Confirmation Protocol for *E. coli* O157:H7

Introduction

The following protocol is used by Hygiena to recover *E. coli* O157:H7 from beef samples that were enriched according to the BAX® System method. The protocol is taken from the USDA-FSIS and Dyna Beads Product Information page for analyzing raw and ready-to-eat meat products. https://www.fsis.usda.gov/wps/wcm/connect/51507fdb-dded-47f7-862d-ad80c3ee1738/MLG-5.pdf?MOD=AJPERES and https://www.thermofisher.com/order/catalog/product/71003?SID=srch-srp-71003

Many culture methods begin with a highly selective enrichment broth to promote growth of *E. coli* O157:H7 and suppress competing background flora. Because the BAX® system amplifies only those DNA fragments that are unique to *E. coli* O157:H7, it is not distracted by competing flora and can reliably detect the target in less time and with less selective enrichment. This difference in enrichments, however, can make it difficult to confirm positive BAX® system results with traditional culture methods, especially when the sample contains low levels of *E. coli* O157:H7.

Various other factors over which the BAX® system has no control can also affect confirmation results. Low levels of *E. coli* O157:H7 in a sample may be lost during refrigeration of the enrichment. Amplicon contamination in a lab might yield an initial positive result that does not confirm. Electrical noise, mislabeling and other errors can sometimes yield a non-confirmable positive result.

Although this confirmation protocol should be effective for most samples, some particularly difficult samples may require additional steps. Contact technical support (800-863-6842) for more information.

Equipment and Supplies

- Incubators, static (36°C (+/-1°C) and 42°C (+/-1°C))
- Micropipettors to deliver 15-1000 µl with sterile disposable filtered micropipette tips
- Mechanical pipettor with 1.0 ml, 5.0 ml, 10.0 ml sterile pipettes
- Inoculating loops, “hockey sticks” or spreaders
- Vortexer (Vortex-Genie 2 #G560 or equivalent)
- UV lamp (366 nm) (Merck #1.13203.0001 or equivalent).
- Sterile disposable 12 x 75 mm polypropylene tubes (e.g. Fisher # 14-956-1B)

- Microcentrifuge and sterile 1.5 ml microcentrifuge tubes
- Sterile 50 ml conical tubes (e.g. Falcon® # 2070) or sterile bottles
- Sterile 40 µm Cell Strainer (Falcon® # 2340)
- MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02)
- OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09)
- Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03)
- Tray, autoclavable, approximately 130 mm x 83 mm (e.g. VWR # 62663-222) for use with the OctoMACS®

Media, Reagents and Cultures

- Modified Tryptone Soya Broth (mTSB)
- Modified Tryptone Soya Broth with Novobiocin (mTSB+n)
- Modified Rainbow® Agar (mRBA) [Rainbow® Agar O157 Biolog Inc., Hayward California, 94545] containing 5.0 mg/L novobiocin, 0.05 mg/L cefixime trihydrate and 0.15 mg/L potassium tellurite
- Tryptic soy agar with 5% sheep blood (SBA)
- SOB + A Medium
- E Buffer, approximately 7 ml per sample (Buffered Peptone Water, Bovine Albumin Sigma # A7906-500G and Tween-20®)
- Disinfectant (Lysol® I. C., 2.0%)
- Dynal® # 710.04 anti-E. coli O157 antibody-coated paramagnetic beads (Dynal Inc., Lake Success, NY 11042)
- *E. coli* O157:H7 strain 465-97 (positive control used throughout method)
- *E. coli* ATCC strain 25922 (optional negative control for the latex agglutination assay)
- Selective agars (Rainbow® Agar O157 [Biolog Inc., Hayward California, 94545] containing 10 mg/L novobiocin plus 0.8 mg/L potassium tellurite, CT-SMAC agar or equivalent).
- **Note:** *Since not all E. coli O157:H7 are tellurite-resistant, it is important to include other selective plating media, such as Cefixime and Rhamnose supplemented Sorbitol MacConkey agar (CR-SMAC) (Oxoid Inc., Ontario, Canada) or Fluorocult E. coli O157:H7 Agar (Merck KgaA, Darmstadt, Germany).*
- Cefsulodin (Sigma #C8145)

- Blood agar - Tryptic soy agar with 5% sheep blood.
- Dynabeads® anti-*E. coli* O157:H7 antibody-coated paramagnetic beads (Thermo Fisher Scientific
- #71003 - 50 tests or #71004 - 250 tests)
- *E. coli* O157:H7 latex agglutination test kit (REMEL RIM® E. coli O157:H7 Latex Test Kit or equivalent).

