AOAC Official Method 997.11
Escherichia coli O157:H7 Counts in Foods
Hydrophobic Grid Membrane Filter (ISO-GRID) Method Using SD-39 Agar and Serological Confirmation

First Action 1997
Final Action 2001

(Applicable to enumeration of *E. coli* O157:H7 from meats, poultry, dairy foods, infant formula, liquid eggs, mayonnaise, and apple cider.)

Caution: *See* Appendix B, safety notes on handling microorganisms. Sterilize contaminated equipment and media before disposal or reuse.

See Table **997.11A** for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle

Hydrophobic lines in grid pattern act as barriers to spread of colonies, thereby dividing membrane filter surface into separate compartments of equal and known size. After incubation, number of squares occupied by colonies is enumerated and converted to most probable number (MPN) value of organisms.

SD-39 agar contains selective agents that inhibit growth of gram-positive bacteria and some gram-negative bacteria. Elevated incubation temperature also inhibits some gram-negative bacteria. Differential reactions (lysine decarboxylase positive, glucuronidase negative, sorbitol negative) distinguish between presumptive *E. coli* O157:H7 and most other remaining gram-negative bacteria. Confirmation of presumptive positive results is based on absence of yellow colony pigmentation when incubated on Tryptone soy agar, and on positive agglutination reactions with O157 and H7 antisera.

Critical control points are: (*1*) proper adjustment of pH in preparation of SD-39 agar, and (*2*) close control of incubation temperature of 44.0–44.5°C.

B. Apparatus, Culture Media, and Reagents

(**a**) *Hydrophobic grid membrane filter*.—Membrane filter, 0.45 μm porosity, imprinted with nontoxic hydrophobic material in grid pattern. ISO-GRID (QA Life Sciences, Inc., 6645 Nancy Ridge Dr, San Diego, CA 92121, USA), or equivalent, meets these specifications.

(**b**) *Filtration units*.—For hydrophobic grid membrane filter, with 5 μm mesh pre-filter to remove food particles during filtration. Use 1 unit/test. ISO-GRID (QA Life Sciences, Inc.), or equivalent, meets these specifications.

(**c**) *Pipets*.—1.0 mL serological with 0.1 mL graduations (1.1 or 2.2 mL milk pipets are satisfactory), 5.0 and 10.0 mL serological with 0.1 mL graduations.

(**d**) *Blender*.—Multi-speed model with low-speed operation at 10 000–12 000 rpm, and 250 mL glass or metal blender jars with covers. Use 1 jar/test.

(**e**) *Vacuum pump*.—Water aspirator vacuum source is satisfactory.

(**f**) *Manifold or vacuum flask*.

(**g**) *Peptone diluent*.—Dissolve 1.0 g peptone (gelatin hydrolysate peptone) in 1 L H_2O. Dispense into dilution bottles so final volume is 90 ± 1 mL or 99 ± 1 mL after autoclaving 15 min at 121°C.

(**h**) *Peptone/Tween 80 (PT) diluent*.—Dissolve 1.0 g peptone (gelatin hydrolysate peptone) and 10.0 g Tween 80 in 1 L H_2O. Dispense into dilution bottles so final volume is 90 ± 1 mL or 99 ± 1 mL after autoclaving 15 min at 121°C.

(**i**) *SD-39 agar*.—5.0 g Proteose peptone, 3.0 g yeast extract, 5.0 g NaCl, 10.0 g L-lysine·HCl, 2.5 g D-glucose, 20.0 g sorbitol, 1.5 g $MgSO_4 \cdot 7H_2O$, 38 mg Na monensin (Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178, USA), 0.5 g Na glucuronate, 7.5 mg novobiocin, 120 mg phenol red, 50 mg 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt, and 15.0 g agar diluted to 1 L with H_2O. (SD-39 agar, QA Life Sciences, Inc., or equivalent.)

