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Notice of Change

Chapter **new**, revised**,** or archived: MLG 5C Appendix 3.00

Title: PCR Platform Instructions, Data Analysis, and Control Results Interpretation for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-time PCR Assay

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Description and purpose of change(s):

PCR Platform Instructions, Data Analysis, and Control Results Interpretation for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-time PCR Assay

Procedure Outline

A3 5C.1 Introduction
A3 5C.2 Safety Precautions
A3 5C.3 Equipment, Reagents and Media
- A3 5C.3.1 Equipment and Materials
- A3 5C.3.2 Media and Reagent

A3 5C.4 Quality Control
- A3 5C.4.1 General
- A3 5C.4.2 Sample Enrichment Controls
- A3 5C.4.3 DNA Extraction Control Preparation
- A3 5C.4.4 PCR Controls
- A3 5C.4.5 IMS Plating Controls

A3 5C.5 Sample Preparation and Primary Enrichment
A3 5C.6 Screening Procedure using Real-time PCR
- A3 5C.6.1 DNA Extraction from Overnight Enrichments
- A3 5C.6.2 Real-time PCR Procedure

A3 5C.7 Isolation Procedure
- A3 5C.7.1 Immunomagnetic Separation and Culture Plating

A3 5C.8 Identification and Confirmation
- A3 5C.8.1 Serological Agglutination Procedure
- A3 5C.8.2 Optional Presumptive PCR Assay

A3 5C.9 Culture Storage
A3 5C.10 ABI® 7500 FAST PCR Platform Instructions
A3 5C.11 Analyzing the Data
A3 5C.12 Interpretation of Control Results
A3 5C.13 Selected References

A3 5C.1 Introduction

This procedure provides instructions for using the ABI® 7500 FAST PCR Platform for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges. Photos provided are from software version 2.04/2.05. Details include analysis of the data and interpretations for the control results.

A3 5C.2 Safety Precautions

Similar to *E. coli* O157:H7, non-O157 STEC serotypes are human pathogens with a low infectious dose. The use of gloves, protective laboratory coats and eye protection is for all post enrichment viable culture work. Work surfaces must be disinfected prior to and immediately after use. Laboratory personnel must abide by CDC guidelines for manipulating Biosafety Class II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. All available Safety Data Sheets (SDS) shall be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the materials should read all SDS sheets.

A3 5C.3 Equipment, Reagents and Media

A3 5C.3.1 Equipment and Materials

a. Balance, sensitivity ± 0.1 g
b. Incubators, static 42 ± 1°C and 35 ± 2°C
c. Micropipettors for culture plating to deliver volumes ranging from 15-1000 µl with sterile disposable filtered tips
d. Micropipettors for Real-time PCR to deliver volumes ranging from 0.50 µl to 1,000.00 µl with sterile, filtered tips
e. Applied Biosystems® ABI 7500 FAST PCR platform
f. Applied Biosystems® 7500 FAST MicroAMP™ Fast Optical 96-well Reaction Plate with Barcode (Part# 4346906)
g. Applied Biosystems® 7500 FAST MicroAMP™ Splash Free 96-Well Base (Part# 4312063)
h. Applied Biosystems® 7500 FAST Optical Adhesive Film (Part# 4311971)
i. Applied Biosystems® TaqMan® Environmental Master Mix 2.0 (Part# 4396838)
j. VITEK® 2 system
k. GN cards for VITEK® 2 system (bioMerieux Vitek, Inc.)
l. Heating block (95-99°C) or thermocycler for DNA preparation step
m. Vortexer
n. Centrifuge that holds microcentrifuge tubes and is capable of speeds up to 16,000 x g
o. Centrifuge plate adapter for the centrifugation of 96-well PCR plates
p. Disposable, sterile pipettes for volumes 1.0 ml and for 5.0 ml.

q. Sterile, inoculating loops, "hockey sticks" or spreaders, and needles
r. Rotating tube agitator with clips to hold microcentrifuge tubes
s. Sterile, disposable 12 x 75 mm polypropylene or polystyrene tubes
t. Sterile microcentrifuge tubes (1.5 - 2.0 ml)
u. Sterile 50 ml conical tubes
v. Sterile 40 µm Cell Strainer
w. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02)
x. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09)
y. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03)
z. Tray, autoclavable, approximately 130 mm x 83 mm for use with the OctoMACS®
aa. Sterile filter or non-filter bags
bb. Optical density reader

A3 5C.3.2 Media and Reagents

a. Modified Tryptone Soy Broth (mTSB)
b. 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
c. Cefixime trihydrate
d. Tryptic soy agar with 5% sheep blood [Sheep Blood Agar (SBA)]
e. 1.0 N Hydrochloric Acid (HCl)
f. Physiological saline solution (0.85% NaCl)
g. 1X Tris-EDTA (TE) Buffer
h. E Buffer, approximately 7 ml per sample (See Media and Reagents Appendix 1, Buffered Peptone Water, Bovine Albumin Sigma and Tween-20®)
i. Disinfectant (Lysol® I. C., 2.0%)
j. SDIX RapidChek® CONFIRM STEC Immunomagnetic Separation (IMS) Kit with anti-O26 antibody-coated paramagnetic beads, anti-O103 antibody-coated paramagnetic beads, anti-O111 antibody-coated paramagnetic beads, anti-O145 antibody-coated paramagnetic beads, anti-O45 antibody-coated paramagnetic beads, and anti-O121 antibody-coated paramagnetic beads
k. RNase free, DNase free PCR Certified Water

l. TaqMan® Environmental Master mix 2.0, Applied Biosystems Catalog Number 4396838
m. Biochemical test kit and system, GN cards (VITEK® 2 system, bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395)
n. Primer and probe sequences detailed in MLG Chapter 5C Appendix 4 Primer and Probe Sequences and Reagent Concentrations for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-time PCR Assay, Integrated DNA Technologies (Coraville, Iowa)
o. Abraxis non-O157 STEC Latex Agglutination Test (LAT) Kits or equivalent specific for serogroups O26, O45, O103, O111, O121 and O145

A3 5C.4 Quality Control

A3 5C.4.1 General

a. Unless otherwise stated, weight and volume ranges and minutes have a tolerance of ± 2%.
b. All media, plates and buffers shall be warmed to 18-35°C prior to use.
c. The top six non-O157 STEC control strains shall meet the following genetic characteristics: *stx*+ and *eae*+. Such strains can be obtained through reference culture collection centers including but not limited to the American Type Culture Collection (ATCC), the STEC Center at The Michigan State University and the *E. coli* Reference Center at The Pennsylvania State University. Non-O157 strains (*stx*+, *eae*+) must be used by FSIS Laboratories to prepare the DNA template positive PCR control. However, for safety considerations, toxin-attenuated or toxin-negative strains that have an appearance on mRBA typical of the non-O157 STEC may be used as controls on plating media for serological agglutination testing. The following non-O157 STEC control strains shall be used when stated in the method:

    i. *E. coli* O26, which shall be *stx* positive and *eae* positive
    ii. *E. coli* O45, which shall be *stx* positive and *eae* positive
    iii. *E. coli* O103, which shall be *stx* positive and *eae* positive
    iv. *E. coli* O111, which shall be *stx* positive and *eae* positive
    v. *E. coli* O121, which shall be *stx* positive and *eae* positive

vi. *E. coli* O145, which shall be *stx* positive and *eae* positive

Note: In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses.

A3 5C.4.2 Sample Enrichment Controls

Include with each sample batch, a positive growth control (*E. coli* O157:H7 strain other reference strain that is stx+, eae+) inoculated into a meat matrix free of the target analyte, and an uninoculated media (mTSB) control.

A3 5C.4.3 DNA Extraction Control Preparation

Include with each sample batch; extract DNA from the positive growth control sample using the same extraction method as used to process the sample batch. This shall serve as positive DNA extraction controls.

A3 5C.4.4 PCR Controls

a. ***stx/eae* screen PCR**
   - DNA template from bioluminescent *E. coli* O157:H7 (DNA extraction *stx/eae* positive control)
   - DNA template from a cocktail of top six STEC cultures (PCR positive control) analyzed in triplicate
   - No Template Control (NTC)

b. **Serogroup-specific screen PCR (O antigen gene cluster)**
   - DNA template from a cocktail of top six STEC cultures (PCR positive control) analyzed in triplicate
   - NTC

c. **Optional *stx/eae* presumptive PCR / *stx/eae* confirmatory PCR**
   - DNA template from a cocktail of top six STEC cultures (PCR positive control) analyzed in triplicate
   - NTC

d. **Optional serogroup-specific presumptive PCR (O antigen gene cluster) / Serogroup-specific confirmatory PCR (O antigen gene cluster)**
   - DNA template from a cocktail of top six STEC cultures (PCR positive control) analyzed in triplicate
   - NTC

To prepare PCR positive control template for the ABI® 7500 FAST, grow the top six STEC cultures on SBA and incubate at 35 ± 2°C for 18-24 h. Colonies shall be used to create a culture suspension in PCR certified water corresponding to approximately 10^9 CFU/ ml. In one tube, 1.0 ml from each suspension shall be added to 4.0 ml of PCR certified water to create a 10.0 ml cocktail of all six strains. This will provide approximately a 10^8 CFU/ml cocktail using each strain. One hundred microliter aliquots of the suspension are then transferred to PCR tubes or microcentrifuge tubes and heated at 95-99°C for 10 minutes on a thermocycler or heating block. The tubes shall be centrifuged at 10,000 x g for 3 minutes to pellet cellular debris. The supernatant shall be used as the PCR positive control for all PCR assays. DNA control template can be prepared as a batch, transferred to smaller volume tubes, and stored at ≤-20°C for 1 year. Additional information on controls and troubleshooting based on control reactions is included in Section A3 5C.12 Interpretation of Control Results.

A3 5C.4.5 IMS Plating Controls

Streak the serogroup(s) of interest (based on serogroup-specific PCR results) onto mRBA and incubate along with the samples that have been treated with the IMS procedure.

