17.4.01B AOAC Official Method 996.10
Enterohemorrhagic Escherichia coli (EHEC) O157:H7 Detection in Selected Foods
Assurance® Polyclonal Enzyme Immunoassay Method
First Action 1996
Revised First Action 1999
Final Action 1999

(Applicable to detection of enterohemorrhagic E. coli [EHEC] O157 including strain H7 in dairy foods, meats, poultry products, fruits, nutmeats, seafood, pasta, and liquid eggs.)

See Table 996.10 for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle

Proprietary antibodies with high specificity to EHEC antigens are bound to microwell plates. Appropriately enriched test samples and positive controls are added to plates. Any EHEC antigens present will bind to microwells, forming antibody–antigen complex. Nonreactive material is washed away. Alkaline phosphatase antibody conjugate is added, and, after incubation, unbound conjugate is washed away. The substrate, p-nitrophenylphosphate, is added and absorbance of resulting colored product is read spectrophotometrically at 405–410 nm.

B. Reagents

(a) Wash solution concentrate.—2% Polyoxyethylene 20 sorbitan monolaurate (Tween 20) in H₂O.
(b) Substrate.—p-nitrophenylphosphate solution.
(c) Positive control.—Stabilized, inactivated EHEC antigen including O157:H7.
(d) Conjugate solution.—Specific antibodies to EHEC conjugated to alkaline phosphatase.
(e) Stop solution.—20% Ethylenediaminetetraacetic acid (EDTA) in H₂O (w/v).
(f) Antibody-coated microwells.—Microwell strips, each well coated with EHEC antibody, 96-well holder, and cover.
(g) Modified TSB with novobiocin.—Mix 30.0 g Trypticase soy broth, 1.5 g bile salt No. 3, and 1.5 g K₂HPO₄ (anhydrous) in 1 L deionized H₂O. Autoclave mixture and let cool to room temperature. Add 0.2 mL novobiocin solution just before adding food. Novobiocin solution (100 mg/mL) = dissolve 100 mg novobiocin in 1.0 mL deionized water. Filter, sterilize, using a 0.2 µm filter and syringe. May be stored for 60 days in dark bottle at 2–8°C.
(h) Diagnostic reagents.—For culture confirmation of presumptive positive EIA tests, see chapter 4 in Bacteriological Analytical Manual, current edition, AOAC INTERNATIONAL, Gaithersburg, MD 20877, USA; and Microbiology Laboratory Guidebook, U.S. Department of Agriculture–Food Safety Inspection Service, Athens, GA 30604, USA.

Items (a)–(f) are available as Assurance® EHEC Enzyme Immunoassay test kit from BioControl Systems, Inc., 12822 SE 32nd St, Bellevue, WA 98005, USA.

C. Apparatus

(a) Incubators.—Maintaining 36 ± 1°C and 41.5 ± 0.5°C.
(b) Water bath.—Maintaining 100 ± 2°C or flowing steam autoclave set at 100°C can be used.
(c) Microplate washer or plastic squeeze bottle.—For washing microwell strips.

(d) Microplate reader.—Photometer with 405–410 nm filter, for reading microwell plates. May include optional printer.

D. General Instructions

Store all reagents at 2–8°C when not in use. Let reagents warm to room temperature before use. Include duplicate positive and one blank test wells with each run of test samples. Use separate pipet for each test portion and reagent to avoid cross-contamination. Use kit reagents and components as an integrated unit and do not mix with components from other manufacturing batches or sources. Use dedicated trough or glassware for each reagent to avoid cross-contamination. Do not use reagents after expiration date. Do not reuse microwells.

E. Preparation of Test Samples

(a) Enrichment.—Aseptically weigh 25 g test portion into 225 mL mTSB+n. If larger test portion sizes are analyzed, proportionately increase volume of mTSB+n to maintain 1:9 dilution ratio. Mix well. Incubate overnight (18–28 h) at 35–37°C.
(b) Preparation of sample for EIA analysis.—Following incubation, mix bottle contents thoroughly. Let particulate matter settle 1 min and transfer 1.0 mL into test tube. Retain the sample and mTSB+n mixture for confirmation of presumptive positive results.

Submerge 1.0 mL aliquot in boiling H₂O bath 20 min to inactivate microorganisms. Cool tubes to 25–37°C before testing. Boiled test samples can be stored at 2–8°C up to 4 days prior to testing. (Note: Tubes that have been stored must be thoroughly mixed on a Vortex mixer before addition of contents to microwell.)