Procedure

Note: *Store the stock vial of Dynabeads® at 2-8ºC, but make sure that the reagents and sample enrichments are pre-warmed to room temperature (>18ºC) before use to ensure optimal recovery.*

Note: *Steps a.- i. may be performed in a sequence that is convenient to the laboratory personnel.*

a. Prepare E Buffer by mixing 0.5 g Bovine Albumin and 50 µl Tween-20® into 100 ml Buffered Peptone Water (BPW). Filter sterilize (0.2 µm) and store at 2-8°C.
b. Remove mRBA plates from 2-8°C storage, allowing 4 plates for each screen-positive culture and each control, with the exception of 2 plates for the fluorescent *E. coli* O157:H7 control. Be sure that plates have no visible surface moisture at the time of use. If necessary, dry plates (e.g. for up to 30 minutes in a laminar flow hood with the lids removed) prior to use. Dried plates that are not used should be labeled "dried", placed in bags and returned to 2-8°C.
c. Remove a bottle of E Buffer from 2-8°C storage. Decant 7 ml of E Buffer for each culture and each control into a sterile tube or bottle and allow it to warm to at least 18oC. (Return the stock E Buffer to 2-8°C.)
d. For each positive control and screen-positive culture to be analyzed, keep in order and label 50 ml conical centrifuge tubes so that the positive control is first, followed by all cultures. Maintain this order for subsequent steps.
e. For each positive control and screen-positive culture, label two sterile 1.5 ml microcentrifuge tubes (for step g and step t), one 50 ml conical centrifuge tube and four 12 x 75 mm capped tubes (one for step p). For each set of 12 x 75 mm tubes, label one tube and add 0.9 ml E Buffer to three of the four tubes (step q).
f. Prepare the Dynal® #710.04 E. coli O157:H7 immunomagnetic bead suspension by

following Table 1 below. Be sure to include the positive control in the total number of cultures. Use the bead suspension immediately (step g), or hold at 2-8°C. Return the stock vial of Dynal® #710.04 E. coli O157:H7 immunomagnetic beads to 2-8°C.

g. Vortex the bead solution briefly (2-3 seconds), then add 50 µl to a labeled microcentrifuge tube (from step e), one for the control and screen-positive culture. Use immediately or hold these tubes at 2-8°C.

h. Place a 40 µm Cell Strainer on a labeled 50-ml conical centrifuge tube (from step e). Pipet 5 ± 1 ml of each control and enrichment culture into the respective Cell Strainer and collect at least 1.0 ml of filtrate.

i. Do not proceed with more than the number of tubes that the OctoMACS® magnet(s) will hold. Transfer 1.0 ml of a filtrate (step h) to the corresponding microcentrifuge tube containing the immunomagnetic bead suspension (step g) and place in the clips of the LabQuake® tube agitator. Rotate the tubes for 10-15 min at 18-30°C.

j. Attach the OctoMACS® Magnet to the Multistand.

k. Position a tray on the base of the Multistand so that it will collect the filtrate passing through the columns. Add approximately 300 ml of 2% Lysol® disinfectant to cover the bottom of the tray.

l. Label and place the appropriate number of Large Cell Separation columns on the OctoMACS® Magnet. Insert columns from the front making sure the column tips do not touch any surfaces. Leave the plungers in the bags at this time to maintain sterility.

m. Transfer at least 0.5 ml E Buffer to the top of each column and let the buffer run through.

n. Resuspend, then transfer each culture and control from step i. to its corresponding column.

o. After a culture or control has drained through, wash the column by applying 1.0 ml of E Buffer to each column and allow to drain. Repeat 3 more times for a total of 4 washes.

p. After the last wash has drained, remove the column from the OctoMACS® Magnet

and insert the tip into an empty labeled 12 x 75 mm tube (from step e.). Apply 1.0 ml of E Buffer to the column, and using the plunger supplied with the column, immediately flush out the beads into the tube. Use a smooth, steady motion to avoid splattering. Cap the tubes. Repeat this for each column. If the OctoMACS® magnet is to be used for a second set of cultures, it must be decontaminated as described in step u, below. Repeat steps j-s for the additional cultures.

q. Make a 1:10 dilution of each treated bead suspension by adding 0.1 ml of the bead suspension to a 12 x 75 mm labeled tube containing 0.9 ml E-Buffer. Make a 1:100 dilution by adding 0.1 ml of the 1:10 dilution to a 12 x 75 mm labeled tube containing 0.9 ml E Buffer.

r. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (1:10 dilution and 1:100 dilutions) onto a labeled mRBA plates. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate.

s. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 ± 2°C.

t. OPTIONAL Acid Treatment: For each sample, transfer 450 µl of the undiluted bead suspension (MACS column eluant) to an empty labeled microcentrifuge tube. Add 25 µl of 1N hydrochloric acid (HCl) to this bead suspension and vortex briefly. This will bring the pH to 2.0-2.5 using E-buffer. Note: The fluorescent E. coli O157:H7 control sample is excluded as it does not grow following acid treatment.

u. Place the microcentrifuge tubes containing the acid treated suspension on a LabQuake® Agitator and rotate tubes for 1 hour at 18-30°C temperature.

v. After 1 hour, dilute the suspension by adding 475 µl of E-buffer.

w. Vortex briefly to maintain beads in suspension and plate 0.1 ml of the neutralized suspension onto a labeled mRBA plate. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate.

x. Add 0.1 ml of the suspension to a labeled tube containing 0.9 ml E-buffer and vortex briefly. This shall represent a 1:10 dilution of the acid-treated cell suspension. Plate

0.1 ml of the diluted suspension onto an appropriately labeled mRBA plate.

y. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 ± 2°C.

z. Optional: Streak *E. coli* ATCC strain 25922 to TSA with 5% Sheep's Blood Agar for use as a latex negative control.

aa.. Decontaminate the OctoMACS® Magnet by applying 2% Lysol® I. C. disinfectant directly to the surface. After approximately ten minutes, rinse with deionized or tap water. Allow the unit to air-dry or use absorbent paper towels to dry the unit.