A3 5C.5 Sample Preparation and Primary Enrichment

Follow MLG 5C for sample preparation and enrichment details.

A3 5C.6 Screening Procedure using Real-time PCR

A3 5C.6.1 DNA Extraction from Overnight Enrichments

a. Transfer 1.4 ± 0.1 ml of enrichment to a sterile microcentrifuge tube.
b. Centrifuge at 1,500 x g for 1 minute to pellet large debris (supernatant will still not be clear at this low speed but should no longer have large particles of meat).
c. Transfer the supernatant to a new sterile microcentrifuge tube. It is essential to ensure that none of the pelleted debris is carried over with the supernatant.
d. Centrifuge the supernatant at a setting of 10,000 *x* g for 5 minutes.

e. Discard the supernatant and add 500 ± 50 µl of 0.85% saline solution (PSS) to the pellet. Resuspend the pellet by either vortexing or using the pipet tip.
f. Centrifuge the tubes at a setting of 10,000 x g for 3 minutes. Remove the supernatant as completely as possible without disturbing the pellet.
g. Resuspend the pellet in 90 ± 9.0 µl of 1X TE Buffer. Use the pipet tip to thoroughly resuspend the pellet.
h. Heat at 97 ± 2ºC for 15 ± 1 minutes. Allow to cool at room temperature for approximately 2 minutes.
i. Centrifuge the tubes at a setting of 16,000 x g for 4 minutes.
j. Transfer the supernatant to a new sterile microcentrifuge tube without disturbing the pellet.
k. If the template is to be used within 24 hours, store at 2-8°C. For long-term storage, store at ≤ -20º C.

A3 5C.6.2 Real-time PCR Procedure

Following DNA extraction from 15-24 h incubated enrichments, Real-time PCR will be used as a screen for the presence of *stx* toxin genes and the *eae* intimin gene. Samples with positive results (both gene targets) on the initial *stx/eae* PCR screen will be tested by three additional Real-time PCR assays to determine if a top six serogroup (O26, O45, O103, O111, O121 or O145) is present.

Note: The Real-time PCR assay described in this method has been optimized and validated specifically for the ABI® 7500 FAST. Use of other Real-time PCR platforms may require optimization with other probe quencher and reporter dyes. Additionally, the ABI® Environmental Mastermix has been validated and optimized for use on the ABI® 7500 FAST and would require additional optimization with reagent volumes and assay cycling parameters if using other Real-time PCR platforms. The assays have been validated for use with the Standard Chemistry, not the Fast Chemistry, on the ABI® 7500 FAST.

a. Set up the Real-time PCR assay on the ABI® 7500 FAST as described in Section A3 5C.10 ABI® 7500 FAST PCR Platform Instructions

b. For the initial *stx* and *eae* PCR screen, prepare master mix with component volumes described in MLG 5C Appendix 4 Primer and Probe Sequences and Reagent Concentrations for non-O157 STEC Real-time PCR Assay.
c. Once the PCR master mix is prepared, aliquot 20.0 µl of master mix for each reaction into the wells of a sterile, 7500 FAST MicroAMP™ Fast Optical 96-well Reaction Plate or ABI® 7500 well strips. Keep the 96 well plate on the ABI® 7500 96-Well Base to ensure the PCR plate does not touch the bench.
d. Add 5.0 µl of DNA template from the DNA extraction step to the appropriate well. Follow Section A3 5C.4.4 for PCR controls to use for quality control. Seal the plate with optical adhesive film and centrifuge the plate briefly at approximately 2000 x g for 30 seconds prior to loading onto the ABI® 7500 FAST platform. This will ensure that no air bubbles are at the bottom of the wells.
e. Load the plate into the ABI® 7500 FAST and begin the assay.
f. At the end of the run, view the results to determine if samples are positive for *eae* and *stx* targets. Prior to the end of the run (45 cycles), a gene target is considered positive when the fluorescence crosses over the threshold that is auto-determined by the instrument. The auto-threshold determination is the default setting on the ABI® 7500 FAST and is automatically determined based on the mean baseline fluorescence. When the sample crosses the threshold for a particular target, a Cycle Threshold (Ct) value is given by the instrument. When the sample does not cross the threshold for a specific target, the instrument reports the sample as "Undetermined" for that target. The 16S rRNA internal control should be positive for every sample. The no template control (PCR certified water) should not yield a product (Ct>45) but may produce a trace 16S result in the 35-45 cycle threshold range; either case is acceptable. However, amplification of any other target other than 16S, or amplification of 16S below 35 cycles in the no template control well indicates cross-contamination, resulting in an invalid run. Consult Section A3 5C.4 Quality Control for additional controls and sample interpretation.

The dye channels for the ABI® 7500 FAST are as follows:
FAM = *stx*
VIC = *eae*
Cy5 = Internal Control (16S rRNA)
See Table 1 for PCR result interpretation.

Table 1. Result interpretation for the *stx* and *eae* PCR assays.

	stx PCR Negative	*stx* PCR Positive
eae PCR Negative	* Negative * STOP Report Result	* Negative * STOP Report Result
eae PCR Positive	* Negative * STOP Report Result	Continue with Serogroup PCR

Samples negative for *stx* and/or *eae* targets are considered negative for non-O157 STEC. DNA extraction templates from any samples positive for both *stx* and *eae* targets will be further analyzed by PCR amplification of the serogroup specific gene targets using three multiplex Real-time PCR reactions.

g. For the serogroup-specific PCR screen, set up the Real-time PCR assay on the ABI® 7500 FAST as described in A3 5C.10 ABI® 7500 FAST PCR Platform Instructions and prepare master mix with component volumes

described in MLG 5C Appendix 4 Primer and Probe Sequences and Reagent Concentrations for non-O157 STEC Real-time PCR Assay.

h. Once the PCR master mix is prepared, aliquot 20.0 µl of master mix for each reaction into the wells of a sterile, 7500 FAST MicroAMP™ Fast Optical 96-well Reaction Plate or ABI® 7500 well strips. Keep the 96-well plate on the ABI® 7500 96-Well Base to ensure the PCR plate does not touch the bench. Add 5.0 µl of DNA template from the DNA extraction step to the appropriate well. Follow Section A3 5C.4.4 for PCR controls to use for quality control. Seal the plate with optical adhesive film and briefly centrifuge the plate at approximately 2000 x g for 30 seconds prior to loading onto the ABI® 7500 FAST platform. This will ensure that no air bubbles are at the bottom of the wells. Load the plate into the ABI® 7500 FAST and begin the assay.

i. At the end of the run, view the results to determine if samples are positive for the serogroup-specific targets. Prior to the end of the run (45 cycles), a gene target is considered positive when the fluorescence crosses over the threshold that is auto-determined by the instrument. The auto-threshold determination is the default setting on the ABI® 7500 FAST and is automatically determined based on the mean baseline fluorescence. When the sample crosses the threshold for a particular target, a Cycle Threshold (Ct) value is given by the instrument. When the sample does not cross the threshold for a specific target, the instrument reports the sample as "Undetermined" for that target. The no template control (PCR certified water) should not yield a product (Ct>45) but may produce a trace 16S result in the 35-45 cycle threshold range; either case is acceptable. However, amplification of any other target other than 16S, or amplification of 16S below 35 cycles in the no template control well indicates cross-contamination, resulting in an invalid run. The dye channels with their respective targets on the ABI® 7500 FAST are as follows:
FAM = Serogroup O26, Serogroup O45 and Serogroup O145
VIC = Serogroup O103, Serogroup O111 and Serogroup O121
Cy5 = Internal Control (16S rRNA)
The 16S rRNA internal control should be positive for every sample.

j. A sample that is negative for the serogroup targets is considered negative for the top six non-O157 STECs and the analysis for that sample stops. A sample with positive PCR results for *stx, eae* and serogroup(s) is

considered a potential positive and the isolation procedure shall be continued.

A3 5C.7 Isolation Procedure

Samples that are potentially positive by PCR screen results shall be plated onto modified Rainbow Agar (mRBA) following IMS. In the isolation procedure, IMS beads shall be used for the specific serogroup identified by the serogroup PCR reaction (i.e. anti-O26 will be used for samples with screen results positive for O26, anti-O45 for O45 PCR positive reactions, anti-O103 for O103 PCR positive reactions or anti-O121 for O121 PCR positive reactions, anti-O111 for O111 PCR positive reactions and/or anti-O145 for O145 PCR positive reactions). A post-IMS acid treatment step has been added to reduce background flora on the mRBA plate. Following the one hour acid treatment step, samples are diluted 1:1 with E-buffer and 0.1 ml is spread plated onto mRBA. Additionally, the suspension is diluted 1:10 and 0.1 ml is spread plated onto mRBA.

A3 5C.7.1 Immunomagnetic Separation and Culture Plating

a. Remove mRBA plates from 2-8°C storage, allowing 4 plates for each screen-positive culture and one plate for each serogroup control strain. 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.
b. For each screen-positive culture, label two sterile microcentrifuge tubes (for step d and step m), one 50 ml conical centrifuge tube (for step c) and four 12 x 75 mm capped tubes (for steps i and j). For three of 12 x 75 mm tubes, add 0.9 ml E-Buffer and label one tube as 1:10, one tube as 1:100 and one tube as acid 1:10.
c. **Sample preparation from overnight enrichment:** For each serogroup that the sample is positive, transfer approximately 2-5 ml from overnight enrichment through a 40 µm Cell Strainer into a 50 ml conical centrifuge tubes.
d. **Binding of paramagnetic antibody beads to specific serogroup:** Transfer 50.0 µl (or volume recommended by the manufacturer) of appropriate immunomagnetic capture beads determined by the serogroup PCR screen results (O26, O45, O103, O111, O121 or O145) to a sterile,

labeled microcentrifuge tube. Next, add 1 ml of enrichment filtrate to the appropriately labeled tube.

e. Place the microcentrifuge tubes containing enrichments and capture beads on LabQuake® Agitator and rotate tubes for 10-15 minutes at 18-30°C (or time recommended by the manufacturer).
f. For each sample, place one MACS® Large Cell Separation Columns onto the OctoMACS® Separation Magnet. Fill the tray below the separation magnet with disinfectant. Prime each separation column with at least 0.5 ml of E-buffer and allow the liquid to pass completely through before adding sample.
g. **Binding of beads to magnetic columns:** Once the liquid has passed through the column, add the 1.0 ml of enrichment plus IMS beads to each appropriately labeled column and allow liquid to completely pass through.
h. **Wash steps (4X):** Add 1.0 ml of E-buffer to each column allowing the liquid to pass completely through. Repeat 3 more times for a total of 4 washes.
i. **Elution step:** 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. 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.
j. 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.
k. 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.
l. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 ± 2°C.
m. **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.
n. Place the microcentrifuge tubes containing the acid treated suspension on a LabQuake® Agitator and rotate tubes for 1 hour at 18-30°C temperature.

o. After 1 hour, dilute the suspension by adding 475 µl of E-buffer.
p. 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.
q. 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.
r. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 ± 2°C.