F. Enzyme Immunoassay Procedure

(1) Prepare wash solution by adding 1.0 mL wash solution concentrate, B(a), to 100 mL H₂O (sufficient to wash 48 wells). Label container. Wash solution is stable for 30 days at 2–8°C.
(2) Install 405–410 nm filter in microwell plate reader.
(3) Fit required number of microwell strips into holder, allowing for 2 positive controls and one blank. Reseal unused microwells in foil pouch. Carefully record positive controls, blank, and test sample positions in holder.
(4) Vortex mix samples and positive control before pipetting. Pipet 100 µL positive control, B(c), into each positive control well. Leave blank well empty.
(5) Cover microplate and incubate 30 min at 35–37°C. Do not stack anything on microwell holder during incubation.
(6) Wash each well 3× using alternative (a) or (b) below.
(a) Completely remove contents of wells with microwell washer. Immediately fill wells with 250 µL wash solution. Repeat 2×. Avoid overfilling or underfilling wells (to prevent ineffective washing). Effective washing is critical to obtaining accurate data.
(b) Remove contents of well by inverting and vigorously tapping plate. Wash wells 3 times using precleaned wash bottle containing wash solution and completely fill each well.
(7) Immediately after removal of last wash, mix conjugate solution, B(d), by gently inverting bottle several times. Add 100 µL conjugate solution to each well, including control and blank wells. Cover and incubate 30 min at 35–37°C.
(8) Wash each well 3× as in step (6).
(9) Immediately after removal of last wash, add 100 µL substrate solution to each well, including control and blank wells. Cover and incubate 30 min at 35–37°C.

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Table 996.10  Interlaboratory study results for detection of *E. coli* O157:H7 in selected foods by assurance enzyme immunoassay

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>Type</th>
<th>MPN, cfu/g</th>
<th>Total</th>
<th>Pres&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Conf&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BAM culture</th>
<th>EIA performance rates&lt;sup&gt;c&lt;/sup&gt;, %</th>
<th>Incidence of false negatives among total positive test portions&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Incidence of false positives among total negative test portions&lt;sup&gt;d&lt;/sup&gt;</th>
<th>% Agreement&lt;sup&gt;h&lt;/sup&gt;</th>
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<td>27</td>
<td>27</td>
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</table>

<sup>a</sup> Pres = presumptive data; conf = confirmed data.

<sup>b</sup> Chi square, as defined by McNemar is \((|a–b|–1)^2/(a+b))\), where \(a\) = test portions positive by EIA and negative by BAM culture method, and \(b\) = test portions negative by EIA and positive by BAM culture method.

<sup>c</sup> Sensitivity and specificity rates for BAM culture method are by study definition 100%. Incidences of false negative and false positive for BAM method are by study definition 0%.

<sup>d</sup> Sensitivity rate = total number of analyzed positive test portions among “known” positive test portions/laboratory divided by total number of “known” positive test portions/laboratory, where “known” positive is defined as samples confirmed positive by reference method.

<sup>e</sup> Incidence of false negative = (100 – sensitivity rate).

<sup>f</sup> Specificity rate = total number of analyzed negative test portions among “known” negative test portions/laboratory divided by total number of “known” negative test portions/laboratory, where “known” negative is defined as test samples confirmed negative by reference method and negative controls.

<sup>g</sup> Incidence of false positive = (100 – specificity rate).

<sup>h</sup> Rate reflects number of confirmed determinations that were equivalent between EIA and BAM culture methods.

<sup>i</sup> N/A = statistical analysis not applicable. Methods give equivalent results.

<sup>j</sup> Uninoculated control samples are by definition “known” negatives in study. Sensitivity rates not calculated.
G. Reading

Immediately after incubation, read absorbance at 405 or 410 nm. Calibrate microwell plate reader by adjusting blank well to zero absorbance at 405–410 nm, then read 2 positive control wells and test wells. Standardize reader by adjusting absorbance (A) of blank well to zero. Read 2 positive control wells before reading sample wells. (Note: Certain samples may read <0; this is not uncommon and indicates a negative result.)

If reading is delayed, add 50 μL stop solution, B(e), to each well and read results within 1 h.

H. Interpretation of Test Results

(a) Control value.—Positive control reading should be 0.8–2.5 A units. Readings not within this range may indicate problems with washing procedure.

(b) Cutoff value.—Calculate average value of 2 positive control readings (in A units) and multiply by 0.25 to determine cutoff value. Test portions with readings ≥cutoff value are presumptively positive.

(c) Negative results.—Test portions with A less than cutoff value are negative.

I. Confirmation of Positive EIA Test Portions

Presumptively positive samples must be confirmed using culture methods as described in chapter on Escherichia coli O157:H7 in the current edition of Bacteriological Analytical Manual, AOAC INTERNATIONAL, Gaithersburg, MD 20877, USA; or Microbiology Laboratory Guidebook, U.S. Department of Agriculture–Food Safety Inspection Service, Athens, GA 30604, USA. Isolate from previously refrigerated enrichment broth.

Reference: J. AOAC Int. 80, 530(1997).

Revised: June 2000