United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health Science
Laboratory QA/QC Division
950 College Station Road
Athens, GA 30605

Laboratory Guidebook
Notice of Change

Chapter **new**, revised**,** or archived: MLG 5B 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

Effective Date: 6/4/12

Description and purpose of change(s):

This procedure was added to describe the PCR platform requirements and expected method control reactions.

The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use. Method validation is necessary to demonstrate the equivalence of alternative tests. FSIS provides guidance at:
http://www.fsis.usda.gov/PDF/Validation_Studies_Pathogen_Detection_Methods.pdf

Procedure Outline

A5B.1 Introduction

This protocol is for use by the FSIS Field Service Laboratories in operation of the ABI® 7500 FAST PCR platform. This procedure provides instructions for using the ABI® 7500 FAST PCR Platform described in the MLG Chapter 5B Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products. Photos provided are from software version 2.04/2.05. Details include analysis of the data and interpretations for the control results.

A5B.2 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:

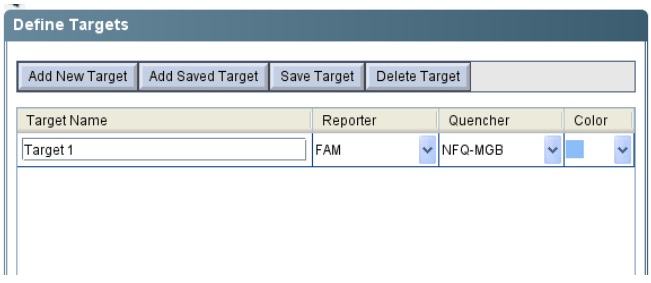


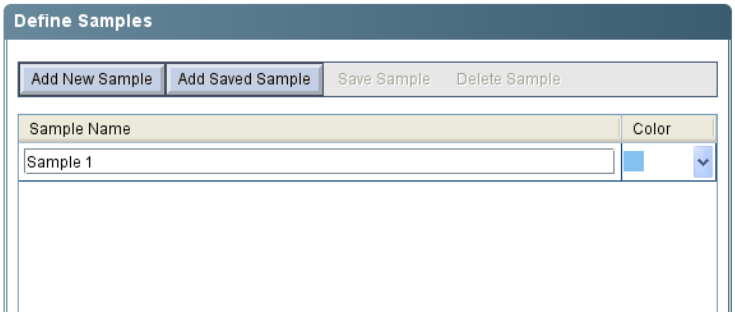
Table 1. *stx* and *eae* Screen and Confirmatory PCR

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

Table 2. Serogroup-specific Screen and Confirmatory PCR

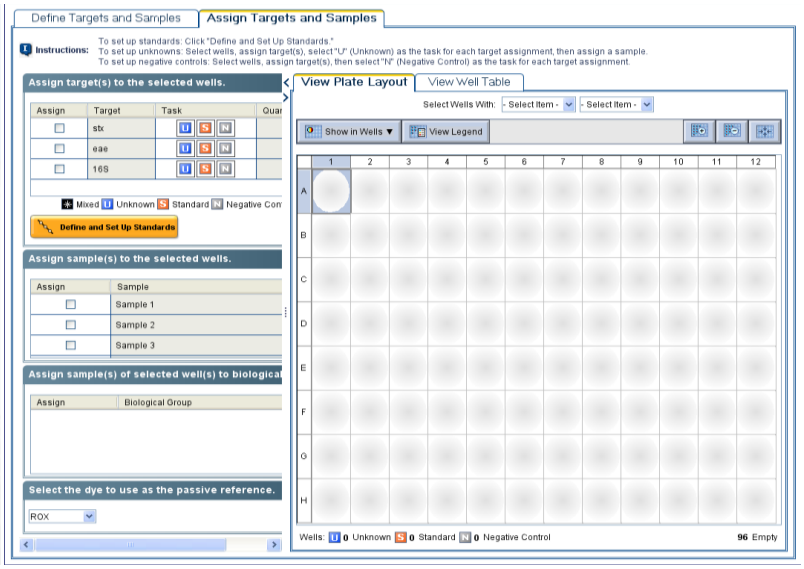
Target	Dye	Quencher
O26	FAM	None
O111	VIC	None
O45	FAM	None
O121	VIC	None
O103	VIC	None
O145	FAM	None
16S	Cy5	None

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.