A3 5C.8 Identification and Confirmation

Following 20-24 h incubation of mRBA, plates will be examined for colonies that agglutinate with antisera specific for the serogroup of interest. Colony colors from representative strains of each serogroup are listed in MLG 5C Appendix 2 Morphologies of Representative Strains from Top Six non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Grown on mRBA. However, the coloration of colonies described in MLG 5C Appendix 2 may vary based on proximity to other competitor colonies or medium discoloration due to competitor colony growth. Since the morphologies of the targeted STEC colonies may vary widely among strains and serogroups, test at least one colony from each identified colony morphology found on the mRBA plate. Samples that have no growth or only contain agglutination negative colonies on mRBA are negative for non-O157 STEC. Any sample with agglutination positive colonies for the serogroup of interest is a presumptive positive for non-O157 STEC. Agglutination positive colonies shall be streaked onto SBA for confirmation on the following day. An optional PCR assay may be performed on the agglutination positive colonies from mRBA to verify the sample is presumptive positive. Following 18-24 h incubation of the SBA, agglutination-positive colonies shall be confirmed with Real-time PCR and biochemical identification. The confirmatory PCR shall include the *stx* and *eae* multiplex PCR assay and the serogroup specific multiplex PCR assay. If no colony picks isolated from the mRBA confirm by PCR and VITEK® 2, the sample is negative for non-O157 STEC.

A3 5C.8.1 Serological Agglutination Procedure

a. Use an inoculating loop or needle to transfer a portion of an isolated colony from the mRBA plate to serological agglutination reagent. Follow manufacturer's instructions on procedure and interpretation.
Control Reactions: A reference strain from the serogroup of interest plated on mRBA shall be used as the positive culture control. For optional PCR screen from colonies isolated on mRBA, refer to Section A3 5C.8.2 Optional Presumptive PCR Assay.
b. Transfer the remainder of an agglutination positive colony from the mRBA plate onto SBA for further biochemical and genetic confirmation. Streak up to 5 agglutination positive colonies onto SBA plates. Incubate plates at 35 ± 2°C for 18-24 hours.
c. Following SBA incubation, perform the agglutination test again on colonies from the SBA plate.
d. To confirm agglutination-positive colonies using PCR, prepare a DNA template by resuspending an agglutination positive colony from the SBA plate in 50 µl of PCR certified water and heating the suspension for 10 minutes at 95-99°C.
e. From the DNA template preparation, perform the *stx* and *eae* multiplex PCR assay and the serogroup specific PCR assay as previously described in Section A3 5C.6.2. For the serogroup specific PCR assay, it is necessary to only perform the multiplex assay for the serogroup of interest. Note: Each PCR assay shall include a NTC negative control and a positive control for each gene target. Refer to Section A3 5C.4 Quality Control for details on expected controls.
f. Additionally, perform biochemical identification (VITEK® 2) on agglutination positive colonies from the incubated SBA. A positive isolate shall be identified biochemically as *E. coli*.
g. If the isolate is agglutination positive for top six STEC serogroups, PCR positive for *stx, eae,* and top six serogroup genes and biochemically identified as *E. coli*, the sample is positive for non-O157 STEC.
h. If the isolate and any additional colony picks from mRBA are ultimately determined to be PCR negative for *stx, eae,* top six serogroup genes, the sample is negative for non-O157 STEC.

A3 5C.8.2 Presumptive PCR Assay

A PCR test may be performed directly on agglutination positive colonies from the mRBA to verify presumptive positive colonies using the following procedure.

a. Transfer the remainder of an agglutination positive colony from the mRBA plate into 50 µl of PCR certified water (for up to 5 colonies). Inoculate a SBA plate from each suspension and then heat the suspension for 10 minutes at 95-99°C.
b. Incubate inoculated SBA plates at 35 ± 2°C for 18-24 hours.
c. From the DNA template preparation, perform the *stx* and *eae* multiplex PCR assay and the serogroup specific PCR assay as described in Section A3 5C.6.2. For the serogroup specific PCR assay, it is only necessary to perform the multiplex assay for the serogroup of interest. Note: Each PCR assay shall include a NTC negative control and a positive control analyzed in triplicate for each gene target. Refer to section A3 5C.4. Quality Control for details on expected controls.
d. The sample is considered negative if any of the 3 PCR targets (*stx*, *eae* or serogroup) are negative AND the internal control is positive (16S Ct < 30). The inoculated SBA plates can then be discarded. If the internal control is negative (16S Ct >30), analyze colonies from the streaked SBA the next day.
e. If an agglutination positive colony is positive for O group, *stx* and *eae* targets, the sample is considered a presumptive positive for non-O157 STEC. Refer to Section A3 5C.8.1 steps c-h for confirmation of the isolates as non-O157 STEC.

A3 5C.9 Culture Storage

For storage requirements of the fluorescent *E. coli* O157:H7 strain or the currently designated control strain, refer to MLG 5C Detection, Isolation and Identification of Top Seven Shiga Toxin-Producing *Escherichia coli* (STECs) from Meat Products and Carcass and Environmental Sponges. Store other "working" non-O157 *E. coli* stock cultures on nutrient agar slants. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at 35 ± 2°C, and then store them at 2-8°C. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the

opportunity for contamination. For long term storage, freeze cultures using cryo-beads i.e. Cryostor™ or lyophilize.

A3 5C.10 ABI® 7500 FAST PCR Platform Instructions

1) Turn on the ABI® 7500 FAST machine and computer.
2) If you are just looking at the data without the machine on, select "Continue without connecting to machine" to proceed.
3) Open the ABI® software and log on as guest.
4) Choose the advanced set-up button on the left.

5) Enter the name of the file according to the laboratory naming scheme (i.e. MF12345).
6) Ensure that items appear as below [instrument: 7500 FAST; experiment: Quantitation-Standard Curve, reagents-Taqman Reagents), except change the ramp speed from FAST to Standard chemistry.

7) Click on the plate set-up tab on the left.

8) Click on "Define Targets and Samples" tab. Enter the targets for the assays listed in Table 1 and Table 2:

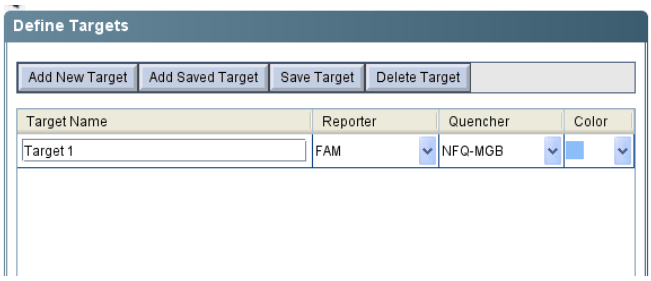


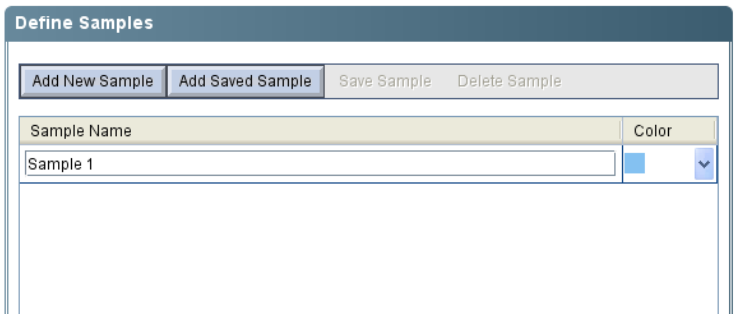
Table 1. *stx* and *eae* Screen and Confirmatory PCR

Target	Dye	Quencher
stx	FAM	None
eae	VIC	None
16S	Cy5	None

Table 2. Serogroup-specific Screen and Confirmatory PCR

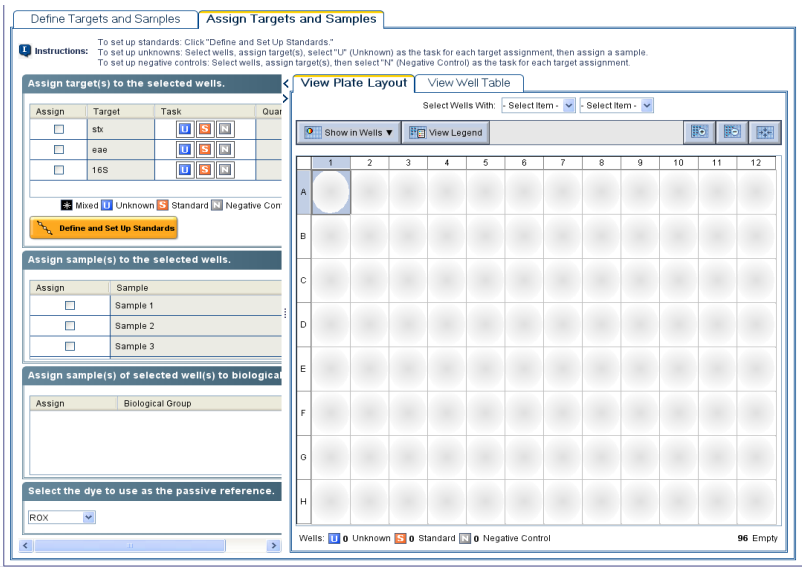
Target	Dye	Quencher
O26	FAM	None
O111	VIC	None
O45	FAM	None
O121	VIC	None
O103	VIC	None
O145	FAM	None
16S	Cy5	None

9) Under "Define Samples", click on the number of samples button and add samples until you have enough for all your assays, including control samples.

