AOAC Official Method 996.10
Escherichia coli O157:H7 in Selected Foods

Enzyme Immunoassay (EIA)
First Action 1996
Revised 1999
Final Action 1999
Revised 2002

[Applicable to detection of *Escherichia coli* O157:H7 (EHEC) in dairy foods, meats, poultry products, fruits, nutmeats, seafood, pasta, and liquid eggs using an 18–28 h enrichment protocol. Applicable to detection of *E. coli* O157:H7 in raw and cooked beef using an 8 h enrichment protocol.]

Caution: EHEC are pathogenic bacteria. Symptoms of infection include bloody diarrhea and cramping, little or no fever, and hemolytic uremic syndrome. Sterilize contaminated equipment and media before disposal or reuse.

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

A. Principle

In Assurance® Enzyme Immunoassay (EIA), antibodies with high specificity for *E. coli* O157:H7 antigens are bound to microwell plates. Enriched test broths and positive controls are added to the plates. If *E. coli* O157:H7 antigens are present, they bind to the antibodies in the microwells, forming antigen–antibody complexes. Nonreactive material is washed away. Specific antibody conjugated to alkaline phosphatase is added and binds to these complexes, and, after incubation, unbound conjugate is washed away. The substrate *p*-nitrophenylphosphate is added, and absorbance of the resulting colored product is read spectrophotometrically at 405–410 nm.

B. Media and Reagents

(**a**) *Wash solution concentrate*.—2% Polyoxyethylene 20 sorbitan monolaurate (Tween 20) in water.

(**b**) *Liquid substrate*.—*p*-Nitrophenylphosphate solution, 4.33mM; 1.6 mg disodium salt/mL.

(**c**) *Positive control*.—Stabilized, inactivated *E. coli* O157:H7 antigen.

(**d**) *Conjugate solution*.—Specific antibodies to *E. coli* O157:H7 conjugated to alkaline phosphatase.

(**e**) *Stop solution*.—20% Ethylenediaminetetraacetic acid (EDTA) in water.

(**f**) *Antibody-coated microwells*.—Microwell strips, each well coated with *E. coli* O157:H7 antibody; 96-well holder, and plastic cover.

(**g**) *For 8 h enrichment*.—*8 h EHEC enrichment medium (BioControl EHEC8™)*.—Prewarm 225 mL sterile deionized water at 42°C overnight. On the day of use, aseptically transfer 10.6 g BioControl EHEC8 powder or one packet of premeasured single test dose medium into the sterile water. Mix gently to dissolve powder completely.

(**h**) *For 18–28 h enrichment*.—*Modified Trypticase (tryptic) soy broth (TSB) with novobiocin (mTSB + N)*.—Mix 30.0 g TSB (dehydrated), 1.5 g bile salts No. 3, and 1.5 g anhydrous dipotassium phosphate in 1 L water. Sterilize by autoclaving at 121°C for 15 min. Prepare novobiocin solution by dissolving 100 mg novobiocin in 1 mL H_2O. Sterilize with 0.2 µm filter. On day medium is used, add 0.2 mL novobiocin solution to 1 L mTSB.

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

C. Apparatus

(**a**) *Incubators*.—Maintaining 36 ± 1°C and 42 ± 0.5°C.

(**b**) *Micropipets*.—Accurately dispensing 0.1 and 4.0 mL.

(**c**) *Microplate washer*.—For washing microwell strips. Plastic squeeze bottle can be used.

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

(**e**) *Vortex mixer*.—For mixing test broth tubes.

(**f**) *Water bath*.—Maintaining 100°C. Alternatively, flowing steam autoclave set at 100°C or dry heat block may be used.

(**g**) *Top loading balance*.—For weighing test portions. Measuring up to 1000 g, sensitivity of ± 0.1 g.

(**h**) *Stomacher*.—IUL Instruments masticator or equivalent for macerating test portions.

