

## GENERAL REFEREE REPORTS

## Committee on Microbiology and Extraneous Materials

### Food Microbiology, Nondairy

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U.S. Food and Drug Administration, 5100 Paint Branch Pkwy, College Park, MD 20740-3835, Tel: 301-436-2008 (Andrews), 301-436-2010 (Hammack), Fax: 301-436-2644, E-mail: wallace.andrews@fda.hhs.gov, thomas.hammack@fda.hhs.gov

### Recommendations

(1) **2000.07**, Modified 5-9-01, *Salmonella* in Foods, Rapid Colorimetric TECRA UNIQUE Test.—Study Director Ian Garthwaite, TECRA International Pty Ltd, 13 Rodborough Rd, Frenchs Forest, NSW 2100, Australia, Tel: 61(0)-2-8977-3032, Fax: 61(0)-2-9453-3422, E-mail: ian.garthwaite@tecrea.net. The TECRA UNIQUE *Salmonella* test was approved First Action Method **2000.07**. However, TECRA has modified the module design so that the test may be read both visually and with the aid of a reader, thereby allowing the test to be read both manually and automatically. A study was conducted to validate the modified UNIQUE method for both manual and automated use. No significant differences were observed between the UNIQUE assays (either in the original format or following minor modification in both manual and automated operation) and the current *Bacteriological Analytical Manual* (BAM) reference method (1). A report to validate this minor modification has been submitted for review.

The Study Director reports that the original First Action Method, **2000.07**, including application to fruit juices (adopted as Revised First Action in 2002) has gained wide acceptance in the food industry. The Study Director recommends that First Action Method **2000.07** be adopted Final Action, and the co-General Referees concur. Continue study.

(2) **H66**, Determination of *Escherichia coli* in Flesh Foods Using a Visual Immunoassay with a Modified Culture Procedure.—Study Director Ian Garthwaite. Resource allocations have limited the progression of this study through the precollaborative study phase. Plans are to expand this study to incorporate immunocapture of the target organism. Continue study.

(3) **H71**, *Staphylococcus aureus* in Foods, TECRA STAPH AUREUS Visual Immunoassay.—Study Director Ian Garthwaite. A precollaborative study of this visual immunoassay (VIA) method was conducted in which the TECRA *Staphylococcus aureus* Visual Immunoassay (TECRA STADIA) was compared to AOAC Official Method **975.55** (2) for the recovery of *S. aureus* in 1200 test portions, representing 20 food types (ham, raw ground beef, cooked

chicken, crab meat, surimi, raw fish filets, mushrooms, potato salad, lettuce, skim milk powder, ice cream, cheddar cheese, liquid milk, infant formula, chocolate éclair, chocolate-coated candy, uncooked macaroni, powdered egg, egg custard, and vanilla pudding).

The alternative method was effective in detecting *S. aureus* in all of the food matrixes used in this study. Of the 1200 test portions examined, 698 were confirmed positive by the alternative method, whereas 332 were confirmed positive by the reference method. Overall, the alternative method gave an increased proportion of positive results when compared to the reference method in its recovery of *S. aureus* from foods inoculated at the low level of contamination. Chi square analysis at the 5% level showed that there was a significant improvement in recovery of *S. aureus* from these test portions when compared to the reference method. For the test portions inoculated at the high level of contamination, the 2 methods were statistically equivalent. Inclusivity and exclusivity studies were all confirmed to be suitable with all strains of target and nontarget analyte organisms tested.

The precollaborative study has been approved by the Official Methods Board, and a collaborative study has been conducted. Continue study.

(4) **995.22**, Modified 2/6/01, *Listeria* spp., TECRA Visual Immunoassay for Environmental Surfaces.—Study Director Ian Garthwaite. This method was approved First Action in 1995 and Final Action in 1998 for the detection of *Listeria* spp. in dairy foods, seafoods, poultry, meats (except raw ground chuck), and leafy vegetables. TECRA plans to extend the applicability of the method to detect *Listeria* spp. on environmental surfaces. The precollaborative study has been completed, and the protocol for the collaborative study has been approved by the Methods Committee. Continue study.

(5) **H17**, *Listeria* in Selected Foods by TECRA Unique *Listeria* Method.—Study Director Ian Garthwaite. The precollaborative and collaborative study protocols have received approval from the Methods Committee and are in progress. Continue study.

(6) **995.22**, Modified 5/23/01, TECRA Enrichment for *Listeria* in Foods.—Study Director Ian Garthwaite. This method is now in Official Final Action status; therefore, it is recommended that this topic be discontinued.

(7) **998.06**, Modified 3/13/03, Validation Study to Demonstrate Equivalence of a Minor Modification to **998.09** with the Reference Culture Method.—Study Director Ian Garthwaite. This assay, using Rappaport-Vassiliadis (RV) [R10] medium as a single selective enrichment broth, has Final Action approval (Official Method **998.09**). TECRA developed a protocol (TECRA ULTIMA) that includes a test portion additive that allows the direct analysis of RV [R10] medium in the VIA without subsequent post-enrichment in M

broth. The TECRA ULTIMA protocol, as an additional option within the method, was granted modified First Action status in 2004. Continue study.

(8) **2000.07**, *Modified 2/15/00, TECRA Unique Salmonella Test*.—Study Director Ian Garthwaite. This assay was approved First Action for all foods, except raw flesh foods. TECRA plans to extend the applicability of the method to all foods using a single module incubation temperature of 41.5°C. The protocols for the precollaborative and collaborative studies have received approval from the Methods Committee. The precollaborative study is nearing completion, and a report will be submitted soon. Continue study.

(9) *Modified 996.06, Evaluation of VIDAS Listeria (LIS) Immunoassay*.—Study Director Karen M. Silbernagel, AOAC INTERNATIONAL, 481 N. Frederick Ave, Suite 500, Gaithersburg, MD 20877, Tel: 301-924-7077, Fax: 301-924-7089, E-mail: ksilbernagel@aoac.org. It is recommended that this topic be deleted; it is a duplicate of Topic 35, which is to be continued.

(10) *H-16, Improved Analysis of Food Samples for Total Escherichia coli Populations to Determine Whether 10<sup>4</sup>C FU/g Action Levels Have Been Exceeded*.—Study Director Michael A. Grant, U.S. Food and Drug Administration (FDA), 22201 23rd Dr, SE, Bothell, WA 98021-4421, Tel: 425-402-3179, Fax: 425-483-4966, E-mail: mgrant@ora.fda.gov. Continue study.

(11) **2003.11**, *3M Petrifilm Staph Express for Staphylococcus aureus in Meat, Seafood, and Poultry*.—Study Director Wendy A. McMahon, Silliker, Inc., Research Center, 160 Armory Dr, South Holland, IL 60473, Tel: 708-756-3210, Fax: 708-756-0049, E-mail: wendy.mcmahon@silliker.com. Official Method **2003.11** was adopted First Action for the specific and exclusive analysis of cooked diced chicken, cured ham, smoked salmon, and pepperoni. No adverse comments have been received by the Study Director. Thus, the Study Director recommends that this First Action method be adopted Final Action, and the co-General Referees concur.

(12) **996.08**, *VIDAS SLM Method for the Detection of Salmonella in Foods*.—Study Directors Wendy A. McMahon and Ronald L. Johnson, bioMérieux, Inc., 595 Anglum Rd, Hazelwood, MO 63042-2320, Tel: 314-506-8182, Fax: 314-731-8276, E-mail: ron.johnson@na.biomerieux.com. This method has been adopted Final Action; therefore, it is recommended that this topic be discontinued.

(13) **2001.09**, *Salmonella in Selected Foods by Immuno-Concentration Salmonella (ICS) and Enzyme-Linked Immunofluorescent Assay (ELFA)*.—Study Directors Wendy A. McMahon and Ronald L. Johnson. This method was approved First Action for selected foods in 2001 and subsequently approved First Action for foods in general in 2004. No adverse comments have been received; therefore, the Study Directors recommend that the First Action method be adopted Final Action, and the co-General Referees concur.

(14) *H7, Clostridium botulinum Toxins A, Proteolytic B, and E, ELCA Enzyme Immunoassay*.—Study Director Wendy

A. McMahon. There has been no activity in this topic for a prolonged period; therefore, the co-General Referees recommend that this topic be discontinued.