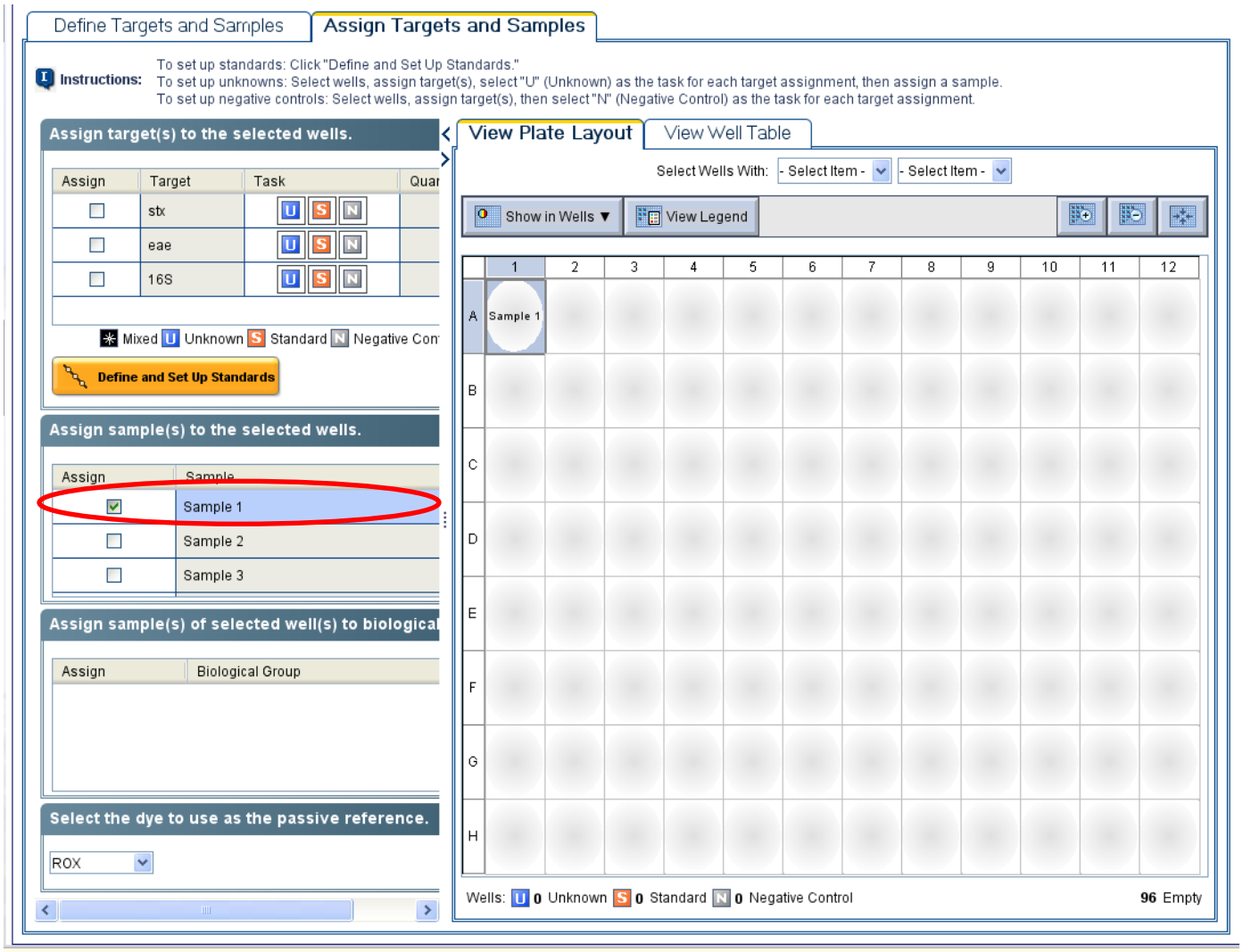


10) After you change the sample name (or do this after the run) and choose any color changes (optional) you want, click on the "Assign Target and Samples" tab.

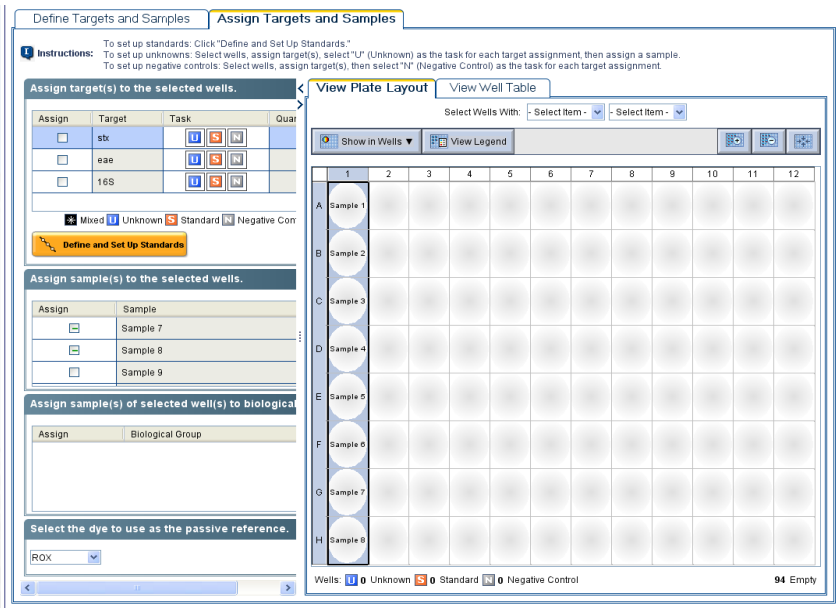
Highlight the well that you want to place the sample in.



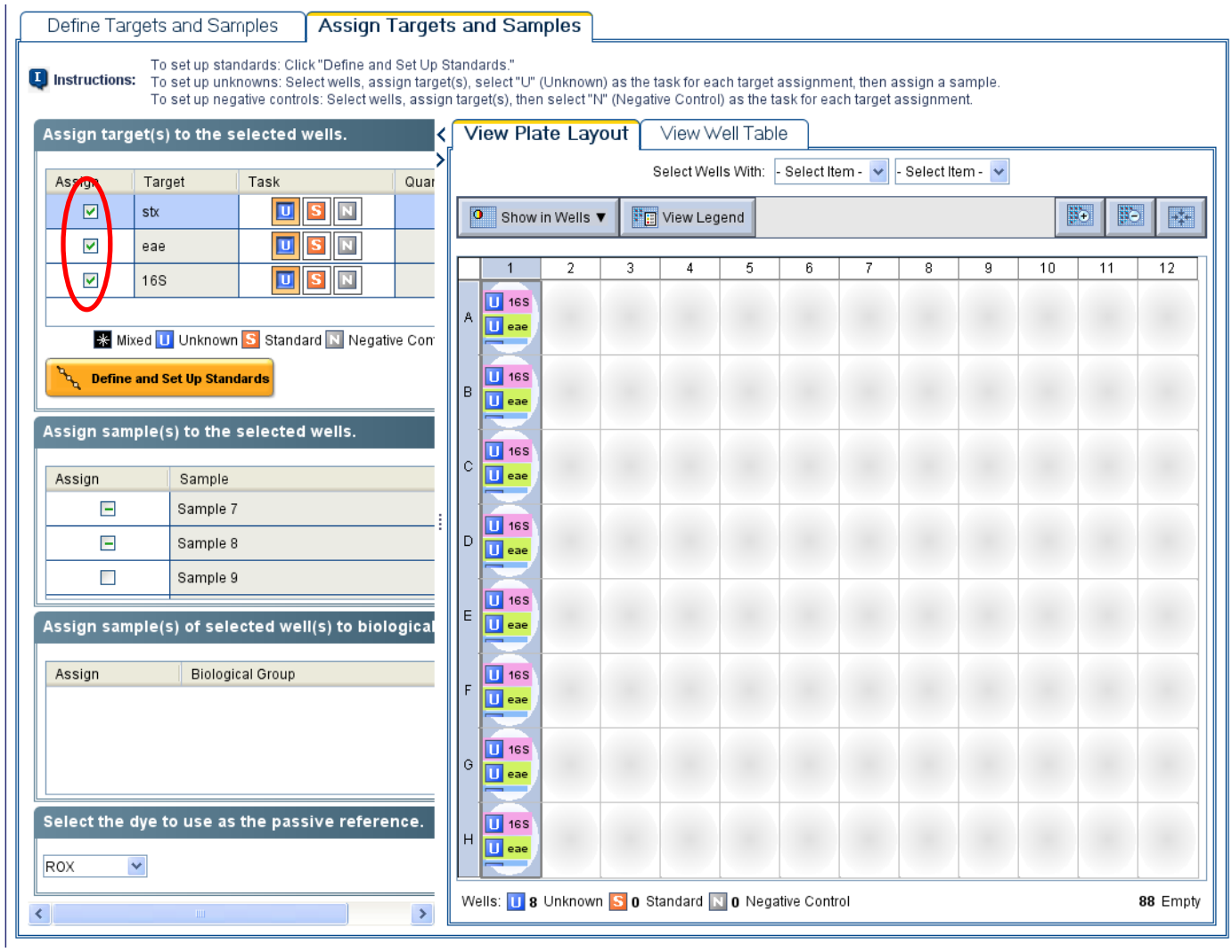
Select the sample from the assign samples box by checking the box. Repeat until all samples are placed in the correct locations.