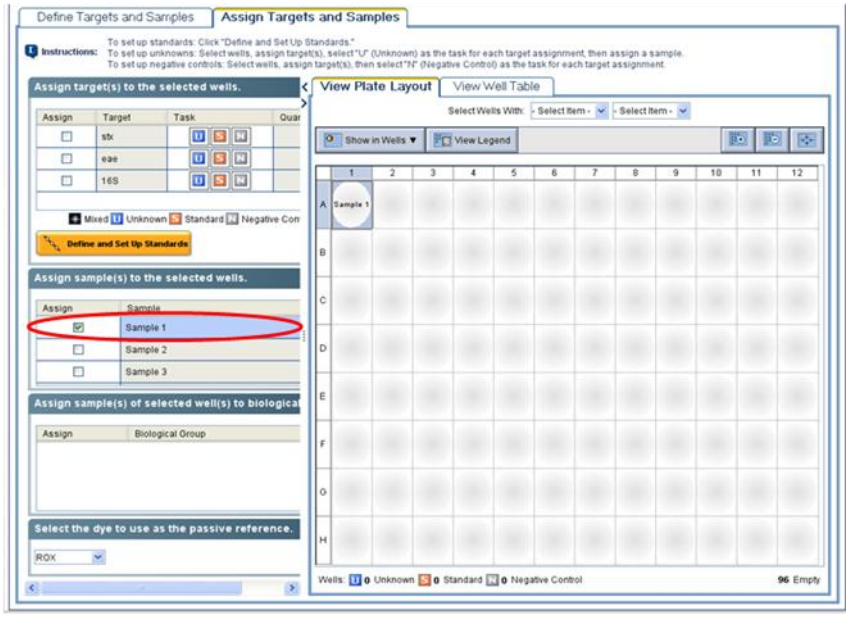


10) After you change the sample name (or do this after the run) and choose any color changes (optional) you want, click on the "Assign Target and Samples" tab.

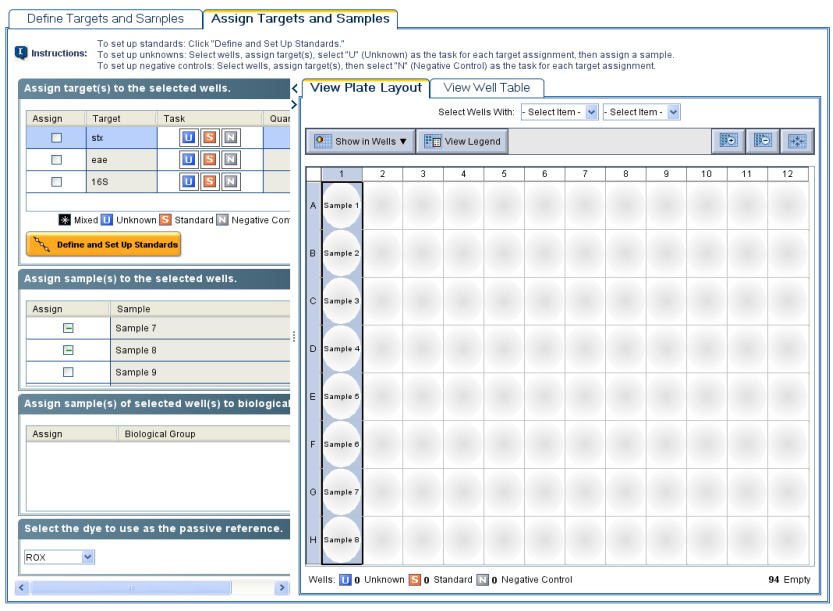
Highlight the well that you want to place the sample in.