(15) **2001.07**, *Salmonella in Selected Foods by Immuno-Concentration (ICS) and Selective Plate (BS, HE, SMID) Procedure*.—Study Directors Wendy A. McMahon and Ronald L. Johnson. This method was approved First Action for selected foods in 2001 and subsequently approved First Action for foods in general in 2004. No adverse comments have been received; therefore, the Study Directors recommend that the First Action method be adopted Final Action, and the co-General Referees concur.

(16) **2001.08**, *Salmonella in Selected Foods by Immuno-Concentration (ICS) and Selective Plate (BS, HE, XLD) Procedure*.—Study Directors Wendy A. McMahon and Ronald L. Johnson. This method was approved First Action for selected foods in 2001 and subsequently approved First Action for foods in general in 2004. No adverse comments have been received; therefore, the Study Directors recommend that the First Action method be adopted Final Action, and the co-General Referees concur.

(17) **2004.03**, *Evaluation of VIDAS Salmonella (SLM) Immunoassay Method with Rappaport-Vassiliadis (RV) Medium for the Detection of Salmonella in Foods*.—Study Directors Wendy A. McMahon and Ronald L. Johnson. No adverse comments have been received; therefore, the Study Directors recommend that the First Action Method be adopted Final Action, and the co-General Referees concur.

(18) **2002.08**, *Detection of Botulinum Toxins A, B, E, and F from Culture Supernatants, Amplified ELISA Procedure*.—Study Director, Joseph L. Ferreira, Centers for Disease Control and Prevention, 1600 Clifton Rd, NCID, Mailstop G-29, Atlanta, GA 30333, Tel: 404-639-0896, Fax: 404-639-4290, E-mail: jferreira@cdc.gov. Continue study.

(19) **2002.10**, *ISO vs AOAC Reference Culture Methods for the Detection of Motile and Nonmotile Salmonella in Selected Foods*.—Study Director Philip T. Feldsine, BioControl Systems, Inc., 12822 SE 32nd St, Bellevue, WA 98005, Tel: 425-603-1123, Fax: 425-603-0070, E-mail: ptf@biocontrolsys.com. This method has been adopted Final Action; therefore, it is recommended that this topic be discontinued.

(20) **2002.07**, *SimPlate Total Plate Count Indicator (TPC-CI) for the Enumeration of Total Aerobic Microorganisms in Foods*.—Study Director Philip T. Feldsine. This method was adopted Final Action in 2004; therefore, it is recommended that this topic be discontinued.

(21) **2002.11**, *SimPlate Yeast and Mold Color Indicator (Y & M-CI) Method for the Enumeration of Yeasts and Molds in Foods*.—Study Director, Philip T. Feldsine. This method was adopted Final Action in 2004; therefore it is recommended that this topic be discontinued.

(22) **996.10**, *Modified 9/21/00, Assurance Enzyme Immunoassay for the Detection of Escherichia coli O157:H7 in Ground Beef*.—Study Director, Philip T. Feldsine. This method was adopted First Action in 1996 and Final Action in 1998. A method applicability modification was submitted to

revise the enrichment protocol to allow for an 8 h enrichment for raw and cooked beef products only. This modification was adopted revised First Action in 2002 and Final Action in 2004. Thus, it is recommended that this topic be deleted.

(23) **2005.06**, *SimPlate CEC Quantitative Method for Coliforms and Escherichia coli in Foods*.—Study Director Philip T. Feldsine. This method has been adopted First Action. Continue study.

(24) **996.14**, *Assurance Polyclonal Enzyme Immunoassay for the Detection of Listeria monocytogenes and Related Listeria Species in Selected Foods*.—Study Director Philip T. Feldsine. This method for selected foods was adopted First Action in 1996 and Final Action in 1998. Subsequently, the applicability of the method was expanded to include the monitoring of environmental surfaces. This method for environmental surfaces was adopted First Action in 2001 and Final Action in 2003. Thus, it is recommended that this topic be discontinued.

(25) **996.09**, *Visual Immunoprecipitate Assay for the Analysis of Ground Beef for Escherichia coli O157:H7*.—Study Director Philip T. Feldsine. This method was adopted First Action in 1996 and Final Action in 1998. A method applicability modification was submitted to revise the enrichment protocol to allow for an 8 h enrichment for raw and cooked beef products only. This modification was adopted revised First Action in 2002 and Final Action in 2004. Thus, it is recommended that this topic be deleted.

(26) **997.03**, *Visual Immunoprecipitate Assay for the Detection of Listeria monocytogenes and Related Listeria Species in Selected Foods*.—Study Director, Philip T. Feldsine. This method was adopted First Action in 1997 and Final Action in 1999. Subsequently, the applicability of this method was expanded to include the monitoring of environmental surfaces. This method for environmental surfaces was adopted First Action in 2001 and Final Action in 2003. Thus, it is recommended that this topic be discontinued.

(27) **2000.14**, *Twenty-Hour REVEAL Screening Test for Detection of Escherichia coli O157:H7 in Selected Foods and Environmental Surfaces*.—Study Director Mark Mozola, Neogen Corp., 620 Leshner Pl, Lansing, MI 48912, Tel: 517-372-9200, Fax: 517-372-0108, E-mail: mmozola@neogen.com. Continue study.

(28) **2000.13**, *Eight-Hour REVEALS Screening Test for the Detection of Escherichia coli O157:H7 in Selected Foods*.—Study Director Mark Mozola. Continue study.

(29) **2003.01**, *Enterobacteriaceae in Foods, Dry Rehydratable Film Method*.—Study Director Deborah A. McIntyre, rtech Laboratories, MS 0075, PO Box 64101, St. Paul, MN 55164-0101, Tel: 651-481-2636, Fax: 651-486-0837. This method received Final Action status in 2005; therefore, it is recommended that this topic be discontinued.

(30) **2004.02**, *VIDAS Listeria monocytogenes II (LMO2) Immunoassay for the Detection of Listeria monocytogenes in Foods*.—Study Directors Deborah A. McIntyre and Ronald L. Johnson. Continue study.

(31) **2003.09**, *BAX System with Automated Detection of Salmonella in Foods*.—Study Director Deborah A. McIntyre. This method received Final Action status in 2005; therefore it is recommended that this topic be discontinued.

(32) **2003.07**, *3M Petrifilm Staph Express Count Plate Method for the Enumeration of Staphylococcus aureus in Selected Processed and Prepared Foods*.—This method received Final Action status in 2005, therefore; it is recommended that this topic be discontinued.

(33) **2003.12**, *BAX System for Detection of Listeria monocytogenes in Foods*.—Study Director Deborah A. McIntyre. This method received Final Action status in 2005; therefore, it is recommended that this topic be discontinued.

(34) **2000.15**, *Coliform Counts in Foods, Dry Rehydratable Film Method*.—Study Director Deborah A. McIntyre. This method is in Final Action status; therefore, it is recommended that this topic be discontinued.

(35) **2004.06** (formerly **996.06**, Modified; *Hg135*), *VIDAS Listeria (LIS) Immunoassay for the Detection of Listeria Species in Foods Using Demi-Fraser and Fraser Enrichment Broths*.—Study Directors Deborah A. McIntyre and Ronald L. Johnson. Continue study.

#### AOAC Research Institute Studies

Since last year's General Referee Report (3), the following studies have been approved by the AOAC Research Institute:

(1) *DuPont Qualicon BAX<sup>®</sup> System PCR Assay for Screening Salmonella in Selected Foods: BAX<sup>®</sup> Q7 Instrument Modification*.—The BAX<sup>®</sup> System (Qualicon, Inc., ESL Bldg 400, Rt 141 and Henry Clay, Wilmington, DE 19880) uses the polymerase chain reaction (PCR) to amplify a specific fragment of bacterial DNA, which is stable and unaffected by growth environment. The fragment is a genetic sequence that is unique to *Salmonella*, thus providing a highly reliable indicator that the organism is present. The automated BAX System then uses fluorescence detection to analyze PCR product for positive or negative results. The system combines primers, polymerase, and nucleotides needed for PCR into a single tablet. The specificity of a PCR assay is determined by the DNA sequences of the primers used. The basis for primer design for the BAX assay is a collection of 1572 strains of *Salmonella*, which have been analyzed by ribotyping.