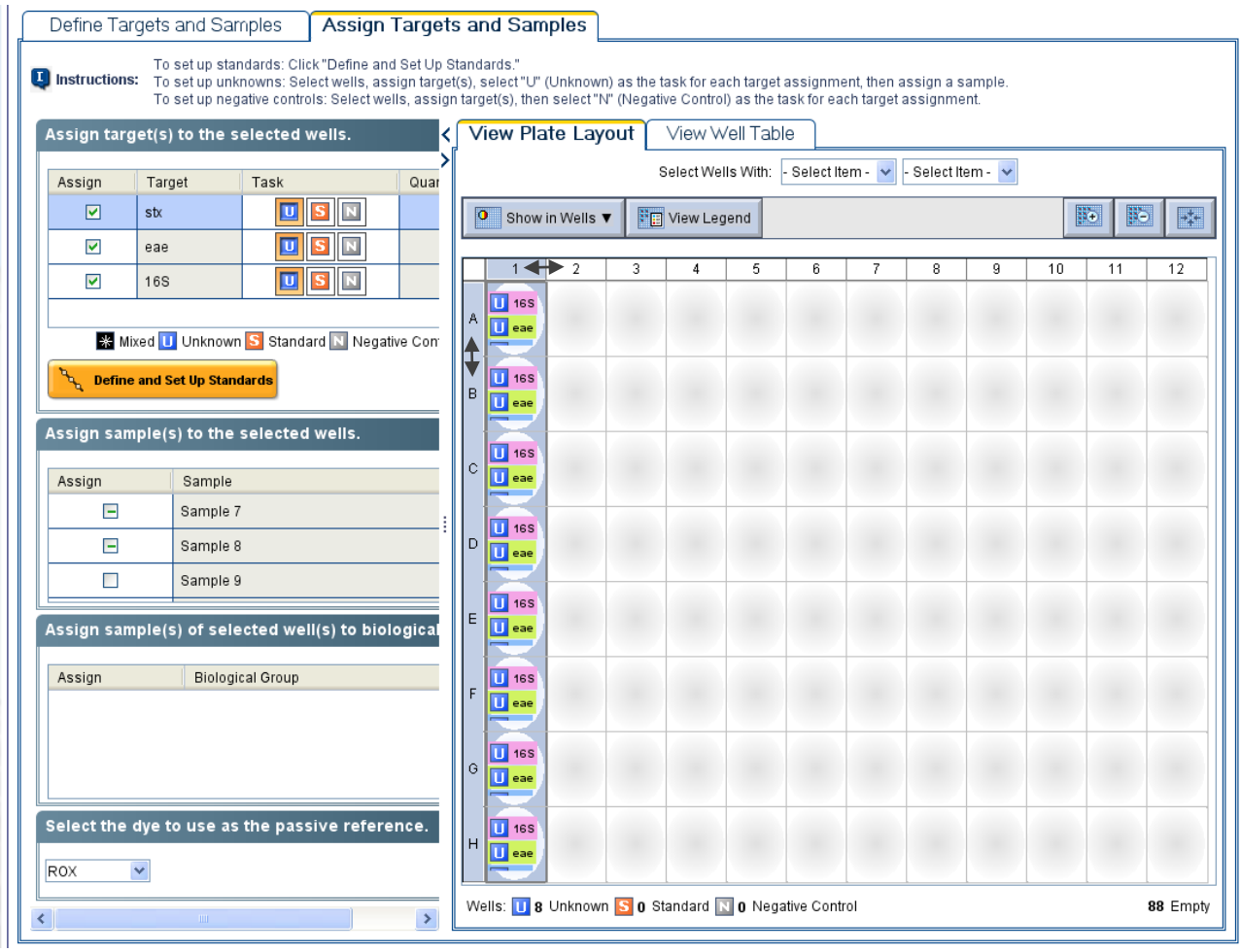
Highlight all wells that contain samples, hold down the CTRL button to add multiple wells.



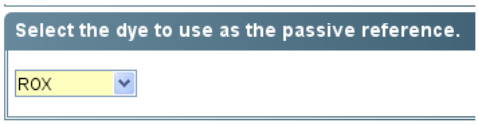
After you assign all of your samples to the wells, select the targets that you want from the assign targets box. For example, add stx, eae and 16S to each sample.



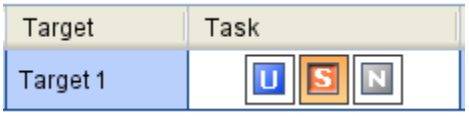
In order to see all of the targets and sample names in each well, you may stretch the wells using the arrows. This will allow all the contents of the well to be seen.



11) Make sure the box at the very bottom of the page says passive reference dye set to "ROX".



12) **(OPTIONAL)** If running a standard curve, click the orange S box before assigning the wells. Otherwise, all samples are unknown which is the default so you should not have to check that parameter.



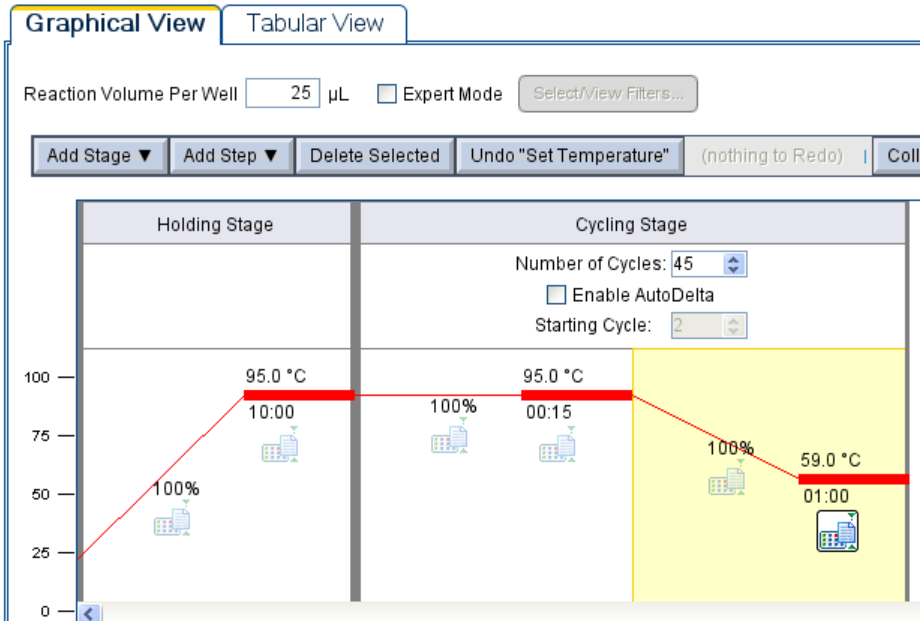
13) Click on the left tab set-up run method.

Remove the first holding step by right clicking that box and deleting. Change the temperatures and cycles to the parameters defined in Table 3.

Table 3. PCR Parameters (stx and eae; serogroup-specific)

Number of Cycles	Temperature	Time
1 (Holding Stage)	95°C	10 minutes
45 (Cycling Stages)	95°C	15 seconds
	59°C	1 minute

To change parameters, click on the temperature and change to the appropriate setting for that step. Next, click on the time and change to the appropriate setting for that step.



14) At the top of the graph, change reaction volume per well to 25 µl.

Reaction Volume Per Well 25 µL

15) Prepare your PCR assay plate at this step. See MLG 5C Appendix 4 Primer and Probe Sequences and Reagent Concentrations for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-time PCR Assay for PCR master mix component volumes. To determine the volume of the PCR components needed, add the number of sample reactions along with controls and at least one additional reaction.

16) After you have the master mix and template added to the appropriate wells, seal the plate with the MicroAmp Optical Adhesive Film and centrifuge the plate briefly to ensure no bubbles are at the bottom of each well.

17) On the ABI® 7500 FAST instrument, open the holder for the plate by pressing the indention next to the power button. Change the holder from strips to 96-well plate if needed. If you use the strips, you MUST put extra strips in the holder on the opposite side of the tube holder and they must be pushed into the grooves

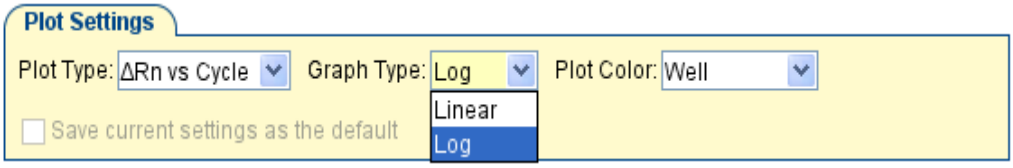
perfectly to avoid crushing your tubes. If you are using more than 3 columns of reactions (24 reactions), it is more economical to use the 96-well plates and seals.

18) Set the plate or strips into the holder and re-close using the indention on the right.
19) In the software interface, the temperature cycling graph should still be up. Click the Green button labeled "Start Run".

20) Store the experiment run data in a location accessible to the laboratory.
21) The start run button will turn from green to red and it will show the amount of time left in the program.
22) Do not edit or click anything while it is running. There should be a graph showing all the raw data as the program proceeds.
23) After it is finished, take out your plate, close the door and turn off the machine.
24) Press save at the top of the screen. If you want to analyze the data at a location with the ABI® 7500 Software, move the .eds file to the designated folder on the server and open within the program. Otherwise, click the green analyze button at the top.

A3 5C.11 Analyzing the Data

1) To see the amplification curves, choose target: stx, eae and/or 16S at the bottom left options panel. It starts by showing all 3 in the log phase. Change to linear phase.



