AOAC Official Method 995.22
Listeria in Foods
Colorimetric Polyclonal Enzyme Immunoassay Screening Method (TECRA® *Listeria* Visual Immunoassay [TLVIA])
First Action 1995
Final Action 1999

[Method is screening procedure for detection of *Listeria* spp. in dairy foods, seafoods, poultry, meats (except raw ground chuck), and leafy vegetables. Assay is not confirmatory because polyclonal antibodies may cross-react with a small percentage of non-*Listeria* organisms. Enrichments positive by TLVIA method must be streaked on selective media and confirmed by appropriate biochemical and hemolysis tests as described in *Bacteriological Analytical Manual*, current edition, AOAC INTERNATIONAL, Gaithersburg, MD, USA, and in *Microbiology Laboratory Guidebook*, U.S. Department of Agriculture–Food Safety Inspection Service, Athens, GA 30604, USA.]

Caution: Pregnant women and persons who are immunocompromised because of illness, medication, or advanced age should avoid handling this organism.

See Table **995.22** for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle

TLVIA detects *Listeria* antigens from enriched foods, food ingredients, and environmental samples using 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 at 490 ± 10 nm for dual wavelength readers.

B. Apparatus

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

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

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

(**d**) *Boiling H_2O bath*.—Alternatively, autoclave with flowing steam, set at 100°C may be used.

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

(**f**) *Incubator*.—Maintaining 35–37°C and 28–30°C.

(**g**) *Package insert*.

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

(**i**) *Color comparator 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 (*1*) single wavelength reader set to zero (blank) while reading through unreactive substrate well or well with H_2O, or (*2*) dual wavelength reader, with second reference filter set at 490 ± 10 nm and set to zero (blank) while reading an empty well.

C. Media and Reagents

(**a**) *Wash concentrate*.—One vial contains 1.0 g Tris, 6.0 g NaCl, 0.1 g Tween 20, and 2.0 mg thimerosal in 25 mL H_2O.

(**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 contains 0.01% saline, 0.01 g Tris, 1.0 mg Tween 20, and 1.0 mg thimerosal in 6 mL H_2O.

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

(**e**) *Conjugate diluent*.—Two vials (13.5 mL/vial). Contains 0.2 g borate buffer and 2.0 mg thimerosal in H_2O.

(**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 contains 0.1 g acetic acid and 0.003 g H_2O_2 in H_2O.

(**h**) *Stop solution*.—One vial contains 0.15 g NaF in 6 mL H_2O. (*Caution*: Do not pipette stop solution by mouth; avoid contact with skin or eyes. In event of a spill, sweep or pipette contents into a beaker and dilute with water. Dilute remainder of spilled stop solution with water.)

(**i**) *Test portion additive*.—1.0 g Tris, 0.1 g Tween, and 1.0 mg thimerosal in H_2O.

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

(**k**) *Listeria enrichment broth (LEB).*—Prepare trypticase soy broth according to manufacturer's instructions, then add yeast extract, 6.0 g/L, and anhydrous K_2HPO_4, 2.5 g/L. Autoclave 15 min at 121°C on slow exhaust and cool to 20–25°C. Just before use, add following filter-sterilized additives: acriflavine hydrochloride (0.5% stock solution in distilled H_2O), 3 mL/L; nalidixic acid (0.5% stock solution in distilled H_2O), 8 mL/L; and cycloheximide (1% stock solution in 40% ethanol), 5 mL/L. Use of commercially available LEB is acceptable if its formulation is the same as that described.

(**l**) *Fraser broth*.—5.0 g proteose 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 anhydrous KH_2PO_4, 12.0 g anhydrous Na_2HPO_4, 1.0 g esculin, 3.0 g LiCl, and 20 mg nalidixic acid. Suspend ingredients in 1.0 L H_2O. 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 following filter-sterilized reagent additives: 0.1 mL (2.5 mg/mL) acriflavine hydrochloride and 0.1 mL (5% in distilled H_2O) ferric ammonium citrate. Use of commercially available Fraser broth is also acceptable if its formulation is the same as that described.

(**m**) *University of Vermont medium (UVM)*.—5.0 g tryptone, 5.0 g Lab Lemco powder (meat extract), 5.0 g yeast extract, 20.0 g NaCl, 1.35 g anhydrous KH_2PO_4, 12.0 g anhydrous Na_2HPO_4, 1.0 g esculin, and 1.0 mL nalidixic acid [2% solution in 0.1M NaOH (4 g/L)]. Suspend ingredients in 1.0 L H_2O. Autoclave 15 min at 121°C on slow exhaust. Cool to 20–25°C. Before use, add 1.2% filter-sterilized acriflavine hydrochloride (1.0 mL/L UVM). Use of commercially available UVM medium is acceptable if its formulation is the same as that described.