Most chemical and operational parameters of the new instrument/assay kit combination are the same with the new instrument as with the current instrument. All kit reagents are identical to those used in previous AOAC validation studies. All components of the BAX tablets including PCR primers, Taq polymerase, nucleotides, and bulking agents are the same. Kits are thus fully compatible using both the current BAX and Q7 instruments. The only difference between the 2 instruments is the way in which the signal is recorded. Emission of bound SYBR<sup>®</sup> Green is detected by using a photomultiplier tube in the current BAX platform. In the Q7 instrument, a charged coupled device camera is used to detect the emitted light.

In the inclusivity study, all 50 *Salmonella* serotypes were positive with the assay. In the exclusivity study, all 20 non-*Salmonella* spp., grown in brain heart infusion (BHI) broth, were negative with the assay.

An internal laboratory method comparison study was done with frankfurters, raw ground chicken, and orange juice. The alternative method was compared to the USDA reference culture method (4) for frankfurters and raw ground chicken and to the BAM reference culture method (1) for orange juice. There were no significant differences between the alternative and reference methods for the recovery of *Salmonella* from these 3 foods.

Because this was a minor modification, no ruggedness data were required.

(2) *BioControl Assurance GDS™ for the Detection of Salmonella in Selected Foods and Environmental Surfaces*.—GDS (BioControl Systems, Inc.) is an automated gene-based assay that incorporates multiple levels of specificity to ensure highly accurate results. The method uses proprietary probes and specific primers directed against a highly conserved DNA sequence of the target organism. It also uses an immuno-capture device and reagents that concentrate populations of target microorganisms and eliminate potential competitive microflora. The method is designed to be highly selective and does not detect microorganisms that are potential cross-reactors in antibody-based assays.

In the inclusivity study, all 100 *Salmonella* serotypes were positive with the assay. In the exclusivity study, non-*Salmonella* spp. were grown in BHI broth and tested directly in the GDS system, rather than being put through the immuno-capture step first. All 35 cultures were negative with the assay.

Internal and independent laboratory method comparison studies both showed that the alternative method was not significantly different ( $p < 0.05$ ) from the U.S. Department of Agriculture and FDA reference culture methods (1, 4) for raw pork, raw beef, raw ground turkey, raw shrimp, liquid milk, nonfat dry milk, liquid egg, chicken rinse, stainless steel, rubber, and concrete.

For ruggedness testing, the following parameters were examined: (1) sample incubation temperature (34, 36, and 38°C); (2) enrichment time (16, 22, and 26 h); and (3) assay sample volume (15, 20, and 25 µL). Varying the ruggedness parameters did not affect test results.

(3) *Transia Plate Salmonella Gold for the Detection of Salmonella Species in Foods and Feeds*.—This assay (Diffchamb AB, F O Petersons Gata 32, SE-421 31 Vastra Frolunda, Sweden) is based on a sandwich-type immunoassay relying on a polyclonal antibody coated onto the solid phase (wells of a microtiter plate with divisible strips) and a monoclonal antibody labeled with a peroxidase. The monoclonal antibody specifically recognizes a lipopolysaccharide determinant. The enriched test portion is added to the well after a heat inactivation step to release any *Salmonella* antigens that may be present in the culture. After a

3-step protocol, with a total incubation time of 105 min, the plate is read with a spectrophotometer.

For inclusivity testing, 129 *Salmonella* species cultures, representing 101 serovars, were tested with the assay. Of these cultures, 124 were reactive with the assay, resulting in an inclusivity rate of 96.1%. The 5 cultures that were not reactive with this assay were *S. Arizonae* (2 strains), *S. Bergen*, *S. Urbana*, and *S. 47:z4,z23:-*.

With respect to exclusivity testing, all of 72 non-*Salmonella* cultures, grown in buffered peptone water, were nonreactive with the assay.

A method comparison was conducted in the internal laboratory study in which the alternative method was compared with the ISO method (5) for the detection of *Salmonella* species in 400 samples comprising 18 foods (cooked chicken, raw ground beef, sausages, raw shrimp, smoked trout, raw milk, yogurt, cantaloupe, frozen berries and currants, shell eggs, mayonnaise, bean sprouts, fresh pasta, milk chocolate, ground black pepper, cake mix, dry milk-based infant formula, and dry cat food). In the independent laboratory study, 2 food types (raw ground turkey and Brie) were examined. For both studies combined, there was 98.8% agreement between the 2 methods. Moreover, the alternative method demonstrated a sensitivity of 99.3% and a specificity of 97.4%.

With respect to ruggedness testing, variation in the following parameters was studied: (1) heat-treatment time (15, 20, and 25 min); (2) test portion volume (80, 100, and 120 µL); and (3) conjugate volume (80, 100, and 120 µL). Results demonstrated that method performance was not affected by variation in any of these parameters.

(4) *Modified RapidChek System from Cassette to Strip Format for Salmonella Species in Ground Chicken and Deli Turkey*.—This assay (Strategic Diagnostics, Inc., 111 Pencader Dr, Newark, DE 19713-1147) may be used in combination with the proprietary enrichment system for a rapid 24 h test or with currently recommended USDA or FDA media for a 48 h test. After suitable enrichment, the test portion is dispensed into the sample port of the lateral flow device (LFD). The test portion flows through a zone containing antibody-coated colloidal gold reagents specific to *Salmonella* species. If antigens are present in the test portion, they will bind to the antibody conjugates to form an antigen-antibody complex. As this complex migrates through the nitrocellulose matrix, it passes through a zone of anti-*Salmonella* antibody. If antigen is present, the complex is captured in this zone and is visualized by the formation of a red line. A second zone on the membrane is designed to capture any antibody-gold complex not bound in the first zone. As a result, when *Salmonella* antigen is present, the formation of 2 red lines is observed, whereas only 1 line forms when no *Salmonella* organisms are present.

A modification has been made which involves a change in the format of the LFD from a test strip in a cassette to a stand-alone test strip. The test strip is constructed with the same filter and sink pad components but cut to a slightly longer length. Longer plastic backing and a protective

membrane cover are incorporated into the modified test strip to accommodate the increased length while adding some level of protection to the device. This modification uses the same reagents (membrane-bound antibody, antibody-gold reagent, and release pad) as have been used in the cassette version of the test.

The 24 h alternative method detected 17 positive test portions of 20 turkey test portions examined and 19 positive test portions of 20 ground chicken test portions examined. In comparison, the reference methods detected 19 and 15 positive turkey and chicken test portions, respectively. No false-positive results were reported by the alternative method for either the 24 or 48 h enrichment. In all cases, the alternative method performed equivalently or better than the reference method.

(5) *3M Petrifilm Environmental Listeria Plate Method*.—This assay (3M Co., 3M Center, St. Paul, MN 55144-1000) is a dry rehydratable sample-ready culture medium containing selective agents, nutrients, a cold water-soluble gelling agent, and a chromogenic indicator that facilitates the detection and enumeration of *Listeria* species but does not differentiate species from one another. Buffered peptone water (BPW) is used as a repair broth in conjunction with the Petrifilm EL Plate to facilitate the resuscitation of stressed *Listeria* organisms. The environmental test portion is collected using a swab, moistened sponge, or other sampling device. After sample collection, 5 mL of repair broth (BPW) is added to the test portion, and the test portion is mixed and then allowed to remain at room temperature for a minimum of 1 h and up to a maximum of 1.5 h. The test portion is remixed, and 3 mL is plated onto the EL Plate. The gel is allowed to form, and the plate is then incubated for  $28 \pm 2$  h at 35 or 37°C (temperature based on validated references). Red-violet colonies on the plate are considered *Listeria*.

In the inclusivity study, 57 of 59 *Listeria* species isolates were detected as typical colonies. The 2 strains that were not detected produced nontypical (no esculinase reaction) on PALCAM agar. One strain, obtained from ATCC, uses identified as *L. monocytogenes* serotype 3. The other strain, obtained from a private culture collection, was ribotyped as *L. monocytogenes*. Further characterization of these 2 nontypical isolates was not performed. For the exclusivity study, cultures were held in BPW at 25°C for 1 h, as a repair step, before being plated onto the alternative method plate. All of 53 isolates tested were nonreactive on the alternative method plate.