Heat to boiling with stirring until completely dissolved. Do not autoclave. Temper to 45–50°C in water bath. Aseptically pour sufficient volume of SD-39 agar into small weighing boat or into Petri dish to produce an agar depth of at least 3 mm, and let solidify. Check pH with flat-surface combination electrode. Adjust pH, if necessary, to 7.2 ± 0.2 by adding sterile 1M NaOH or HCl. Aseptically pour another small portion of medium and check pH as before. Continue adjustments until pH is within specified range. Dispense ca 18–20 mL portions into 15 × 100 mm Petri dishes. Prepared plates may be stored refrigerated for up to 4 weeks if protected from dehydration. Surface-dry plated medium before use by inverting partly open 15–20 min in 35°C incubator.

(**j**) *Motility agar*.—5.0 g tryptose, 5.0 g NaCl, and 3.0 g agar diluted to 1 L with H_2O. Heat to boiling with constant stirring to dissolve completely. Temper to 45–50°C in water bath. Determine pH and adjust, if necessary, to final pH of 7.3 ± 0.2 as in (**i**). Dispense 4 mL aliquots into 13 × 100 mm test tubes. Sterilize by autoclaving 15 min at 121°C. Let solidify in vertical position (i.e., without slanting tubes). Motility agar may be stored refrigerated for up to 4 weeks if protected from evaporation.

(**k**) *Tryptone soy agar (TSA)*.—15.0 g tryptone, 5.0 g soy peptone, 5.0 g NaCl, and 15.0 g agar diluted to 1 L with H_2O (commercially available dehydrate is satisfactory). Heat to boiling with stirring until agar is completely dissolved. Sterilize by autoclaving 15 min at 121°C. Temper to 45–50°C in water bath. Verify pH and adjust, if necessary, to 7.3 ± 0.2 using technique as in (**i**). Dispense 18–20 mL aliquots into Petri dishes.

(**l**) *Tryptone soy tryptose broth (TSTB)*.—8.5 g tryptone, 10.0 g tryptose, 1.5 g soy peptone, 3.0 g yeast extract, 5.0 g NaCl, 1.25 g anhydrous K_2HPO_4, and 1.75 g D-glucose diluted to 1 L with H_2O. Stir without heating until completely dissolved. Verify pH and adjust, if necessary, to 7.2 ± 0.2. Dispense into bottles or tubes, as required. Sterilize by autoclaving 15 min at 121°C.

(**m**) *Papain stock solution*.—Reconstitute 10.0 g sterile papain powder (available as EZ-Papain from QA Life Sciences, Inc.) with 100 mL sterile H_2O. Unused portion of solution can be stored frozen for up to 3 months.

(**n**) *Cellulase stock solution*.—Reconstitute 20.0 g sterile cellulase powder (available as EZ-Cellulase from QA Life Sciences, Inc.) with 100 mL sterile H_2O. Unused portion of solution can be stored frozen for up to 3 months.

(**o**) *Amyloglucosidase stock solution*.—Reconstitute 20.0 g sterile amyloglucosidase powder (available as EZ-AMG from QA Life Sciences, Inc.) with 100 mL sterile H_2O. Unused portion of solution can be stored frozen for up to 3 months.

(**p**) *Alkaline protease stock solution*.—Reconstitute 20.0 g sterile alkaline protease powder (available as EZ-APUG from QA Life Sciences, Inc.) with 100 mL sterile H_2O. Unused portion of solution can be stored frozen for up to 3 months.

Table 997.11A Interlaboratory study results for confirmed *E. coli* O157:H7 enumeration by ISO-GRID and BAM culture methods