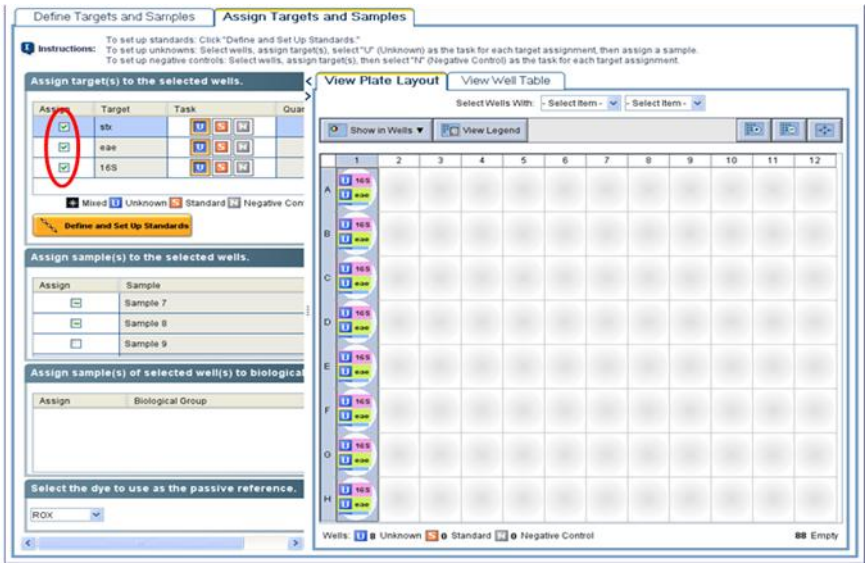
Select the sample from the assign samples box by checking the box. Repeat until all samples are placed in the correct locations.



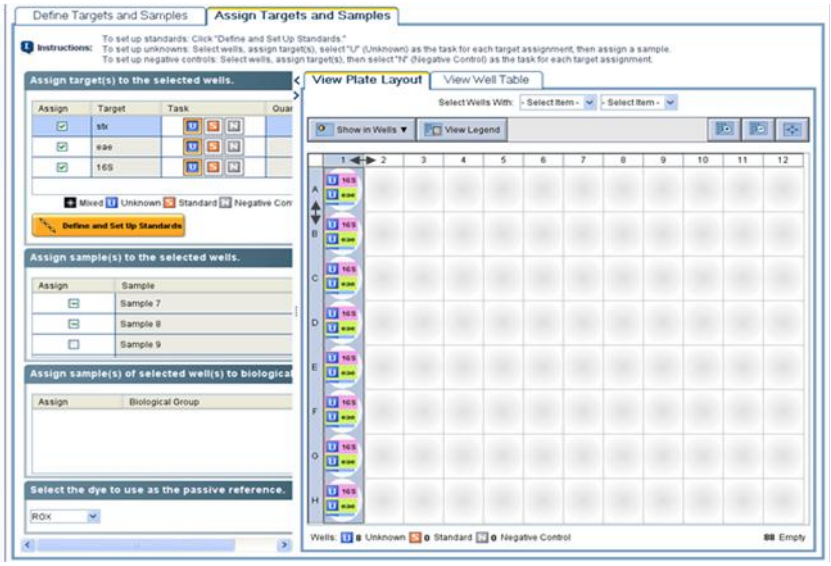
Highlight all wells that contain samples, hold down the CTRL button to add multiple wells.



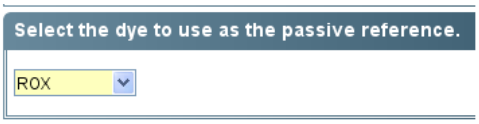
After you assign all of your samples to the wells, select the targets that you want from the assign targets box. For example, add stx, eae and 16S to each sample.