(**i**) *pH meter*

D. General Instructions

Store at 2–8°C when not in use. Let reagents equilibrate to room temperature before use. Include 2 positive control and 1 blank test wells with each run of test samples. Use separate pipets for each test sample and reagent to avoid cross-contamination. Kit reagents and components must be used as an integrated unit and may not be mixed 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 Enriched Broth

(**a**) *Enrichment*.—(*1*) *8 h enrichment*.—Raw and cooked beef only: Aseptically weigh 25.0 g test portion into 225 mL prewarmed (42°C) BioControl EHEC8, **B**(**g**) and masticate 2 min. Incubate for 8 h at 42°C.

(*2*) *18–28 h enrichment*.—Food products: Aseptically weigh 25.0 g test portion into 225 mL mTSB + N, **B**(**h**). 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. For viscous materials (i.e., powdered dairy products) add 2.25 mL steamed (15 min) Triton X-100 per 225 mL mTSB + N at time of test portion addition and prior to incubation.

(**b**) *Inactivation*.—*For either 8 h enrichment or 18–28 h enrichment*.—Gently mix enriched test broth and let food particles settle. Transfer 4 mL enriched broth for 8 h protocol or 1 mL for 18–28 h protocol to test tube. Inactivate broth for 10 min at 100°C. Cool inactivated broths to room temperature before testing on EIA. Inactivated broths can be stored up to 4 days at 2–8°C. Store remaining broths, which have not been inactivated, at 2–8°C for confirmation of presumptive positives.

F. Enzyme Immunoassay Procedure

(*1*) Prepare wash solutions by adding 1.0 mL wash solution concentrate, **B**(**a**), to 100 mL water. Label container. This volume is sufficient to wash 40 wells. Wash solution is stable for 30 days when stored at 2–8°C.

(*2*) Install 405–410 nm filter in microwell plate reader.

(*3*) Fit required number of microwells, **B**(**f**), into holder. Reseal unused microwells. In addition to test wells, allow 3 extra wells for 2 positive controls and 1 blank. Carefully record positions of positive controls, blank, and tests in holder.

Table 996.10. Interlaboratory study results for detection of *E. coli* O157:H7 in raw and cooked beef by EHEC EIA and culture methods

Food type	Level	MPN/g	No. of labs	No. of test portions	Test broths positive			Chi square[b]	Sensitivity rate, %[c]		Incidence of false negatives among total positive samples, %[d]		Specificity rate, %[e]	Incidence of false positives among total negative samples, %[f]	Agreement between EIA and culture methods, %[g]
					EIA Pres.[a]	EIA Conf.[a]	Culture		EIA	Culture	EIA	Culture	EIA	EIA	
Raw ground beef	Low	0.007	10	60	27	28	16	2.2	80	46	20.0	54.0	100	0	52
	High	0.147	10	60	52	55	53	0.0	96	93	4.0	7.0	100	0	85
	Uninoculated	NA[h]	10	60	1	0	0	—	—	—	—	—	—	—	—
Cooked beef	Low	0.004	11	66	26	25	11	4.3	81	36	19.0	64.0	100	0	59
	High	0.014	11	66	39	37	21	6.8	86	49	14.0	51.0	100	0	57
	Uninoculated	NA[h]	11	66	6	0	0	—	—	—	—	—	—	—	—

[a] Pres. = Presumptive positive data, Conf. = culturally confirmed data.

[b] Chi square, as defined by McNemar is $(|a - b| - 1)^2 / (a + b)$ where a = test portions positive by EIA and negative by culture, and b = test portions negative by EIA and positive by culture. A chi square value >3.84 indicates significance at $p < 0.05$.

[c] Sensitivity rate is defined as total number of confirmed positive test portions by EIA divided by total number of confirmed positive test portions by both the EIA and culture methods.

[d] Incidence of false negatives is 100 – sensitivity rate. Low number of total confirmed positives will result in high false negative data.

[e] Specificity rate is defined as total number of analyzed negative test portions by EIA divided by the total number of confirmed negative test portions by both the EIA and culture methods.

[f] Incidence of false positives is 100 – specificity rate.

[g] Rate reflects number of confirmed determinations that were equivalent between EIA and culture.