In the internal laboratory study, the alternative method was compared to the USDA reference method (4) for the detection and enumeration of *Listeria* species on 4 environmental surfaces: stainless steel, plastic (polypropylene), sealed concrete, and ceramic tile. Each of these surfaces was inoculated with a different *Listeria* strain at one of 3 levels (low, medium, and high) in addition to an uninoculated control level. Qualitatively, *Listeria* species were detected with the alternative method at all levels, and, similarly, *Listeria* species were detected at all levels with the reference method. Quantitatively, no differences were observed

between the 2 methods at any level of contamination when plastic and sealed concrete were the test matrixes. For stainless steel, only the low level of contamination yielded statistically equivalent results. For the medium and high levels of contamination on stainless steel, the alternative method yielded significantly higher counts than did the reference method. For ceramic tile, all contamination levels resulted in the alternative method having significantly higher counts than the reference method.

In the independent laboratory study, the 2 methods were compared for the detection and enumeration of *Listeria* species on stainless steel. Direct statistical analysis (2-sample *t*-test) was possible only for the low and medium levels of inoculation. At the low level of inoculation, there was no difference between the 2 methods in the ability to detect and enumerate *Listeria* species. At the medium level of inoculation, there was a significant difference between the 2 methods in favor of the alternative method.

For ruggedness testing, variations in the following parameters were studied: (1) test portion volume (2.8, 3.0, and 3.2 mL); (2) incubation temperature (33, 36, and 39°C); (3) plate position in stack (up to 10); (4) test portion pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0); (5) replication time in repair broth (0, 30, 60, 90, and 120 min); (6) freezer storage time (0, 1, 4, and 7 days); (7) hydration fluid composition (neutralizing buffer, letheen, and sterile water); and (8) source of BPW (Difco, Remel, Hardy, and Edge Biologicals).

For ruggedness parameters 1–4, the following cultures were used: *L. monocytogenes* ATCC 43256, *Enterococcus faecalis* ATCC 29212, and *Enterococcus faecium* ATCC 12952. These cultures were grown in Trypticase soy broth for 18–24 h at 35°C and diluted to achieve individual colonies on the alternative method plates. For parameters 5–8, the following organisms were tested: *L. monocytogenes* ATCC 43256, *L. monocytogenes* ATCC 43257, *L. monocytogenes* 51772, *L. monocytogenes* ATCC 19115, *L. monocytogenes* ATCC 19116, *Listeria* sp. J1-023 (initially classified as *L. monocytogenes* and later as atypical *L. innocua*), *L. innocua* ATCC 33090, *L. innocua* ATCC 49595, and *L. welshimeri* ATCC 35897.

For parameter 1, sample volume variations were tested with inocula of *L. monocytogenes* and cocktails of *L. monocytogenes* plus *E. faecalis* or *L. monocytogenes* plus *E. faecium*. For the test portion volumes examined (2.8, 2.9, 3.0, 3.1, and 3.2 mL), an analysis of variance (ANOVA) showed that there were differences between the counts for 2 of the inocula (*L. monocytogenes* and *E. faecium* plus *L. monocytogenes*). Differences were further evaluated (Dunnett's one-way multiple comparison using 3.0 mL as control) for significant differences for *L. monocytogenes* and *E. faecium* plus *L. monocytogenes* results. The zero fell within the interval at all volumes, (indicating no difference), except for the 3.1 mL inoculum of *E. faecium* plus *L. monocytogenes*.

For parameters 2–7, variations as described previously showed no significant difference in *Listeria* species counts on the alternative method plates.

For parameter 8, 4 sources of BPW were tested for efficacy as repair broth with 10 target organisms (strains of *L. monocytogenes*, *L. innocua*, and *L. welshimeri*) and 2 nontarget organisms (*Bacillus pumilis* and *Enterococcus faecium*). An ANOVA showed that for 8 of the 10 *Listeria* strains, there were no statistical differences in the *Listeria* counts of completed tests when BPW from Difco, Remel, Hardy, and Edge Biologicals was used. For one strain of *L. monocytogenes* and one strain of *L. innocua*, the Remel and Hardy formulations yielded low results, but this was not deemed to be sufficient grounds for making a recommendation against these 2 particular sources of BPW.

(6) *Paradigm PDX-LIB Labor Saver Test for the Detection of Listeria spp. on Environmental Surfaces.*—The Paradigm Diagnostics' *Listeria* Indicator Broth (PDX-LIB) Labor Saver method (Paradigm Diagnostics LLC, 1334 Eckles Ave, St. Paul, MN 55108) is modified from the previously approved PDX-LIB method. PDX-LIB is a specialty medium that is intended to be used as a screening tool for the detection of *Listeria* spp. on environmental surfaces. It is distinct because it is a single-stage enrichment method that can be used as a screening test for *Listeria* spp. Indicator compounds turn the broth from yellow to black by utilizing  $\beta$ -glucosidase that is produced by *Listeria* species. Darkening of the medium, from yellow amber to brown black, after 30 to 48 h incubation at 37°C, indicates the presence of *Listeria*. In the original method, a surface was sampled with a sponge that was placed in a bag containing 6 mL peptone broth. The sponge was massaged and 2 mL peptone broth was transferred to 20 mL PDX-LIB. The PDX-LIB was incubated for 30–48 h. In the modified method, the surface is sampled with a Tecra Enviroswab, and 20 mL PDX-LIB is added to the TECRA tube/sponge assembly, which is then incubated for 30–48 h.

In the inclusivity study, all 67 *Listeria* spp. isolates were positive with the assay. In the exclusivity study, 30 of 37 non-*Listeria* spp. were negative with the assay. All of the 7 isolates that produced false-positive reactions were  $\beta$ -glucosidase positive *Enterococcus* spp. (4 *E. hire* strains, 1 *E. faecalis* strain, 1 *E. gallinarum* strain, and 1 *E. avium* strain).

Internal method comparison studies were done with *L. monocytogenes* (sealed concrete), *L. innocua* (ceramic tile), *L. ivanovii* (stainless steel), and *L. welshimeri* (plastic). The alternative method was significantly more productive ( $p < 0.05$ ) than the USDA reference method on ceramic tile, stainless steel, and sealed concrete. The alternative method was equivalent to the reference method on plastic.

The ruggedness parameters were incubation time (28, 30, 32, 46, 48, and 50 h) and incubation temperature (36, 37, and 38°C). Varying the ruggedness parameters did not affect test results.

(7) *Modified Neogen GeneQuence DNA Probe Assay for Detection of Listeria Species in Selected Foods and Selected Environmental Surfaces.*—This assay (Neogen Corp.) is a DNA probe-based diagnostic in kit format which permits rapid and accurate detection of *Listeria* species in selected

foods. Following sample enrichment, target bacteria are lysed enzymatically at 37°C, and *Listeria*-specific oligonucleotide probes are added for a 60 min hybridization at 45°C. If *Listeria* ribosomal RNA (rRNA) is present in the test portion, the detector probe, labeled with horseradish peroxidase (HRP), and polydeoxyadenylic acid (poly dA)-tailed capture probe will hybridize to the target organism rRNA sequences. Concurrently, base pairing between the poly dA-tailed capture probe and polydeoxythymidylic (poly dT)-coated polystyrene microwells facilitates solid-phase capture of probe-target molecules. Unbound probe is removed by washing, and then substrate chromogen is added to react with HRP to yield a blue color. The reaction is stopped by the addition of sulfuric acid, which changes the color of the substrate from blue to yellow. A microwell plate or microwell strip reader measures the absorbance at A<sub>450</sub>. Absorbance in excess of the threshold value indicates the presence of *Listeria* species in the test portion.

This assay, as originally validated, uses a primary enrichment in selective broth media: (1) red meats and poultry (University of Vermont medium (UVM) broth); (2) dairy products, raw and cooked seafoods [buffered *Listeria* enrichment broth (BLEB) base supplemented with sodium pyruvate] followed, after 4 h incubation, with addition of BLEB supplements; (3) environmental swab samples (UVM broth); and (4) environmental sponge samples (UVM broth). These incubated enrichments are then streaked onto an Oxford plate. Growth from the plate is harvested and resuspended in buffer before testing in the assay. The alternative method uses the following enrichment schemes: (1) red meats and poultry (primary enrichment in UVM broth and secondary enrichment in PALCAM broth); (2) dairy products, cooked seafoods, and fruits and vegetables (primary enrichment in BLEB base supplemented with sodium pyruvate) followed, after 4 h incubation, with addition of BLEB supplements and secondary enrichment in fresh BLEB with supplements; (3) environmental swab samples [*Listeria* Enrichment Single Step (LESS) broth for 30 h]; and (4) environmental sponge samples (LESS broth for 30 h). The DNA hybridization assay itself is unchanged.