Food	Level	Method[a]	Log_{10} mean count/g or mL	Precision estimates[b] r	R	s_r	s_R	RSD_r, %	RSD_R, %
Apple cider	High	ISO-GRID	2.57	0.53	0.73	0.19	0.26	7.27	10.10
		BAM culture	2.05	0.71	2.68	0.25	0.95	12.27	46.17
	Medium	ISO-GRID	2.32	0.28	0.59	0.10	0.21	4.32	9.03
		BAM culture	1.95	0.70	2.31	0.25	0.82	12.64	41.81
	Low	ISO-GRID	1.83	0.44	0.48	0.16	0.17	8.54	9.33
		BAM culture	1.49	0.89	1.69	0.31	0.60	21.10	40.07
	Overall	ISO-GRID	2.23						
		BAM culture	1.83						
Pasteurized milk	High	ISO-GRID	2.84	1.60	1.76	0.57	0.62	19.89	21.86
		BAM culture	1.93	1.77	2.80	0.62	0.99	32.41	51.26
	Medium	ISO-GRID	2.42	1.71	1.58	0.60	0.56	24.98	23.15
		BAM culture	1.58	0.89	1.92	0.31	0.68	19.83	42.88
	Low	ISO-GRID	2.15	1.23	1.13	0.43	0.40	20.19	18.63
		BAM culture	1.24	1.54	2.05	0.54	0.73	43.97	58.74
	Overall[c]	ISO-GRID	2.46						
		BAM culture	1.58						
Cottage cheese	High	ISO-GRID	2.35	1.12	1.24	0.39	0.44	16.82	18.66
		BAM culture	2.10	1.22	2.68	0.43	0.95	20.65	45.28
	Medium	ISO-GRID	2.15	1.44	1.87	0.51	0.66	23.62	30.70
		BAM culture	1.77	0.99	2.38	0.35	0.84	19.73	47.58
	Low	ISO-GRID	1.82	0.83	1.83	0.29	0.65	16.02	35.41
		BAM culture	1.28	0.99	2.03	0.35	0.72	27.40	56.23
	Overall	ISO-GRID	2.11						
		BAM culture	1.71						
Cooked ground pork	High	ISO-GRID	2.04	1.57	1.63	0.55	0.58	27.14	28.18
		BAM culture	1.44	2.21	2.52	0.78	0.89	54.04	61.84
	Medium	ISO-GRID	1.79	0.73	1.18	0.26	0.42	14.35	23.20
		BAM culture	1.41	0.69	2.22	0.24	0.78	17.26	55.46
	Low	ISO-GRID	1.12	0.49	0.59	0.17	0.21	15.51	18.63
		BAM culture	0.83	0.49	1.70	0.17	0.60	20.89	72.50
	Overall[c]	ISO-GRID	1.65						
		BAM culture	1.24						
Raw ground beef	High	ISO-GRID	2.29	0.81	1.06	0.29	0.37	12.55	16.32
		BAM culture	1.18	1.50	2.01	0.53	0.71	44.84	59.98
	Medium	ISO-GRID	1.64	1.13	0.96	0.40	0.34	24.36	20.71
		BAM culture	0.99	1.31	1.33	0.46	0.47	46.91	47.52
	Low	ISO-GRID	1.12	0.46	0.50	0.16	0.18	14.61	15.90
		BAM culture	0.61	0.55	0.72	0.19	0.25	31.98	41.87
	Overall[c]	ISO-GRID	1.73						
		BAM culture	0.93						
Frozen whole egg	High	Direct ISO-GRID	2.95	0.55	1.24	0.19	0.44	6.54	14.83
		Resuscitation ISO-GRID	3.20	0.77	1.21	0.27	0.43	8.54	13.40
		BAM culture	2.72	1.78	2.99	0.63	1.06	23.13	38.83
	Medium	Direct ISO-GRID	1.12	0.50	0.84	0.18	0.30	15.70	26.52
		Resuscitation ISO-GRID	1.25	0.66	0.98	0.23	0.35	18.65	27.71
		BAM culture	1.03	1.04	1.47	0.37	0.52	35.66	50.43
	Low	Direct ISO-GRID	1.19	1.51	1.45	0.54	0.51	44.87	42.85
		Resuscitation ISO-GRID	1.16	0.50	0.91	0.18	0.32	15.19	27.64
		BAM culture	0.83	1.20	1.32	0.42	0.47	50.96	55.93
	Overall[d]	Direct ISO-GRID	1.76						
		Resuscitation ISO-GRID	1.87						
		BAM culture	1.53						

[a] ISO-GRID = ISO-GRID method using SD-39 agar and serological confirmation; BAM culture = modified BAM culture method (*Bacteriological Analytical Manual,* 7th Ed., 1992, AOAC INTERNATIONAL, Gaithersburg, MD, USA); Direct ISO-GRID = direct test method; Resuscitation ISO-GRID = resuscitation test method.