2) To see the threshold automatically determined by the software, check the show threshold box. Auto will be checked since the threshold has been set automatically. A thick line with the threshold for the selected target should come

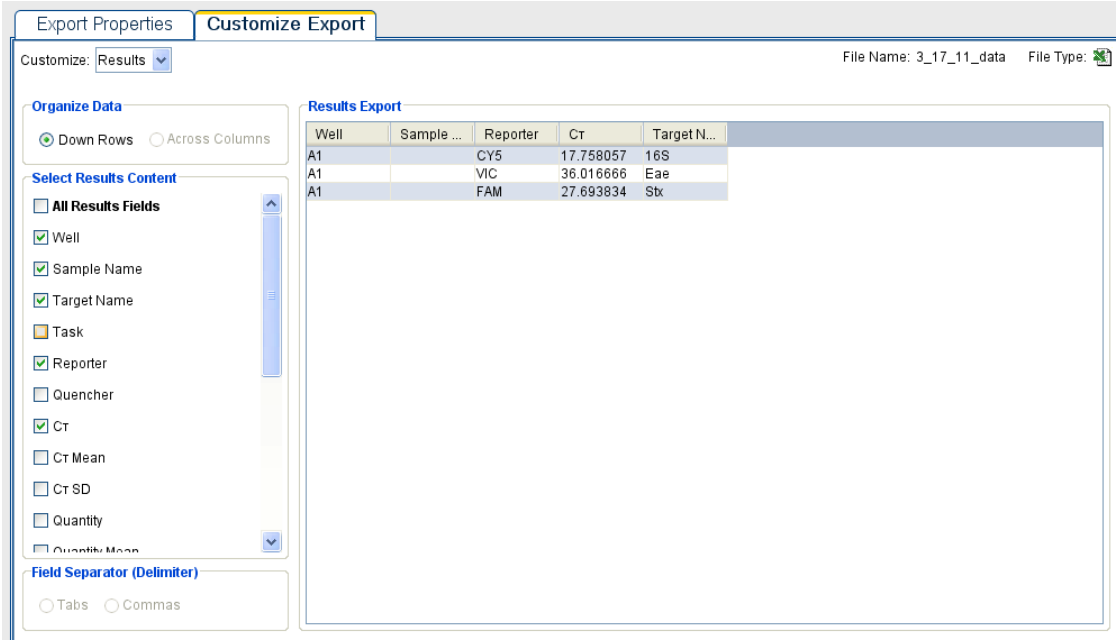
up. To see the threshold line and value, leave as is with all three and it will show three thresholds or click on a single target using the drop-down box.

3) To show curves of the selected samples, click on a well in the 96-well plate diagram. Use the ctrl button to click multiple wells simultaneously.
NOTE: Any time you make a change (ex. sample name or location), click the green analyze button for the changes to take effect.

To view the C_T values, click from the "View Plate Layout" tab to the "View Well Table" tab. If you do not see any C_T values, click the analyze button again.

4) Choose "Group by Target Name" to view results by gene target.

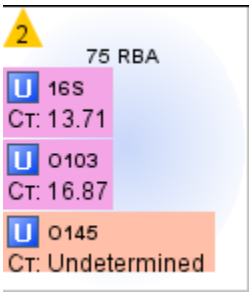
5) To **export** results (make sure you pressed "Analyze" button before this step), click "Export" on the top bar. To export the data for all samples, **make sure that all samples are selected on the graph**. Choose the destination for the file and click "Customize Export" to check which boxes you want exported. To simplify data analyses, select the following items from "Select Results Content": Well, Sample Name, Target Name, Reporter, and C_T.



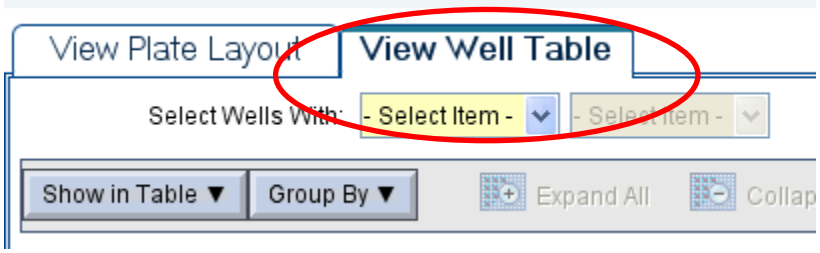
6) **(OPTIONAL)** If you want to save the **picture** of the graph click the button to the right of the print button at the top of the graph and save as a jpg.

7) Data can be analyzed directly from the ABI® 7500 FAST machine or from the exported file. A reported C_T value (i.e. 34.5 for eae) for a target indicates that the sample is positive for that target. If a sample is negative for target, "undetermined" will be reported by the instrument. Follow MLG Chapter 5C Detection, Isolation and Identification of Top Seven Shiga Toxin-Producing *Escherichia coli* (STECs) from Meat Products and Carcass and Environmental Sponges to determine if analyses must be continued on sample(s).

In addition to checking if a sample is positive for a given target based on it having a C_T value <45, you must also check for any flags present in a well. In plate layout view flags are indicated by a yellow triangle as seen below:



To determine specifically which flags are present in a given well click the view well table tab in the analysis window.



The column headers indicate which flags are present

Table 4 describes possible flags encountered during a run.

Table 4. Descriptions of Potential Run Flags

Flag	Description
BADROX	Bad passive reference signal
EXPFAIL	Exponential algorithm failed
NOAMP	No amplification
NOISE	Noise higher than other samples in plate
SPIKE	Noise Spikes
THOLDFAIL	Thresholding algorithm failed

If the flags BADROX, NOISE, or SPIKE occur within a well, the results are invalid and the reaction must be repeated. These flags are possibly caused by evaporation, improper plate sealing, or inaccurate volumes.
NOAMP will occur in well in which none of the targets is detected; this is expected in the no template control. EXPFAIL is expected in samples negative for a given target. No action is taken for either of the preceding flags.
If THOLDFAIL flag is present the threshold should be adjusted manually for the target based on the positive control. The appropriate place to set the threshold is within the exponential phase of the amplification plot. Consult the Applied Biosystems 7500/7500 FAST Guidebook for Standard Curve Experiments provided with the PCR platform for troubleshooting information. The figures below show the different phases of the amplification plot as well as appropriate threshold position.