For inclusivity testing, 52 strains, representing all 6 *Listeria* species, were tested with the assay. All 52 strains produced positive results with the assay from all 3 enrichment protocols (BLEB to BLEB enrichment, UVM to PALCAM enrichment, and LESS enrichment). With respect to exclusivity testing, all 32 strains of non-*Listeria* organisms, grown in Trypticase soy broth, were nonreactive with the assay.

Validation studies with 14 different food types (deli turkey, deli ham, frankfurters, raw ground beef, raw frozen ground pork, smoked salmon, cooked crab meat, ice cream, pasteurized milk, Brie, Parmesan cheese, lettuce, frozen peas, soy flour) were conducted to compare the performance of this alternative method with the USDA (4) or FDA (1) reference culture methods, depending on the food matrix. In the testing of 780 food test portions, overall sensitivity of the alternative method was 95.6% (false-negative rate of 4.4%), while that of

the reference culture methods was 87.1% (false-negative rate of 12.9%). Specificity of the alternative method was 100% (false-positive rate 0%). Agreement between the alternative method and the reference culture methods was 96.6% for the USDA method and 85.4% for the FDA method.

With respect to validation of the modified method for use with environmental surfaces, 6 surfaces (stainless steel, plastic, cast iron, ceramic tile, sealed concrete, and painted wood) were used. A total of 110 hybridization assays from the 6 surfaces was positive, while a total of 112 test portions was confirmed positive from the Neogen 1-Step Enrichment Broth cultures (2 additional positives on sealed concrete). Thus, the sensitivity of the assay, relative to its own enrichment, was calculated at 98.2% (110/112). There was a total of 62 positive test portions by the USDA method. Thus, the productivity of the hybridization assay, relative to the reference culture method, was estimated at 177% (110/62). All positive hybridization assays were confirmed by plating from the LESS broth cultures. Thus, the specificity of the assay in these trials was 100%.

Ruggedness testing was performed as part of the original submission for this method. Variation in the following parameters was assessed: number of mixing steps of the lysed test portion and the probe/hybridization solution, volume of pre-mixed probe/hybridization solution, number of wash steps, hybridization incubation temperature, and hybridization incubation time. Of these assay parameters evaluated, the volume of hybridization/probe solution was the most critical for achieving accurate results.

(8) *VIDAS Listeria species Xpress (LSX) with Ottaviani Agosti Agar for the Detection of Listeria Species in Meat Products, Dairy Products, and Eight Environmental Surfaces.*—The VIDAS *Listeria Xpress* (LSX, bioMérieux, Inc.) is an enzyme-linked fluorescent immunoassay (ELFA) designed for use with the automated VIDAS or mini-VIDAS instruments for the specific detection of *Listeria* species. The instrument performs all of the assay steps automatically. A small aliquot of the test portion is placed into the reagent strip. The test portion is then cycled in and out of the solid-phase receptacle (SPR) for a specific length of time. *Listeria* antigens present in the test portion will bind to the anti-*Listeria* monoclonal antibodies, which are coated on the interior of the SPR. Unbound test portion components are washed away. Alkaline phosphatase-labeled antibodies are cycled in and out of the SPR and will bind to any *Listeria* antigens captured on the SPR wall. Further wash steps remove unbound conjugate. During the final detection step, the substrate, 4-methyl-umbelliferyl phosphate, is cycled in and out of the SPR. The bound enzyme conjugate catalyzes the hydrolysis of this substrate into a fluorescent product, 4-methyl-umbelliferone, the fluorescence of which is measured at 450 nm. When the assay is completed, the results are analyzed automatically by the instrument, and a test value is generated for each test portion. This value is then compared to an internal reference (threshold), and each result is interpreted as positive or negative.

With respect to inclusivity testing, all of 50 strains of *Listeria* species were reactive with the assay. For exclusivity testing, 70 non-*Listeria* strains were grown in tryptone broth with yeast extract. All strains were nonreactive with the assay.

The alternative method was validated for use in detection of *Listeria* species in meat (raw pork, frozen raw ground beef, frankfurters, roast beef, and ham), and dairy products (Camembert cheese, cheddar cheese, yogurt, pasteurized milk, and ice cream) as well as for detection of *Listeria* species on 8 environmental surfaces (stainless steel, plastic, rubber, ceramic, wood, sealed concrete, cast iron, and air filter material). The USDA method (4) was used as the reference method for meat and environmental surfaces and AOAC Official Method 993.12 (2) was used as the reference method for dairy products.

Internal and independent laboratory method comparison studies were performed. The alternative method was significantly more sensitive than the USDA method for raw pork, plastic, rubber, ceramic, and cast iron. Moreover, the alternative method was significantly more sensitive than the AOAC method for Camembert cheese. Confirmation of presumptive results with Ottaviani Agosti agar was equivalent to confirmation with those agars used by the respective reference methods (Oxford agar with the AOAC method and modified Oxford agar with the USDA method).

For ruggedness testing, variation in the following parameters was examined: test portion volume (450, 500, and 550  $\mu$ L); boiling times (10, 15, and 20 min); and test portion temperature (10, 25, and 50°C). There was no significant effect on method performance by a variation in any of these parameters.

(9) *Neogen GeneQuence Test for the Detection of Listeria monocytogenes in Raw and Processed Meats, Raw and Processed Seafood, Dairy Products, and Fruits and Vegetables.*—The GeneQuence *Listeria monocytogenes* Test (Neogen Corp.) is a DNA probe-based diagnostic kit, which permits rapid and accurate detection of *L. monocytogenes* in selected foods. Following sample enrichment, target bacteria are lysed enzymatically at 37°C and *L. monocytogenes*-specific oligonucleotide probes are added for a 60 min hybridization incubation at 45°C. If *L. monocytogenes* ribosomal RNA (rRNA) is present in the test sample, the detector probe, labeled with HRP, and poly dA-tailed capture probe will hybridize to the target organism rRNA sequences. Concurrently, base pairing between the poly dA-tailed capture probe and poly dT-coated polystyrene microwells facilitates solid-phase capture of probe-target molecules. Unbound probe is removed by washing, and then substrate chromogen is added to react with HRP to yield a blue color. The reaction is stopped by the addition of sulfuric acid, which changes the color of the substrate from blue to yellow. A microwell plate or microwell strip reader (A<sub>450</sub>) measures absorbance. An absorbance in excess of the threshold value indicates the presence of *L. monocytogenes* in the test sample. Positive assay results must be confirmed by standard culture methods.

In the inclusivity study, all 50 *L. monocytogenes* isolates were positive with the assay. In the exclusivity study, all 30 non-*L. monocytogenes* species were grown in Trypticase soy broth and were negative with the assay.

Internal and independent laboratory method comparison studies both showed that the alternative method performed as well as the reference culture methods. In tests of 910 food samples (cooked crab meat, raw shrimp, ice cream, pasteurized milk, Brie, Parmesan cheese, cottage cheese, lettuce, frozen peas, soy flour, and deli roast beef), overall sensitivity of the DNA hybridization method was 92.7% (false-negative rate 7.3%), while that of the reference culture methods was 89.0% (false-negative rate 11.0%). Specificity of the DNA hybridization assay was 100% (false-positive rate 0%). Agreement between the DNA hybridization method and the reference culture procedures was 95.4% for the USDA reference method (4) and 87.3% for the FDA reference method (1). The DNA assay is able to detect as low as 1 colony-forming unit (CFU) *L. monocytogenes* in 25 g food samples. Results can be obtained as early as 48 h after the start of test portion enrichment.