[b] Precision estimates were calculated after conversion of data to log_{10}; $r = 2.8 \times s_r$; $R = 2.8 \times s_R$; s_r = repeatability standard deviation; s_R = reproducibility standard deviation; RSD_r = repeatability relative standard deviation; RSD_R = reproducibility relative standard deviation.

[c] Difference between methods is significantly different at the 5% level.

[d] Difference between resuscitation test method and reference method is significantly different at the 5% level.

(**q**) *E. coli O157 antiserum*.—Available from BD Laboratories, 7 Lorton Cir, Sparks, MD 21152, USA.

(**r**) *E. coli H7 antiserum*.—Available from BD Laboratories.

C. Preparation of Test Suspension

(**a**) *Liquid egg*.—Thoroughly mix test sample with sterile spoon or spatula. Prepare 1:10 dilution by aseptically weighing 11 g egg material into sterile wide-mouth, screw-top bottle. Add 99.0 mL PT diluent, **B**(**h**), and 1 tbsp of sterile glass shot. Thoroughly agitate 1:10 dilution to ensure complete distribution of egg material in diluent by shaking each bottle rapidly 25×, each shake an up-and-down movement of ca 30 cm in ≤7 s. Let bubbles escape. If enzyme treatment is needed (*see* Table **997.11B** for diluents and enzyme treatments), combine 5.0 mL 1:10 dilution with 1.0 mL appropriate enzyme stock solution and mix by pipetting up and down or by shaking gently. Incubate solution 20–30 min in water bath at 35–37°C. Correct for additional dilution by filtering 1.2 mL enzyme-treated suspension.

(**b**) *Other liquid foods*.—Thoroughly mix contents of container. To prepare 1:10 dilution, aseptically transfer 10.0 mL test portion into 90.0 mL peptone diluent, **B**(**g**), or PT diluent, **B**(**h**), (*see* Table **997.11B**) in sterile wide-mouth, screw-top bottle. Mix by shaking 25× through 30 cm arc in ≤7 s. If enzyme treatment is needed (*see* Table **997.11B**), combine 5.0 mL 1:10 dilution with 1.0 mL appropriate enzyme stock solution and mix by pipetting up and down or by shaking gently. Incubate solution 20–30 min in water bath at 35–37°C. Correct for additional dilution by filtering 1.2 mL enzyme-treated suspension.

(**c**) *Powdered foods*.—Thoroughly mix test sample using sterile spoon or spatula. Prepare 1:10 dilution by aseptically weighing 10 g test portioin into sterile wide-mouth, screw-top bottle. Add 90.0 mL peptone, **B**(**g**), or PT, **B**(**h**), diluent (*see* Table **997.11B**) and shake rapidly 25×, each shake an up-and-down movement of ca 30 cm in ≤7 s. Let bubbles escape. If enzyme treatment is needed (*see* Table **997.11B**), combine 5.0 mL 1:10 dilution with 1.0 mL appropriate enzyme stock solution and mix by pipetting up and down or by shaking gently. Incubate solution 20–30 min in water bath at 35–37°C. Correct for additional dilution by filtering 1.2 mL enzyme-treated suspension.

(**d**) *Other foods*.—Prepare 1:10 dilution by aseptically weighing 10 g test portion into sterile blender jar. Add 90.0 mL peptone, **B**(**g**), or PT, **B**(**h**), diluent (*see* Table **997.11B**), and blend 2 min at low speed (10 000–12 000 rpm). If enzyme treatment is needed (*see* Table **997.11B**), combine 5.0 mL 1:10 dilution with 1.0 mL appropriate enzyme stock solution and mix by pipetting up and down or by shaking gently. Incubate solution 20–30 min in water bath at 35–37°C. Correct for additional dilution by filtering 1.2 mL enzyme-treated suspension.