Figure 1. Different Phases of the Amplification Plot

(a) Plateau Phase (b.) Linear Phase (c.) Exponential Phase

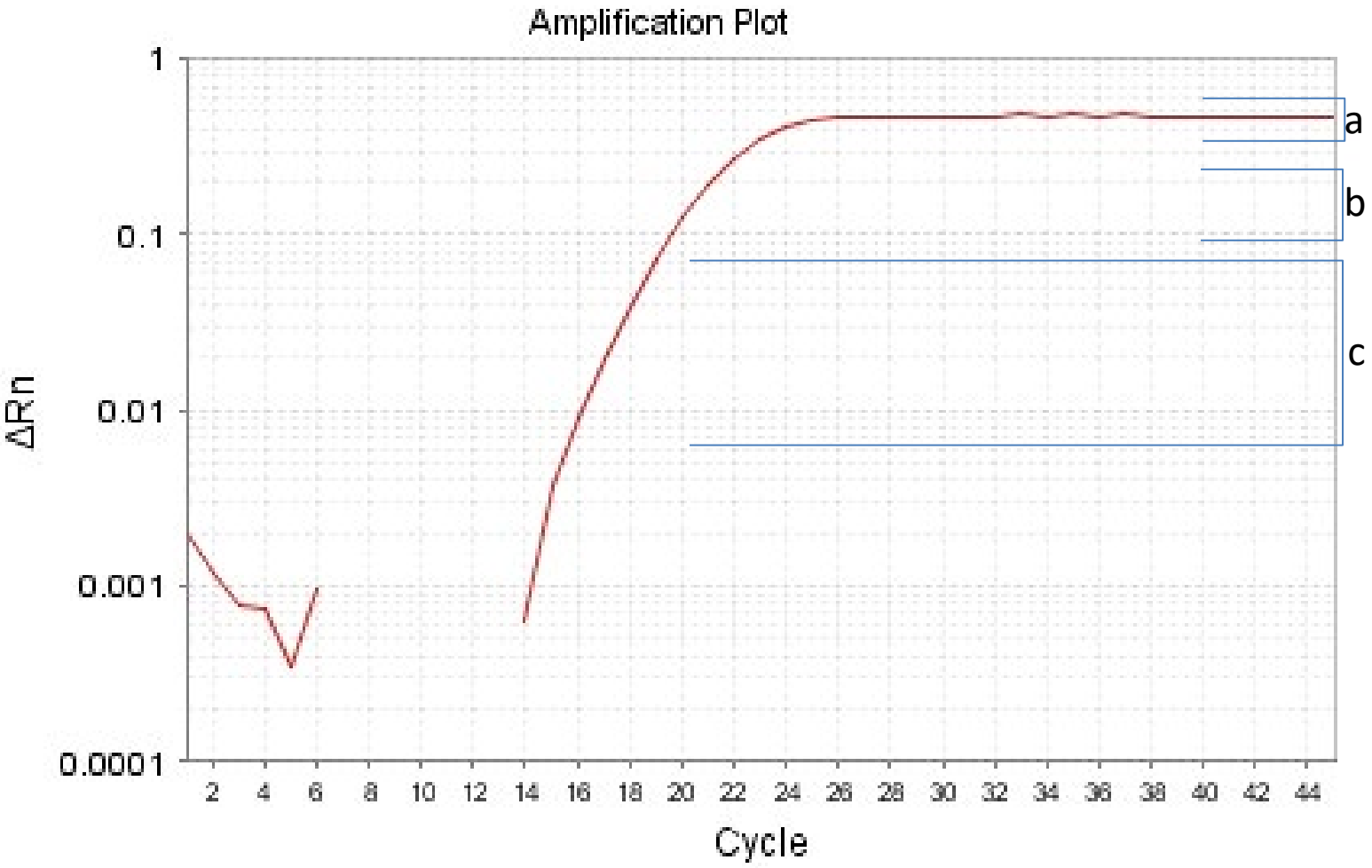
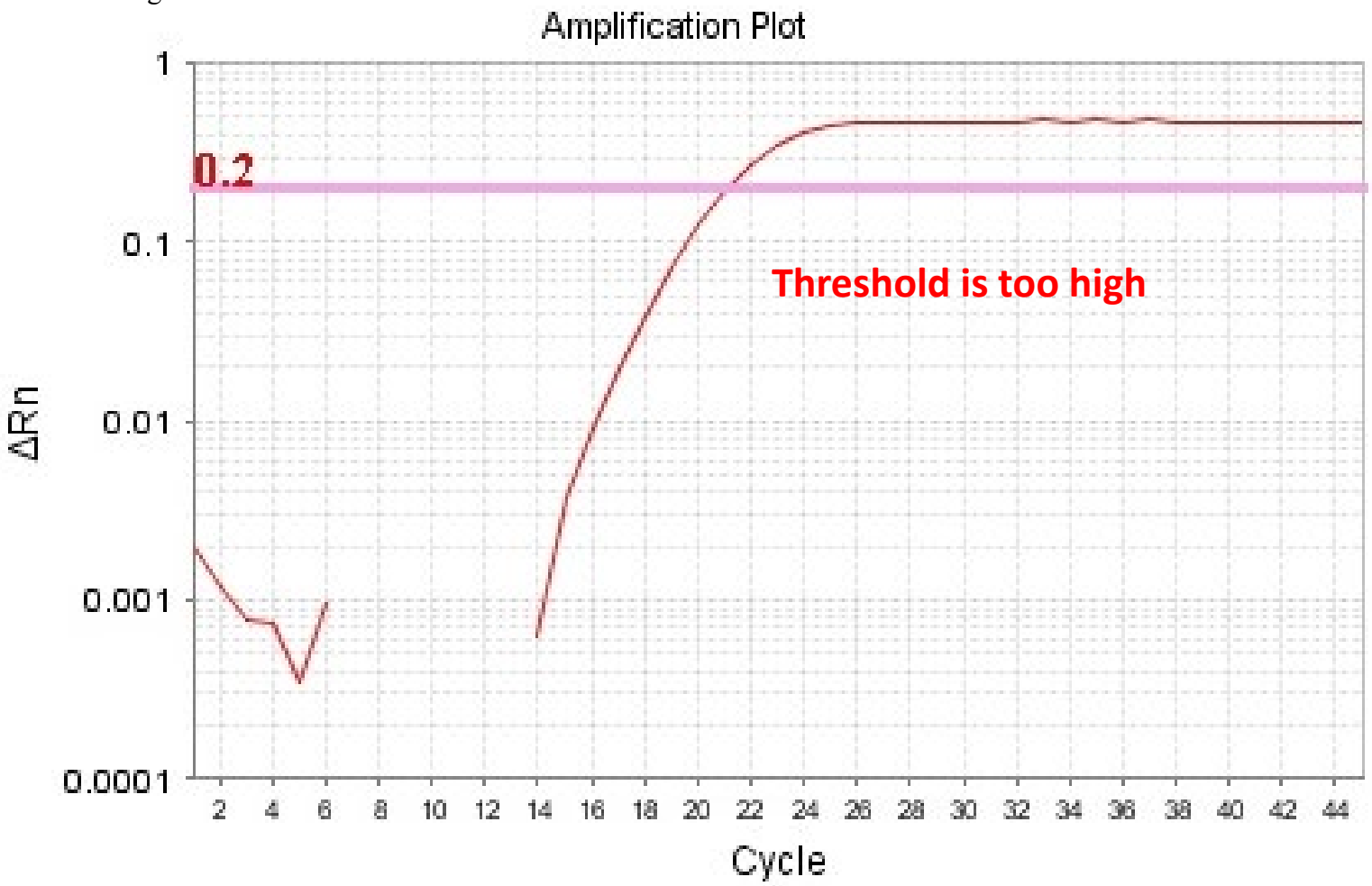
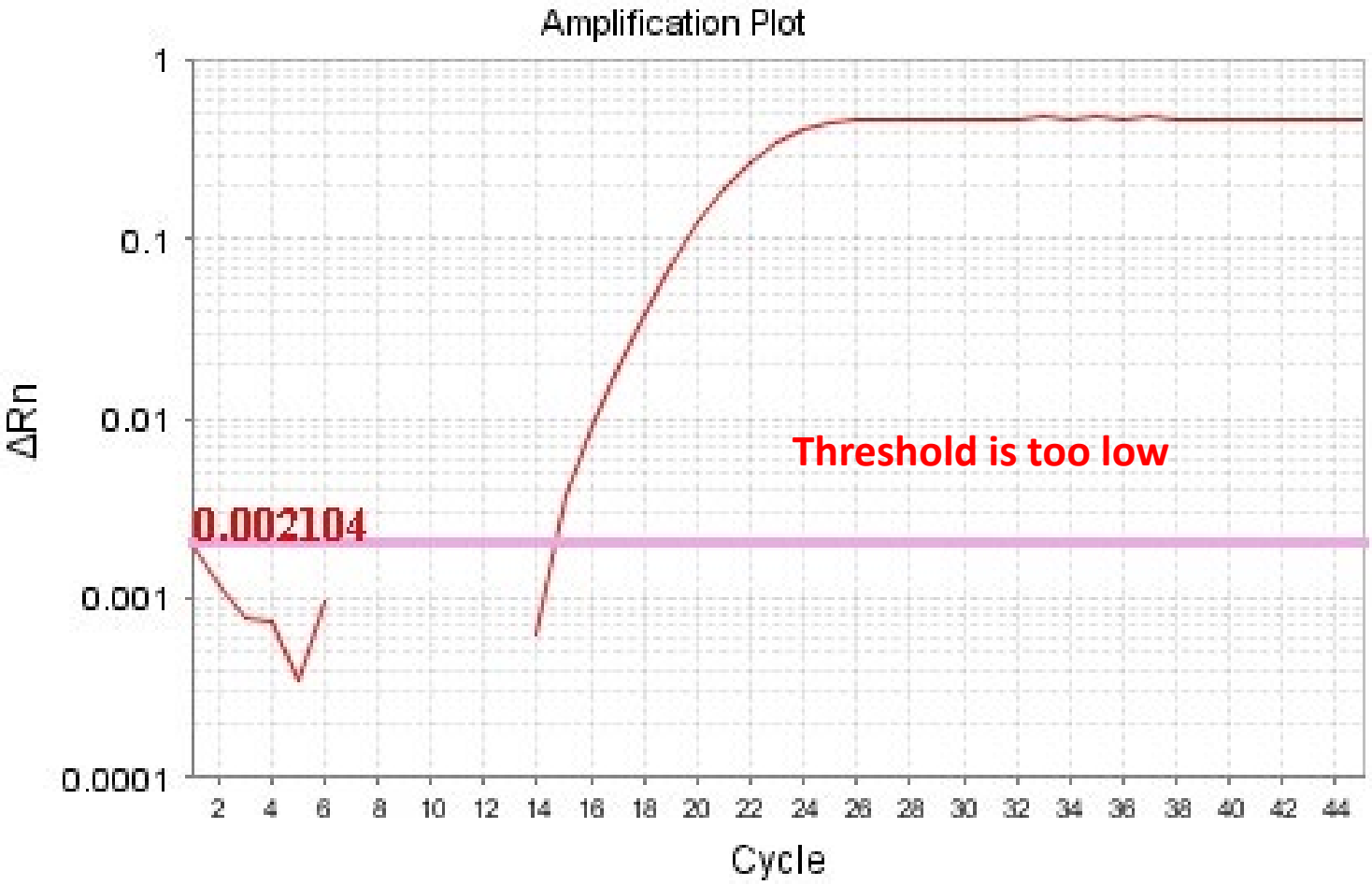
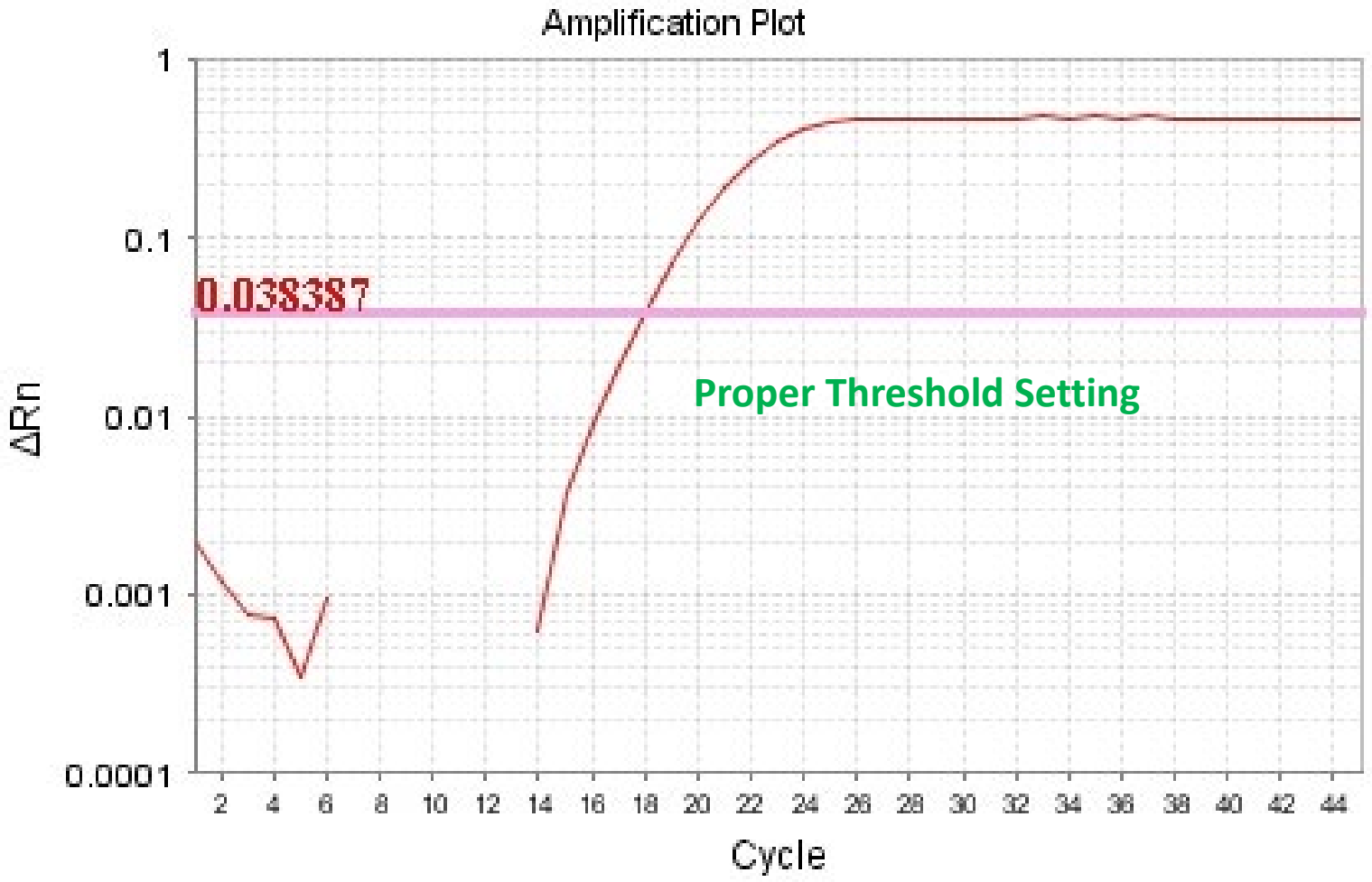