For ruggedness testing, the following parameters were examined: (1) variation of the number of mixes (none, 5, and 10 times) of the lysed sample and the probe/hybridization solution; (2) variation in the premixed probe/hybridization solution volume (0.1, 0.125, and 0.15 mL); (3) variation in the number of wash steps (4, 5, 6 times); (4) variation in the hybridization incubation temperature (42, 45, and 48°C); and (5) variation in the hybridization incubation time (45, 60, and 75 min).

For parameter 1, mixing the lysed sample and the probe/hybridization solution 10 times was more effective than either 0 or 5 mixes, so it is now recommended that the lysed sample and probe/hybridization solution be mixed 10 times. For parameter 2, variation of the premixed probe/hybridization solution volume had an effect on results in that a false-positive reaction occurred with *L. innocua* at a volume of 0.1 mL. A volume of 0.125 mL is recommended for the probe/hybridization solution. Changes in parameter 3 (number of wash steps) had no effect on the results. For parameter 4, varying the hybridization temperature had an effect on test results. A temperature of 48°C adversely affected the results, while temperatures of 42 and 45°C did not (45°C is the recommended temperature). Finally, parameter 5 (hybridization time) had no effect on the performance of the test.

(10) *Marshfield Clinic Test for the Detection of E. coli O157:H7 in Raw Ground Beef*.—The Marshfield Clinic method, marketed as the LightCycler *E. coli* (*eae*) Detection Kit (Roche Applied Science, 9115 Hague Rd, PO Box 50414, Indianapolis, IN 46250-0414) is a system composed of kits from several manufacturers to allow for the rapid growth, capture and concentration, DNA extraction, and PCR detection of *E. coli* O157:H7 organisms within 8 h. The raw ground beef test portion is stomached with BPW and incubated for 4–5 h at 42°C to enable *E. coli* O157:H7 organisms to recover and multiply. After incubation, the

enrichment media containing the raw ground beef test portion is placed into a thermally controlled pot on the MATRIX MicroScience Ltd. PATHATRIX Instrument (MATRIX Microscience, Inc., 651 Corporate Circle, Suite D, Golden, CO 80401). Magnetic beads, coated with antibodies to *E. coli* O157 are added to each test portion and a preprogrammed run is started. The PATHATRIX instrument continuously circulates through the test portion through a closed loop system of tubing. As the test portion is circulated, it is passed over a magnet, and the beads are captured. After a test portion is circulated for 1 h, debris is removed from the beads by a wash step. The beads are then eluted into a wash vessel and concentrated in a magnetic rack. The excess liquid is removed, and the beads are suspended into 50–100 µL buffer. The DNA is then extracted from any *E. coli* O157:H7 attached to the beads using The ShortPrep foodproof I Kit (Roche Applied Science). The suspended beads are added to the ready-to-use lysis tubes, and mixed. The tubes are then heated at 95°C for 10 min and cooled at room temperature for 1 min. The test portions are mixed on a vortex mixer and centrifuged. A 5 µL of the extracted DNA is amplified by PCR and detected fluorescently using primers and hybridization probes specific to the *eae* gene of *E. coli* O157:H7. A competitive internal control is included in each PCR reaction to verify the ability of PCR to occur in each reaction. The presence of amplification of the internal control in a test portion negative for *E. coli* O157:H7 confirms that the test portion is negative.

In the inclusivity study, all 50 *E. coli* O157:H7 cultures were positive with the assay. In the exclusivity study, 30 cultures, other than *E. coli* O157:H7, were grown in BHI broth. The following cultures were reactive with the assay: *E. coli* O55:H7 (strain 1); *E. coli* O55:H7 (strain 2); *E. coli* O157:nonmotile, *E. coli* O145:nonmotile (strain 1); and *E. coli* O145:nonmotile (strain 2). Reactivity of these isolates was eliminated when these cultures were grown in BPW for 4–5 h, as prescribed by the Marshfield Clinic test method protocol.

Internal laboratory and independent laboratory method comparison studies both showed that the alternative method had greater sensitivity in both 25 and 375 g test portions (95–100 and 88%, respectively) than did the USDA reference culture method (80–89 and 22%, respectively). The alternative method demonstrated higher specificity than did the USDA method for the analysis of the 25 g test portions in the internal laboratory study (100 and 60%, respectively). The specificity rates were 100% for both the alternative method and the reference culture method in all other studies.

Because certain steps involved in this method have already been performance-tested [PATHATRIX *E. coli* O157 test (AOAC license No. 030202) and ShortPrep foodproof I Kit (AOAC license No. 120301)], independent parameters were selected from parameters not yet studied. These 2 parameters were test portion incubation time (3.5 and 4.0 h) and PCR test portion volume (4, 5, and 6 µL). Assay results from inoculated test portions demonstrated that the performance of this assay was not affected by the described variations in these 2 parameters.

(11) *Envisio E. coli O157 Assay for the Detection of E. coli O157 in Raw Ground Beef*.—This assay (Centrus International, Inc., PO Box 2003, Kingsport, TN 37662) is a rapid detection system that incorporates the use of antibody-coated superparamagnetic nanoparticles in a lateral flow immunoassay format. Ground beef, the single matrix for which this system is approved, is enriched in a selective medium formulated to enhance the growth of *E. coli* O157 relative to the competitive microbial flora. An aliquot of the enriched test portion is treated to reduce the amount of insoluble particles in the test portion, thus improving the consistency of test portion flow in the cassette and reducing the potential for plugging of pipet tips. The test portion is then heated to kill the target analyte. The resulting test portion is then mixed with a suspension of antibody-coated magnetic particles. The detector senses small changes in the magnetic field along the test strip as it passes under a group of 3 sensing coils, wound in alternating directions such that a characteristic signal profile is generated by the presence of magnetic particles bound to test or control lines. The instrument compares the detection signal with a positive threshold value encoded in a data matrix on the individual cassette, and then reports a positive or negative result.

For the inclusivity study, all 74 *E. coli* O157 cultures were positive with the assay. For the exclusivity study, all 45 cultures, grown in Trypticase soy broth, were negative with the assay.

An independent laboratory study was conducted in which the alternative method was compared to the USDA reference culture method (4) for the detection of *E. coli* O157 inoculated at 2 levels into raw ground beef. At both levels, the alternative method detected a significantly higher level of *E. coli* O157 organisms than did the reference method.

With respect to ruggedness testing, variation in the following parameters was investigated: (1) test portion treatment volume (1 and 2 drops); (2) heating temperature (100 and 110°C); (3) cassette development time (25 and 50 min); and (4) stomaching time (30 and 120 s). Results of the ruggedness testing demonstrated that the performance of the method was not affected by the described variations in any of the parameters.

(12) *Modified RapidChek System from Cassette to Strip Format for E. coli O157 (Including H7) in Ground Beef*.—This assay (Strategic Diagnostics, Inc.) uses a lateral flow test strip in combination with the proprietary enrichment broth for a rapid 8 h test or the standard reference broth (mEC broth) for a 20 h test. After suitable enrichment, an aliquot of the broth is transferred from the enrichment bag to a disposable plastic tube. A test strip is then placed horizontally into the test tube. The test portion flows up the test strip via capillary action. It flows through a zone containing antibody-coated colloidal gold reagents specific to *E. coli* O157. If antigens are present in the test portion, they will bind to the antibody-conjugates to form an antigen/antibody complex. As this complex migrates through the nitrocellulose matrix, it passes a zone of anti-*E. coli* O157 antibody. If antigens are present, the complex is captured in this zone and

is visualized by the formation of a red line. A second zone on the membrane is designed to capture any antibody-gold complex not bound in the first zone. As a result, when *E. coli* O157 antigen is present, the formation of 2 red lines is observed. When no *E. coli* O157 is present, only 1 line forms.

A modification has been made which involves a change in the format of the LFD from a test strip in a cassette to a stand-alone test strip. This modification is the same as described for the modified RapidChek for *Salmonella* (see *AOAC Research Institute Study* No. 4).

The 8 h alternative method detected *E. coli* O157 in 19 of the 20 artificially contaminated test portions of raw ground beef, whereas the reference method detected this organism in 6 test portions. No false positives were observed with the modified lateral flow strip for the 8 h enrichment. When the alternative method was combined with the 20 h mEC broth, *E. coli* O157 was detected in 8 test portions. Thus, with both the 8 and 20 h enrichments, the lateral test strip performed equivalently or significantly better than the reference method.