D. Analysis

[*See* Figures **986.32A** and **B** (*see* 17.2.05).] Turn on vacuum source. Place sterile filtration unit on manifold or vacuum flask. Open clamp A. Rotate back funnel portion C. Aseptically place sterile filter, **B**(**a**), on surface of base D. Rotate funnel forward. Clamp shut by sliding jaws L of stainless steel clamp over entire length of flanges B that extend from both sides of funnel C and base D, and rotating moving arm K into horizontal (locked) position.

Aseptically add ca 15–20 mL sterile H_2O to funnel. Pipet 1.0 mL 1:10 dilution (or appropriate volume of enzyme-treated suspension) into funnel. Apply free end of vacuum tubing E to suction hole F to draw liquid through pre-filter mesh G. Aseptically add additional 10–15 mL sterile H_2O to funnel and draw through mesh as before. Close clamp A to direct vacuum to base of filtration unit and draw liquid through filter.

Open clamp A. Rotate moving arm K of stainless steel clamp into unlocked (ca 45° angle) position and slide jaws L off flanges B. Rotate back funnel C.

E. Presumptive Result

Place filter on surface of SD-39 agar plate. Incubate 24 ± 2 h at 44.0–44.5°C. Do not use ultraviolet lamp to examine filter. Examine filter in natural light, under incandescent lighting, or under fluorescent lighting, for presence of orange or pink colonies (presumptive positive *E. coli* O157:H7). Other organisms will produce green or yellow colonies. Occasionally, a mixed square containing both a pink and a green colony will appear purple. This should also be considered presumptive positive.

Total the number of squares containing one or more presumptive positive colonies as described above. This is the presumptive score.

F. Confirmed Result

Select up to 5 presumptive positive squares and subculture to TSA, **B**(**k**), and TSTB, **B**(**l**). Incubate 18–24 h at 36 ± 1°C. Examine TSA for purity and purify, if necessary, by inoculating fresh TSA and TSTB with an isolated colony and incubating as above. Examine purified cultures for pigmentation, and discard any pigmented isolates

Table 997.11B Diluents and enzyme treatments for foods[a]

Food	Diluent	Enzyme
Raw ground beef	PT[b]	None
Cooked ground beef	PT	None
Raw ground turkey meat	PT	None
Cooked ground turkey meat	PT	None
Raw ground pork	PT	None
Cooked ground pork	PT	None
Raw ground lamb	PT	None
Raw fermented sausage	PT	None
Raw milk	PT	Papain
Pasteurized milk	PT	Papain
Ice cream	Peptone[c]	Papain + AMG[d]
Cottage cheese	PT	Papain
Cheddar cheese	PT	Papain
Cream cheese	PT	Papain
Liquid infant formula	Peptone	Papain
Apple cider	Peptone	None
Pasteurized whole egg	Peptone	APUG[e]
Mayonnaise	Peptone	Papain + AMG
Dry infant formula	Peptone	Papain + cellulase

[a] Based on analysis of 1.0 mL 1:10 dilution. Foods tested at dilutions of 1:100 or higher do not usually need enzyme treatment. Also refer to AOAC Official Methods **986.32** (*see* 17.2.05) and **995.21** (*see* 17.2.08) for recommended enzyme treatments of foods not listed in this table.

[b] PT = 0.1% peptone + 1.0% Tween 80 diluent.

[c] Peptone = 0.1% peptone diluent.

[d] AMG = Amyloglucosidase enzyme.

[e] APUG = Alkaline protease enzyme.

without performing serological testing. If all 5 isolates are pigmented, report as less than (reciprocal of dilution factor) *E. coli* O157:H7/g or mL.

(**a**) *Slide agglutination test*.—Perform test on nonpigmented isolates as follows:

(*1*) Mark off 2 areas on clean glass microscope slide using wax pencil.

(*2*) Deposit drop of sterile saline into each area on slide.

(*3*) Emulsify material from TSA culture in both drops of saline, using enough material to make uniform turbid emulsion.

