or glass reagent bottle. Plastic squeeze bottle can be used for washing trays manually.

Reconstitute positive control by transferring 3 mL control diluent, **C**(**c**), to lyophilized positive control, **C**(**b**). Mix thoroughly. Replace stopper and screw cap firmly for storage.

As negative control use control diluent, **C**(**c**), remaining after reconstitution of positive control.

Reconstitute one set of conjugate by adding conjugate diluent, **C**(**e**), to lyophilized conjugate, **C**(**d**). Reconstitute another set as needed. Let dissolve completely at room temperature before use. Replace red stopper and screw cap for storage. Write date of reconstitution on bottle label. Discard reconstituted conjugate after 1 month.

Reconstitute substrate by adding substrate diluent, **C**(**g**), to lyophilized substrate, **C**(**f**). Ensure that contents are dissolved completely. Allow substrate to equilibrate to room temperature before use. Reconstituted substrate is colorless to pale green.

Use sample additive, **C**(**i**), and stop solution, **C**(**h**), as supplied.

(*2*) To the 1 mL aliquot of secondary TLEB or Fraser broth culture from **E**, add 50 µL sample additive, and mix. Heat 10–15 min in boiling water bath or in autoclave with flowing steam set at 100°C. Cool to 25–37°C. Keep unheated broth portion for cultural confirmation.

(*3*) Open pouch, break off required number of wells from test (Removawell) strip, using one well/sample, one well for positive control, and one well for negative control. Place unused wells back into pouch and reseal with resealing strip.

(*4*) Secure desired number of antibody-coated test strips in holder. Press firmly into place.

(*5*) Using new pipet tip for each test, pipet 0.2 mL each heated broth from (*2*) into individual well. Transfer 0.2 mL negative control and 0.2 mL reconstituted positive control to individual wells. Record sample positions on record sheet. Cover tray with plastic film wrap and incubate 30 min at 35–37°C. *Note:* Tray must be covered to prevent evaporation.

(*6*) Wash plate as follows: Ensure that test strips are pressed firmly into holder. Quickly invert tray, emptying its contents into waste container. Remove residual liquid by striking holder firmly several times face down on thick pile of absorbent paper towels. Hold squeeze bottle above plate and using wide nozzle, squeeze and completely fill each well. Do not trap air bubbles in bottom of wells. Wash and completely empty wells 3×. Make sure that plate is empty before proceeding to next step.

(*7*) Add 0.2 mL reconstituted conjugate to each well. Cover tray with plastic film wrap and incubate 30 min at 35–37°C.

(*8*) Empty tray and wash it thoroughly 4× as in step (*6*).

(*9*) Add 0.2 mL reconstituted substrate to each well. Incubate 15 min at room temperature (20–25°C) or until positive control has reached an absorbance >1.0 or color darker than panel No. 4 on color card. Color development tends to concentrate around edges of wells. Therefore, tap sides of plate gently to mix contents before reading result to obtain accurate readings. *Note*: If absorbance of 1.0 is not attained within 30 min, test is invalid. Refer to "Troubleshooting Guide" in package insert.

(*10*) Add 20 µL stop solution to each well. Tap sides of plate gently to mix contents.

### *G. Reading*

Results of test can be determined either visually or spectrophotometrically.

*Visual determination*.—Place holder onto white background and then compare individual test wells with color card. Test is valid if positive control gives green color at least as dark as panel 4 on color card and negative control is within negative range on color card.

Test is considered positive when controls are valid and test has color greater than or equal to the color in panel 3 on color card.

If negative control has color darker than panel 2 or positive control has color lighter than panel 4 on color card, test is invalid.

*Spectrophotometric determination*.—Read test absorbance, *A*, at 414 ± 10 nm using plate reader. Blank single wavelength instrument on well containing 200 µL substrate or water. When using dual wavelength readers, set second reference wavelength at 490 ± 10 nm and blank instrument on air.

Test is valid if *A* of positive control is >1.0 and *A* of negative control is <0.2. Test is considered positive if *A* is ≥0.2. Test is considered negative if *A* is <0.2.

If *A* of negative control is ≥0.2 or if *A* of positive control is <1.0, test is invalid.

### *H. Confirmation of Positive TLVIA Samples*

Positive results must be confirmed by streaking second enrichment broth from **E** onto Oxford or PALCAM agar plates (BAM). Incubate plates at 35–37°C. Examine plates at 24 and 48 h after incubation for presence of typical *Listeria* colonies. Any suspect colonies must be confirmed biochemically as specified in BAM.

References: *J. AOAC Int.* **86**, 340(2003).

U.S. Food and Drug Administration (1995) *Bacteriological Analytical Manual*, 8th Ed., AOAC INTERNATIONAL, Gaithersburg, MD.