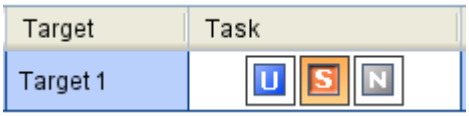
In order to see all of the targets and sample names in each well, you may stretch the wells using the arrows. This will allow all the contents of the well to be seen.



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.



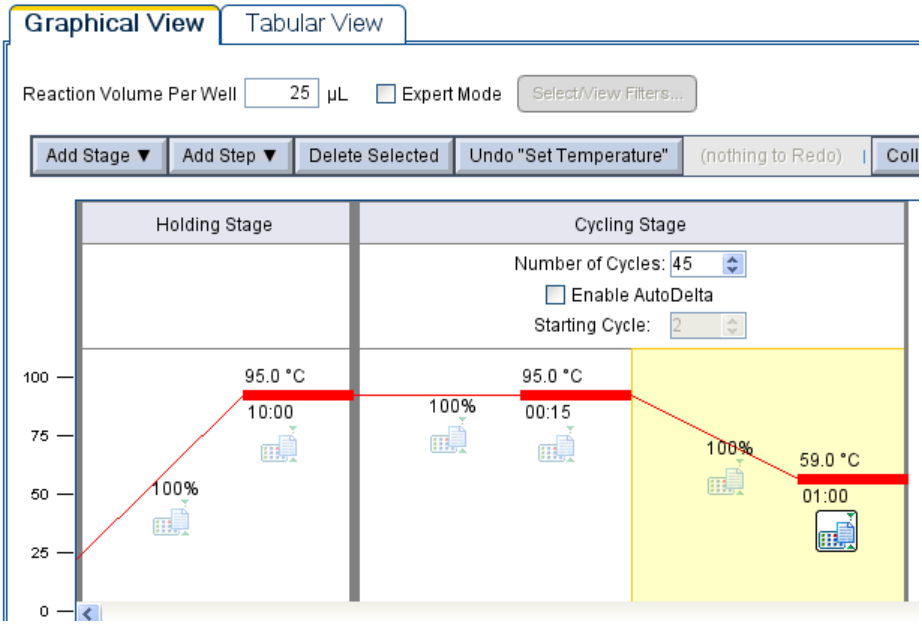
13) Click on the left tab set-up run method.

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

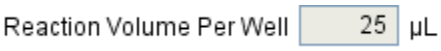
Table 3. PCR Parameters (stx and eae; serogroup-specific)

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.



15) Prepare your PCR assay plate at this step. See MLG 5B Appendix 1 Primer and Probe Sequences and Reagent Concentrations for non-O157 Shiga Toxin-

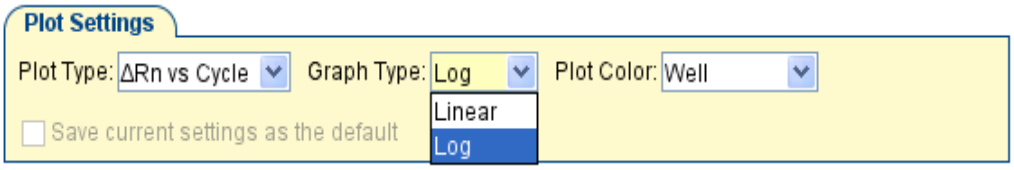
Producing *Escherichia coli* (STEC) Real-time PCR Assay for PCR mastermix component volumes. To determine the volume of the PCR components needed, add the number of sample reactions along with controls and at least one additional reaction.

16) After you have the mastermix 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.

A5B.3 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.