(13) *BBL CHROMagar O157 for the Detection of E. coli O157:H7 in Raw Ground Beef and Unpasteurized Apple Cider*.—This agar (Becton, Dickinson, and Co., 7 Loveton Circle, Sparks, MD 21152) permits the isolation and presumptive identification of *E. coli* O157:H7 in raw ground beef and unpasteurized apple cider through the incorporation of specific chromogenic substrates and inhibitory agents for Gram-positive and Gram-negative organisms other than *E. coli* O157:H7. The chromogenic substrate produces a mauve-colored colony when hydrolyzed by an enzyme present in *E. coli* O157:H7. Bacteria other than *E. coli* O157:H7 may utilize other chromogenic substrates, resulting in blue- to blue-green-colored colonies, or, if none of the chromogenic substrates are utilized, colonies appear as their natural color. The growth of a mauve-colored colony on this agar is considered presumptive for *E. coli* O157:H7.

For inclusivity testing, 54 strains of *E. coli* O157:H7 were enriched in *Enterobacteriaceae* Enrichment Broth (EEB) with antibiotics or in modified Trypticase soy broth with novobiocin. All 54 strains produced mauve-colored colonies on this agar. For exclusivity testing, a total of 32 cultures, other than *E. coli* O157:H7, was enriched in BHI broth. All 32 strains either failed to grow or produced colonies that were not typical of *E. coli* O157:H7.

In the independent laboratory study, the recovery of *E. coli* O157:H7 on this agar was compared to the BAM (1), USDA (4), and ISO (6) plating media using the recommended enrichment broths. Of the 180 food test portions tested, 45 were tested using the BAM method, 45 using the USDA method, and 90 with the ISO method. This agar produced comparable results with the reference methods for both matrixes with a sensitivity of 100% and a specificity of 100%. No false negatives were observed. Moreover, on the basis of a Chi square analysis, there was no statistical difference in recovery among the alternative and 3 reference methods. Method agreement was 100% with the BAM method (apple cider), 85% with the USDA method (ground beef), 95% with the ISO method for ground beef, and 100% for the ISO

method for apple cider. In all cases where method agreement was <100%, the alternative method agar detected more positive test portions than did the reference method media.

With respect to ruggedness testing, 2 *E. coli* O157:H7 strains and one *E. coli* strain were used to study the effects of variations in incubation periods (14, 18, 24, and 28 h) and temperatures (32, 35, and 38°C). For the incubation period of 14 h, the 2 *E. coli* O157:H7 strains were negative at 32°C. At 35 and 38°C, these 2 strains were positive, producing light mauve to mauve-colored colonies. The *E. coli* culture did not grow at any temperature.

For the incubation period of 18 h, one *E. coli* O157:H7 strain produced a positive result at 32°C, while the other strain was colorless to weakly positive. At 35 and 38°C, these 2 strains were positive, producing mauve-colored colonies. The *E. coli* culture did not grow at any temperature.

For the incubation periods of 24 and 28 h, both *E. coli* O157:H7 strains produced mauve-colonies at 32, 35, and 38°C, while the *E. coli* culture produced colorless colonies.

(14) *Two Rapid Membrane Filtration Methods for the Detection of Escherichia coli Exceeding 10<sup>4</sup> CFU/g Action Levels*.—Current FDA regulations require certain foods to be examined for enterotoxigenic *E. coli* and other diarrheagenic strains if the total population of *E. coli* exceeds 10<sup>4</sup> CFU/g. Therefore, a value of 10<sup>4</sup> CFU/g represents an “action level” which triggers additional testing to determine whether a large enough population of diarrheagenic *E. coli* exists to represent a hazard to public health. Because this action level is high, samples can be diluted to 10<sup>3</sup> CFU/g, a level at which it is possible to use membrane filtration (MF) methods for solid samples. The 2 MF methods that were validated use 2 media: m-ColiBlue 24 (CB) and lauryl sulfate tryptose (LST) broth with 5-bromo-4-chloro-3-indolyl beta-D-glucuronide, cyclohexylammonium salt (BCIG). Both media are highly selective for *E. coli*, allowing presumptive determination of *E. coli* levels in 24 h and verified determination in another 1–2 days. CB selects for coliforms by use of several components, and *E. coli* colonies are then differentiated when beta-glucuronidase activity produces blue colonies by cleaving the 5-bromo-4-chloro-3-indolyl moiety. LST/BCIG selects for *E. coli* largely on the basis of incubation at 45.5°C and also differentiates on the basis of target colonies containing glucuronidase.

Inclusivity testing was conducted to determine if typical results are obtained after incubation of *E. coli* cultures on CB or LST/BCIG. Of 50 *E. coli* strains tested, 100% yielded typical results after incubation on CB, and 98% yielded typical results after incubation on LST/BCIG. For exclusivity testing, cultures were grown in Tryptic soy broth plus 0.6% yeast extract. All 50 strains of non-*E. coli* organisms were negative with both MF methods.

The ability of the 2 MF methods to determine whether *E. coli* organisms are present at an action level of 10<sup>4</sup> CFU/g was compared to that of AOAC Official Method 966.24 (4), a most probable number method. The false-positive rate for CB, LST/BCIG, and the reference method was 0% for all 3 methods. The false-negative rate was 2% for both the CB

and LST/BCIG methods and 6% for the reference method. Sensitivity was 98% for both the CB and LST/BCIG methods and 94% for the reference method. Specificity was 100% for all 3 methods. Overall agreement was 98% for both the CB and reference methods and for the LST/BCIG and reference methods. Data analysis by McNemar’s test showed no significant difference for enumerating *E. coli* in any of the 5 food types examined when either CB or LST/BCIG was compared to the reference method.

(15) *RAPID E. coli 2, a Chromogenic Medium for the Differentiation and Enumeration of E. coli and Other Coliform Bacteria in Selected Foods*.—This agar (Bio-Rad Laboratories, 2000 Alfred Nobel Dr, Hercules, CA 94547) relies on the simultaneous detection of 2 enzymatic activities, beta-D-glucuronidase (GLUC) and beta-D-galactosidase (GAL). The medium contains 2 chromogenic substrates. One substrate is specific to GAL and results in blue coloration of colonies positive for this enzyme, and one substrate is specific to GLUC and results in pink coloration of colonies positive for this enzyme. Coliforms, other than *E. coli* (GAL+/GLUC–), form blue to green colonies while *E. coli* (GAL+/GLUC+) form violet to pink colonies. A count of total coliforms can be obtained by adding the number of blue colonies and the number of violet colonies.

For the inclusivity study, a total of 108 *E. coli* and non-*E. coli* coliform bacteria was cultured and plated onto the alternative method agar and incubated at 2 temperatures (37 and 44°C). All non-*E. coli* coliform bacteria produced typical blue colonies, except 2 strains of *Klebsiella oxytoca*, which produced gray colonies (at 44°C only). All *E. coli* strains produced typical colonies at both temperatures. The inclusivity rate was 98% at 44°C and 100% at 37°C. The overall inclusivity rate was 99%. For the exclusivity study, a total of 32 noncoliform cultures was grown in nutrient broth. The cultures were plated on the alternative method agar and incubated at 2 temperatures as described above. At both temperatures, *Shigella sonnei* produced violet colonies. These colonies, however, were pinpoint and with colonial morphology different from that of a typical *E. coli* isolate. The exclusivity rate was 94% at 37 and 44°C.

In internal and independent laboratory studies, the alternative method was compared to Official Method 966.24 (2) for the enumeration of non-*E. coli* coliforms and *E. coli* in various food matrixes. The alternative method compared favorably with the reference method for 11 foods (raw ground beef, raw boneless pork, fermented sausage, processed ham, processed turkey, frozen turkey breast, raw ground chicken, cottage cheese, processed ricotta cheese, raw milk, and dry infant formula). Results with 7 food types (plain yogurt, processed roast beef, chicken nuggets, raw chicken breast, vanilla ice cream, raw fish fillet, and lettuce) did not compare favorably with the reference method, and these food types were removed from the product claim.

With respect to ruggedness testing, variation in the following parameters was studied: (1) incubation temperature (35, 37, 38, 42, 44, and 45°C); (2) incubation period (18 and 26 h); and (3) temperature of poured liquid agar (43 and

48°C). None of these variations had any effect on method performance.