Figure 2. Threshold Positions




Amplification Plot
ΔRn
1
0.1
0.01
0.001
0.0001
0.002104
Threshold is too low
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycle


Amplification Plot
1
0.1
0.038387
0.01
0.001
0.0001
ΔRn
Proper Threshold Setting
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycle

A3 5C.12 Interpretation of Control Results

Various scenarios for control and sample results along with the appropriate actions based on those results are included in Table 5, Figure 3, and Figure 4.

Table 5. Possible positive control results for *stx/eae* screen

Positive control result	sample results	Action
stx(+), eae (+), 16S (-)	all samples neg all targets	Use new primer probe mix and repeat PCR
stx(+), eae (+), 16S (-)	1 or more samples pos for stx/eae	Proceed with O-group PCR for positive samples repeat stx/eae PCR on same run using new primer probe mix on controls/positives
stx(-), eae (+), 16S (+)	all samples negative for stx	Use new primer probe mix repeat PCR, and make sure proper control was used
stx(-), eae (+), 16S (+)	1 or more samples pos for stx/eae	Proceed with O-group PCR for positive samples, repeat stx/eae PCR on same run on + controls making sure correct positive control was used
stx(-), eae (-), 16S (-)	all samples negative for all targets	Rerun PCR
stx(-), eae (-), 16S (-)	1 or more samples pos for stx/eae/16S	Proceed with O-group PCR for positive samples, repeat stx/eae PCR on same run using new positive controls
stx(-), eae (-), 16S (+)	all samples negative for stx/eae	Use new primer probe mix and repeat PCR
stx(-), eae (-), 16S (+)	1 or more positive for stx/eae	Proceed with O-group PCR for positive samples, repeat stx/eae PCR on same run on + controls making sure correct positive control was used
stx(+), eae (-), 16S (+)	all samples eae negative	Use new primer probe mix and repeat PCR
stx(+), eae (-), 16S (+)	1 or more samples eae positive	Use new primer probe mix and repeat PCR
stx(-), eae (+), 16S (-)	all samples stx/16S negative	Use new primer probe mix and repeat PCR
stx(-), eae (+), 16S (-)	1 or more samples is stx/16S positive	Use new primer probe mix and repeat PCR
stx(+), eae (-), 16S (-)	all samples eae/16S negative	Use new primer probe mix and repeat PCR
stx(+), eae (-), 16S (-)	1 or more samples eae/16S positive	Use new primer probe mix and repeat PCR
stx(+), eae (+), 16S (+)	samples negative for 16S and stx/eae	Perform PCR with new DNA extraction templates
stx(+), eae (+), 16S (+)	all samples positive for at least 16S	Run is valid. Proceed as per MLG 5C

Figure 3. Interpretation of sample 16S internal control results.

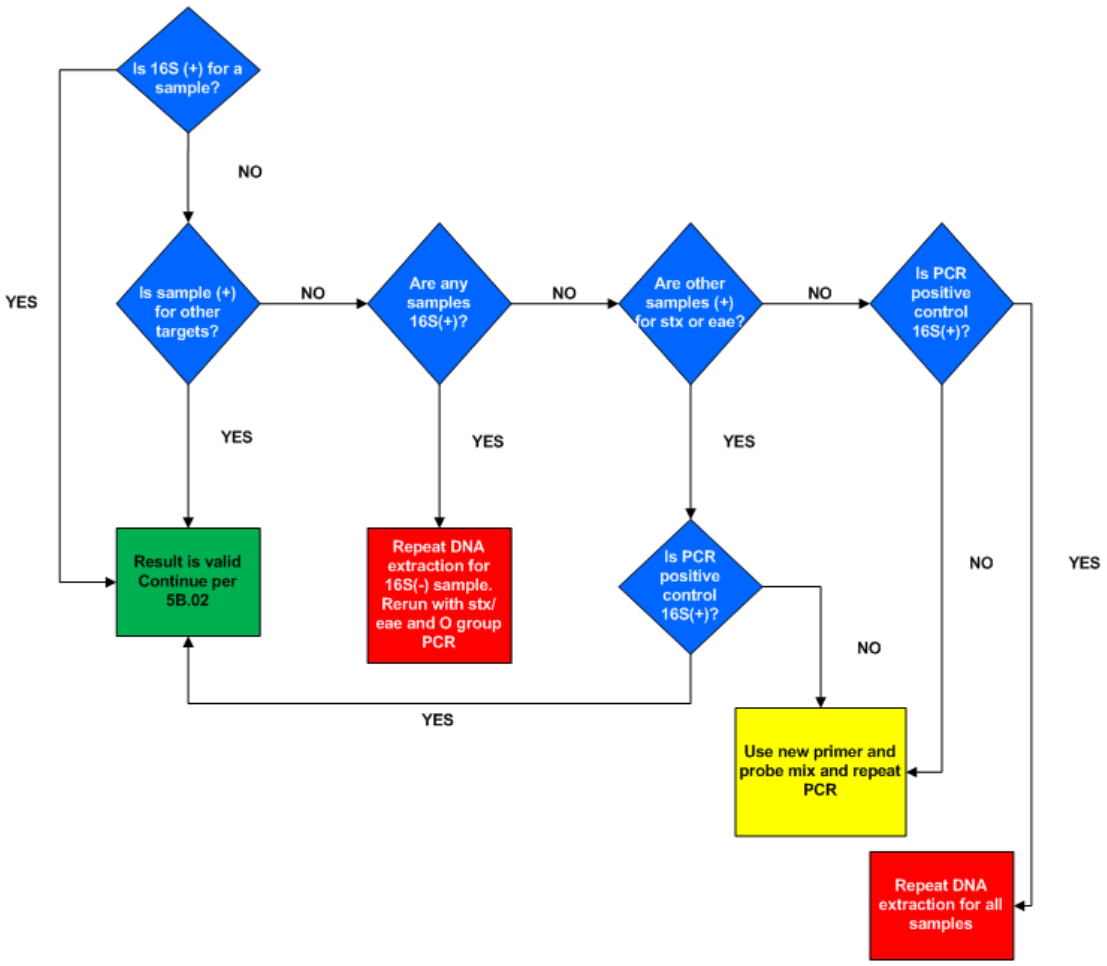
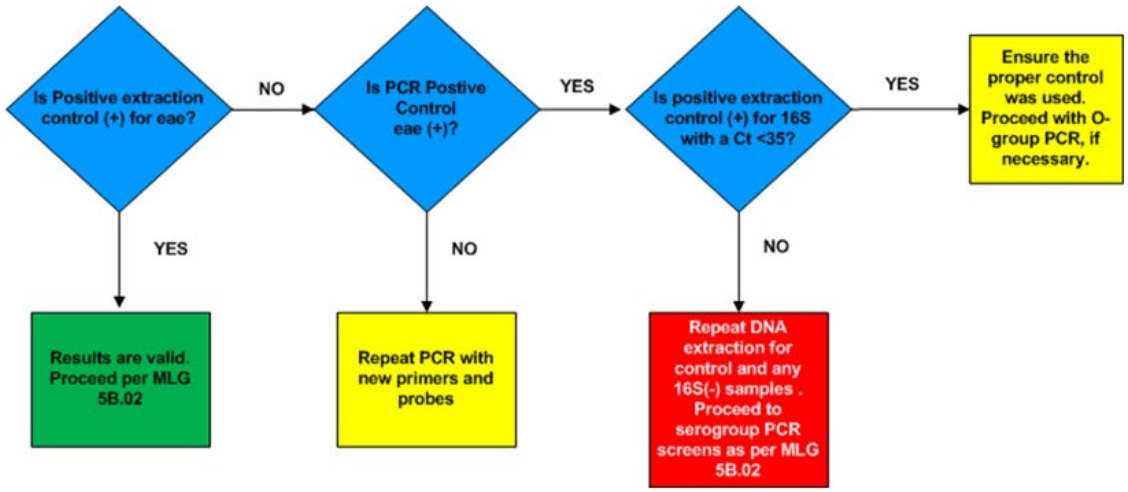


Figure 4. Interpretation of positive extraction control results.



A3 5C.13 Selected References

Applied Biosystems® 7500 Fast Real-time PCR System Guide

Arthur TM, Barkocy-Gallagher GA, Rivera-Betancourt M, Koohmaraie M (2002) Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* on carcasses in commercial beef cattle processing plants. Appl Environ Microbiol. 68(10): 4847-52.

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Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Strockbine NA. (2005) Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. J Infect Dis. 192(8): 1422-9.

Grant, MA (2004) Improved Laboratory Enrichment for Enterohemorrhagic *Escherichia coli* by Exposure to Extremely Acidic Conditions. Appl Environ Microbiol 70(2): 1226-1230.

Possé B, De Zutter L, Heyndrickx M, Herman L. (2008) Novel differential and confirmation plating media for Shiga toxin-producing *Escherichia coli* serotypes O26, O103, O111, O145 and sorbitol-positive and -negative O157. FEMS Microbiol Lett. 282(1): 124-31.

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