[h] NA = Not applicable. This previously screened food lot was verified to be negative for EHEC.

(*4*) Equilibrate enrichment broth to 25–37°C prior to assay. Do not mix inactivated test broths, **E**(**b**). Use a new pipet tip for each test. Pipet 100 µL aliquot of liquid from each test broth into microwell. Do not transfer food particles. Vortex the positive control, **B**(**c**), and pipet 100 µL aliquot into each positive control well. *Leave blank well empty.*

(*5*) Cover microplate with plastic cover provided and incubate 30 min at 35–37°C. Do not stack anything on top of microwell holder during incubation. Do not agitate plate during any incubation step.

(*6*) After incubation, wash each well 3 times using either procedure (**a**) or (**b**) below:

(**a**) *Washing procedure*.—Completely remove contents of well with microwell washer. Immediately fill wells completely with 250 µL wash solution, **F**(*1*). *Note*: Effective washing is critical to obtain accurate data. Avoid overfilling wells to prevent antigen carryover to adjacent nonreactive wells. Avoid underfilling wells to prevent ineffective washing.

(**b**) *Alternative washing procedure*.—Remove contents of well by inverting and vigorously tapping plate. Completely fill each well with wash solution, **F**(*1*), using clean wash bottle. Repeat twice for total of 3 aspiration/wash cycles per step.

(*7*) Immediately after removal of the last wash, mix conjugate solution, **B**(**d**), by gently inverting the bottle several times. Add 100 µL conjugate solution to each well, including control and blank wells. Cover plate with plastic cover and incubate 30 min at 35–37°C.

(*8*) After incubation, wash each well 3 times. Refer to washing procedure instructions, **F**(*6*).

(*9*) Immediately after removal of the last wash, add 100 µL substrate, **B**(**b**), to each well, including control and blank wells. Cover plate with plastic cover and incubate 30 min at 35–37°C. After incubation, *do not wash wells*. Proceed directly to **G**. If reading will be delayed, add 50 µL stop solution, **B**(**e**), to each well. Read within 1 h.

G. Reading

Read control and test well absorbances (A) at 405–410 nm. For valid results, the microwell plate reader must be calibrated against the blank well before test and control wells are read. Standardize reader by reading the blank well and adjusting absorbance to zero. Read the absorbance of each well, starting with the 2 positive controls. *Note*: When reader is standardized to blank well, certain test wells may read <0. This is not uncommon and indicates a negative result.

H. Interpretation of Test Results

(*1*) *8 h enrichment*.—*Positive control value*.—The positive control absorbance readings should be >0.8 A units. Absorbance readings below this value may indicate problems with the washing procedure. Contact BioControl Technical Services for more information.

Positive results.—Test wells with absorbance readings ≥0.3 are considered presumptive positive and need to be confirmed. Test wells with absorbance readings <0.3 are negative.

(*2*) *18–28 h enrichment*.—*Positive control value*.—The positive control absorbance readings should be >0.8 A units. Absorbance readings below this value may indicate problems with the washing procedure. Contact BioControl Technical Services for more information.

Positive results.—Calculate average value of the 2 positive control absorbance readings (in A units) and multiply by 0.25 to establish the cutoff value:

$$\frac{(PC1 + PC2)}{2} \times 0.25 = \text{cutoff value}$$

where PC = positive control absorbance reading (in A units). Repeat positive controls for each test run. Any values above this cutoff value are considered presumptive positive and need to be confirmed. Test wells with absorbance readings lower than the cutoff are negative.

Note: Microwell plate reader linear range is variable depending on manufacturer's specifications. If PC is reported as "over" or a numerical value >2.5, use 2.5 A for calculation purposes.

Confirmation of positive EIA test portions.—Presumptive positive tests must be confirmed using culture methods as described in the current edition of *Bacteriological Analytical Manual*. Isolate from previously enriched broths.

References: *J. AOAC Int*. **80**, 530(1997); **85**, 1037(2002).
Bacteriological Analytical Manual (1998) 8th Ed., AOAC INTERNATIONAL, Gaithersburg, MD.