(*4*) Add one drop O157 antiserum to one of culture drops.

(*5*) Rock slide back and forth for ca 1 min, watching for agglutination to occur. Agglutination should only be recorded as positive if control drop remains smoothly emulsified.

Record agglutination result and discard any isolates that do not react in slide agglutination test with O157 antiserum. If all nonpigmented isolates are also not reactive to O157 antiserum, report as less than (reciprocal of dilution factor) *E. coli* O157:H7/g or mL.

(**b**) *H7 tube agglutination test*.—Perform on nonpigmented O157 positive isolates as follows (always include known positive culture with each series of tube agglutination tests to confirm correct functioning of reagents):

(*1*) Inactivate 1.0 mL TSTB culture by adding 20 μL 37% formaldehyde (commercial full-strength solution). If 20 μL pipet is not available, prepare 1:5 dilution of 37% formaldehyde in saline and transfer 0.1 mL diluted formaldehyde into TSTB culture.

(*2*) Dilute H7 antiserum in saline according to manufacturer's directions. Transfer 0.5 mL diluted H7 antiserum into sterile 13×100 mm glass culture tube.

(*3*) Add 0.5 mL inactivated TSTB culture from (*1*) to the diluted antiserum (*2*). Shake tube gently to mix.

(*4*) Add 0.5 mL saline to remaining 0.5 mL inactivated TSTB culture (*1*) as a negative control. Shake tube gently to mix.

(*5*) Incubate both tubes 60–90 min in water bath at 50 ± 1°C.

(*6*) Carefully remove the tubes from water bath and, without shaking tubes or disturbing contents, examine for evidence of agglutination in tube containing H7 antiserum, but not in negative control tube. If agglutination is present, record as positive result. If result is negative, proceed to (**c**).

(**c**) *Motility test*.—Perform only if H7 agglutination test, (**b**), is negative. Proceed as follows:

(*1*) Inoculate tube of motility agar by stabbing in center of tube. Incubate 18–24 h at 35°C.

(*2*) Examine for evidence of motility. Motile cultures will grow in a diffuse pattern away from area of the stab, both within agar and over agar surface. Nonmotile cultures will grow only in immediate vicinity of the stab.

(*3*) Subculture motile cultures into fresh TSTB medium. Incubate 18–24 h at 35°C, and repeat H7 agglutination procedure, (**b**).

(*4*) Reinoculate nonmotile cultures into fresh motility agar. Incubate and examine as in (*1*) and (*2*).

If culture is still nonmotile after 3 attempts to induce motility, record as "nonmotile" for H7 result. If culture is motile but H7 agglutination test is still negative after 3 attempts, record as negative H7 result.

G. Interpretation of Results

(*1*) Isolates that are unpigmented on TSA and give positive reaction to both O157 and H7 antisera are confirmed as *E. coli* O157:H7.

Determine proportion of the original 5 isolates that meet these criteria (e.g., 3/5), and multiply this fraction by presumptive score, **E**, to obtain confirmed score. Convert confirmed score to most probable number (MPN) index as follows:

$$\text{MPN} = 1600 \times \log_e [1600/(1600 - x)]$$

where x = number of *E. coli* O157:H7 positive squares.

Multiply MPN by reciprocal of dilution factor, round to 2 significant figures, and report as *E. coli* O157:H7/g or mL.

(*2*) Isolates that are unpigmented on TSA, give positive reaction to O157 antiserum, and are nonmotile are confirmed as *E. coli* O157:NM. Determine proportion of the original 5 isolates that meet these criteria and multiply this fraction by presumptive score, **E**, to obtain the confirmed score. Convert confirmed score to MPN index as follows:

$$\text{MPN} = 1600 \times \log_e [1600/(1600 - x)]$$

where x = number of *E. coli* O157:NM positive squares.

Multiply MPN by reciprocal of dilution factor, round to 2 significant figures, and report as *E. coli* O157:NM/g or mL.

Reference: *J. AOAC Int.* **81**, 403(1998).

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