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

Items **B**(**g**)–(**j**) and **C**(**a**)–(**j**) are available as TECRA *Listeria* Visual Immunoassay kit (TLVIA) from TECRA International Pty

Table 995.22 Interlaboratory study results for detection of *Listeria* in foods using TECRA *Listeria* Visual Immunoassay (TLVIA) and culture methods

			Methods									Agreement[d], %	
			Culture			TLVIA visual			TLVIA reader				
Food	Level	MPN/g	*N*[a]	Sensitivity[b]	Specificity[c]	*N*	Sensitivity	Specificity	*N*	Sensitivity	Specificity	TLVIA visual	TLVIA reader
Fish fillets[e]	Low	0.6	50	0.74	—	50	0.78	—	50	0.72	—	84	78
	High	0.39	50	0.80	—	50	0.74	—	50	0.72	—	82	76
	Uninoc.	0	50	—	1.00	50	—	1.00	50	—	1.00	100	100
Ice cream[f]	Low	0.03	55	0.96	—	55	0.96	—	55	0.96	—	100	100
	High	0.21	55	0.96	—	55	0.98	—	55	0.98	—	98	98
	Uninoc.	0	55	—	1.00	55	—	1.00	55	—	1.00	100	100
Lettuce[f]	Low	0.15	55	0.89	—	55	0.84	—	55	0.84	—	94	94
	High	0.93	55	0.95	—	55	0.93	—	55	0.93	—	96	96
	Uninoc.	0	55	—	1.00	55	—	1.00	55	—	1.00	100	100
Chicken[e]	Low	0.09	50	0.82	—	50	0.80	—	50	0.80	—	98	98
	High	0.02	50	0.98	—	50	1.00	—	50	1.00	—	98	98
	Uninoc.	0	50	—	1.00	50	—	1.00	50	—	1.00	100	100
Ground turkey[f]	Low	0.04	55	0.98	—	55	0.96	—	55	0.96	—	98	98
	High	0.23	55	0.96	—	55	1.00	—	55	1.00	—	96	96
	Uninoc.	0	55	—	0.96	55	—	0.96	55	—	0.96	100	100

[a] *N* = Number of test portions included in statistical analysis.

[b] Sensitivity is ratio of correctly classified positives to known positives.

[c] Specificity is ratio of correctly classified negatives to known negatives.

[d] Proportion of test samples that agree between culture methods and TLVIA.

[e] Data from 3 laboratories excluded as technical outliers.

[f] Data from 2 laboratories excluded as technical outliers.

Ltd., PO Box 788, Willoughby, NSW, Australia. Shelf life of test kit is 12 months from date of manufacture when held at 2–8°C.

D. General Instructions

All components in TLVIA kit should be refrigerated 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 of specific matched batch and should not be interchanged with components from other kits. All unused materials must be discarded when new kit is opened. Do not use kit after expiration date.

Reconstituted reagents have 2 month shelf life, except for conjugate that 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. Separate pipet tips must be used for each test portion and kit reagent to avoid cross contamination.

E. Preparation of Test Sample

(**a**) *Dairy foods*.—Aseptically add 25 mL liquid or 25 g solids to 225 mL LEB and blend 2 min at high speed or stomach 2 min. Incubate 24 h at 28–30°C. Transfer 10 mL LEB culture to 9.0 mL Fraser broth and incubate 22–24 h at 28–30°C. Transfer 1.0 mL Fraser broth culture into 13 ×100 mm clean test tube and perform enzyme immunoassay, **F**.

(**b**) *Meat products*.—Aseptically add 25 mL liquid or 25 g solids to 225 mL UVM and blend 2 min at high speed or stomach 2 min. Incubate 24 h at 28–30°C. Transfer 0.1 mL UVM culture to 9.9 mL Fraser broth and incubate 22–24 h at 28–30°C. Transfer 1 mL Fraser broth culture into 13 ×100 mm clean test tube and perform enzyme immunoassay, **F**.

(**c**) *Other foods*.—Aseptically add 25 mL liquid or 25 g solids to 225 mL LEB and blend 2 min at high speed or stomach 2 min. Incubate 24 h at 28–30°C. Transfer 0.1 mL LEB culture to 9.9 mL Fraser broth and incubate 22–24 h at 28–30°C. Transfer 1 mL Fraser broth culture into 13 × 100 mm clean test tube and perform enzyme immunoassay, **F**.

F. Enzyme Immunoassay

(*1*) Prepare following reagents before beginning assay:

Prepare wash solution by diluting wash concentrate, **C**(**a**), to 2 L with H_2O 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**).

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 prior to 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. Let substrate equilibrate to room temperature before use. Reconstituted substrate is colorless to pale green.

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

(*2*) To Fraser broth culture from **E**, add 50 µL test portion additive, **C**(**i**), and mix. Heat 10–15 min in boiling H_2O bath or in autoclave with flowing steam set at 100°C. Do not autoclave. Cool broth to 25–37°C.

(*3*) Open pouch, break off required number of wells from test (Removawell) strip, using one well/test, 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 test positions on record sheet. Cover tray with plastic film wrap to prevent evaporation and incubate 30 min at 35–37°C.

(*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 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 15–20 min at room temperature (20–25°C) or until positive control has reached an absorbance >1.0 or color darker than panel number 4 on color comparator. Color development tends to concentrate around edge 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.

(**a**) *Visual determination*.—Place holder on white background and 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. Test is considered negative when controls are valid and test has color within negative range 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.

(**b**) *Spectrophotometric determination*.—Read test absorbance, *A*, at 414 ± 10 nm using plate reader. Single wavelength instrument should be blanked on well containing 200 µL substrate or H_2O. When using dual wavelength readers, set second "reference" wavelength at 490 ± 10nm 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 Test Portions

Positive results must be confirmed by streaking Fraser broth enrichment from **E** onto LPM and OX agar plates for dairy and other foods or onto modified OX agar plates for meat and meat products. Incubate plates at 30°C for LPM and 35°C for OX and modified OX. Examine plates 24 and 48 h after incubation for presence of typical *Listeria* colonies. Any suspect colonies must be confirmed biochemically.

Reference: *J. AOAC Int.* **79,** 1083(1996).
Revised: June 2000