(16) *Comparison of the Optical Sealing Film Kit to Optical Cap Strips for Use with the Microplate Format of the Warnex Rapid Detection Systems for Salmonella in a Variety of Foods, Listeria Species in a Variety of Foods, L. monocytogenes in a Variety of Foods, E. coli O157 in Ground Beef, and E. coli O157:H7 in Ground Beef.*—The Warnex rapid detection kits (Warnex Diagnostics, Inc., 3885 Industrial Blvd, Laval, Quebec, Canada, H7L 4S3) uses real-time PCR for the detection of the following species: *Salmonella*, *Listeria*, *L. monocytogenes*, *E. coli* O157, and *E. coli* O157:H7. Each detection microplate contains all the reagents required for the detection of these organisms. These assays identify the DNA sequence of pathogens in a series of sequential steps that include sample preparation, enrichment, DNA extraction, and DNA amplification by real-time PCR.

The fluorescence of the molecular beacons occurs once they recognize the targeted bacterial DNA sequence. The amount of fluorescence produced is proportional to the amount of DNA amplified and recognized by the beacon. In the absence of the target bacteria in the food samples, no fluorescence signal is detected. Results are analyzed and recorded automatically with Warnex's proprietary software. This detection procedure takes less than 3 h, after enrichment.

The Warnex detection kits are offered in 2 formats: the microplate format for high-volume analyses, and the Flexiwell format for low-volume, customized analyses. Warnex validated a modification which involved the transition from the use of optical-grade cap strips to the use of the Optical Film Sealing Kit (Bio-Rad Laboratories), which includes the Microseal B Clear Adhesive Seal and Optical Compression Pad. This transition is being made to provide a more secure seal for the detection microplates used with all of the heretofore AOAC Research Institute-validated Warnex detection kits.

Validation studies were undertaken with serially diluted cultures of *Salmonella* Typhimurium, *Listeria* species, *L. monocytogenes*, and *E. coli* O157:H7. A series of microplates, sealed with the currently used optical-grade caps and 3 series of test microplates sealed with 3 different lots of optical sealing film were prepared and analyzed in parallel. The average crossing point ( $C_t$ ), where fluorescence is significant, and standard deviations obtained from the 3 replicates at each dilution, on each microplate, for each pathogen were compared. The results demonstrated that a change to the use of the Optical Film Sealing Kit does not alter method performance.

(17) *BBL CHROMagar Staph aureus for the Enumeration of Staphylococcus aureus in Cooked Roast Beef, Smoked Salmon, and Shell Eggs.*—This agar (Becton, Dickinson, and Co.) is designed for the isolation and enumeration of *Staphylococcus aureus*. Hydrolysis of a chromogenic substrate by *S. aureus* results in the formation of mauve-colored colonies. Selective agents are added for suppression of Gram-negative organisms and for partial suppression of yeasts.

For the inclusivity study, all of 30 strains of *S. aureus* produced a positive result on the alternative method agar at 24 h incubation. For the exclusivity study, 58 strains of species other than *S. aureus* and genera other than *Staphylococcus* were grown in BHI broth. All 58 strains either failed to grow or produced colonies that were easily distinguished (non-mauve) from *S. aureus*.

For the internal and independent laboratory studies, the alternative method was compared to AOAC Official Method 975.55 (2) and ISO Method 6888-1:1999 (7) for the enumeration of *S. aureus* in cooked roast beef, smoked salmon, and shell eggs. Bacterial counts of *S. aureus* on the alternative method agar were compared to those on the reference medium, Baird-Parker agar, at low, medium, and high levels of contamination. On the basis of the paired *t*-test and one-way ANOVA, no statistical difference was noted with the alternative method, compared to the AOAC method, for the enumeration of *S. aureus* in all 3 food types and contamination levels. Compared to the ISO reference method, no statistical difference was observed with the alternative method for any food type or contamination level, with the exception of internal laboratory testing of the smoked salmon at the low level of contamination, where the alternative method recovered more colonies. The repeatability precision estimates of the alternative method were comparable to those of the AOAC and ISO reference methods. The correlation coefficients ranged from 92.6 to 99.4%, demonstrating good correlation for overall levels in all food types and methods.

With respect to ruggedness testing, the effects of variations in incubation periods (18, 22, and 26 h) and incubation temperatures (32, 35, and 38°C) on the performance of the method were studied. One strain of *S. aureus* and one species of *Staphylococcus*, other than *S. aureus* (*S. saprophyticus*) were tested. *S. aureus* was grown in Trypticase soy broth with 10% NaCl and 1% sodium pyruvate, while the *S. saprophyticus* culture was grown in BHI. Following incubation, the *S. aureus* culture was diluted to  $5.0 \times 10^3$  CFU/mL (high level) and  $5.0 \times 10^2$  CFU/mL (low level). *S. saprophyticus* was diluted to  $5.0 \times 10^3$  CFU/mL (high level). At an incubation temperature of 32°C, the incubation of alternative method plates for 18 and 22 h resulted in a significant decrease in the number of *S. aureus* colonies at the high level of inoculation. Incubation at 32°C for 26 h did not result in a significant decrease in the number of *S. aureus* colonies. Incubation at 35 and 38°C produced satisfactory recovery regardless of the incubation time. The colony counts of *S. aureus* and *S. saprophyticus* were not affected by the length of incubation time. Repeatability for each level of either test strain at each incubation period was not significantly different.

(18) *Warnex Rapid Pathogen Detection System for the Quantification of Campylobacter jejuni, C. coli, and C. lari in Poultry Rinses.*—This system (Warnex Diagnostics, Inc.) is based on real-time PCR technology. The system provides rapid detection by identifying the sequence of DNA of *Campylobacter* organisms in a series of sequential steps that

include sample preparation, DNA extraction, and pathogen detection and quantification.

During the detection step, the targeted *Campylobacter* DNA is amplified and detected using specific primers and molecular beacons. These beacons consist of a unique sequence probe for the *Campylobacter* organisms. Once bound to their target, the molecular beacons emit a fluorescent signal that is proportional to the amount of amplified *Campylobacter* DNA. In the absence of the target *Campylobacter* species in the food sample, no fluorescent signal is detected. Quantification results are provided automatically with test kit manufacturer's software that interprets and quantifies the intensity of the fluorescent signal.

The inclusivity study was performed on a total of 72 strains of *C. jejuni*, *C. coli*, and *C. lari*. The assay detected all strains with the exception of one *C. lari* strain.

In the exclusivity study, a total of 30 nontarget strains, including 4 other *Campylobacter* species (*C. fetus*, *C. hyointestinalis*, *C. mucosalis*, *C. sputorum*, and *C. upsaliensis*) was grown in various media (Trypticase soy broth, BHI broth with/without 7% horse blood serum, Man/Rogosa/Sharpe broth, and yeast extract/peptone/dextrose broth) and at various temperatures (25, 30, and 37°C), depending on each strain's growth requirements. All 30 strains were nonreactive with the assay.

The alternative method was compared to the ISO method (8) in both internal (whole chicken rinse and whole turkey rinse) and external (whole chicken rinse) studies. The individual quantification results obtained by the alternative method varied on the average by  $0.153 \pm 0.13 \log_{10}$  CFU/g from those of the reference method. Moreover, the overall comparison of results by the paired *t*-test and 3-way ANOVA showed no statistical difference between the 2 methods.

With respect to ruggedness testing, the effects of the following deviations in 3 method parameters were investigated: (1) increasing the volume of carcass rinse buffer from 0.33 to 0.5 mL/g; (2) decreasing the volume of filtered rinse from 1.6 to 1.2 mL; and (3) inverting the volumes of detection buffer and DNA extract when loading the PCR microplates or Flexiwells (15  $\mu$ L DNA and 10  $\mu$ L DT buffer

instead of 10  $\mu$ L DNA and 15  $\mu$ L DT buffer). The test portions where the volumes of DNA and DT solutions were inverted had the highest results with both the PCR microplates and Flexiwells. The ANOVA and Dunnett's tests confirmed that the modification to the volumes of DNA and DT buffer gave results that were significantly different from the controls. All other modifications tested had no significant effect on results. The 2-way ANOVA revealed no statistically significant difference between the results obtained from PCR microplates or Flexiwells.

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