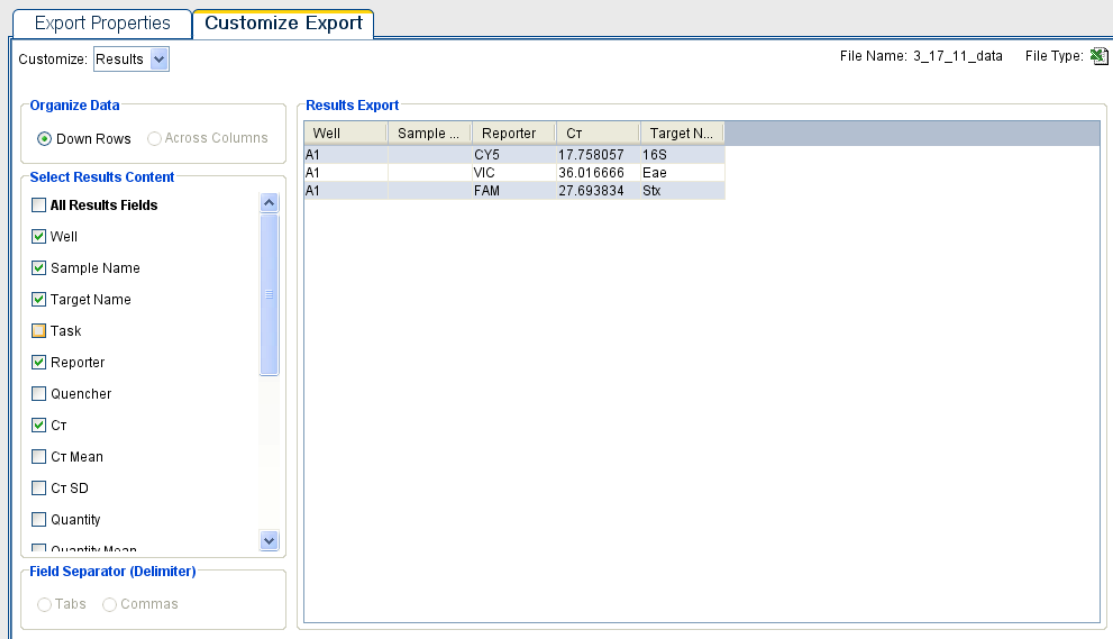
3) To show curves of the selected samples, click on a well in the 96-well plate diagram. Use the ctrl button to click multiple wells simultaneously.
NOTE: Any time you make a change (ex. sample name or location), click the green analyze button for the changes to take effect.

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.

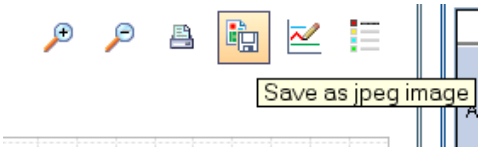
4) Choose "Group by Target Name" to view results by gene target.

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.

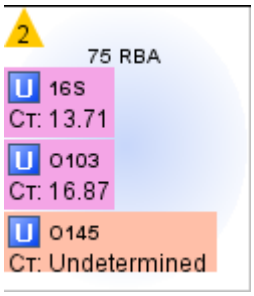


6) **(OPTIONAL)** If you want to save the **picture** of the graph click the button to the right of the print button at the top of the graph and save as a jpg.

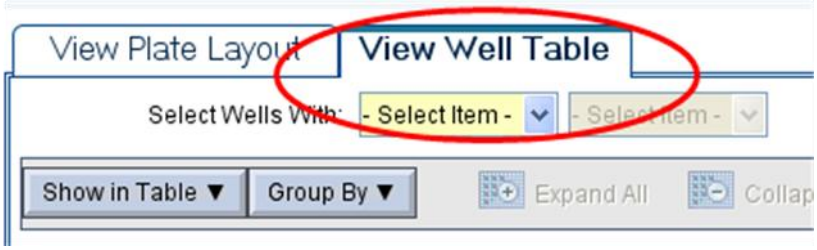


7) Data can be analyzed directly from the ABI® 7500 FAST machine or from the exported file. A reported C_T value (i.e. 34.5 for eae) for a target indicates that the sample is positive for that target. If a sample is negative for target, "undetermined" will be reported by the instrument. Follow MLG Chapter 5B Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products 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

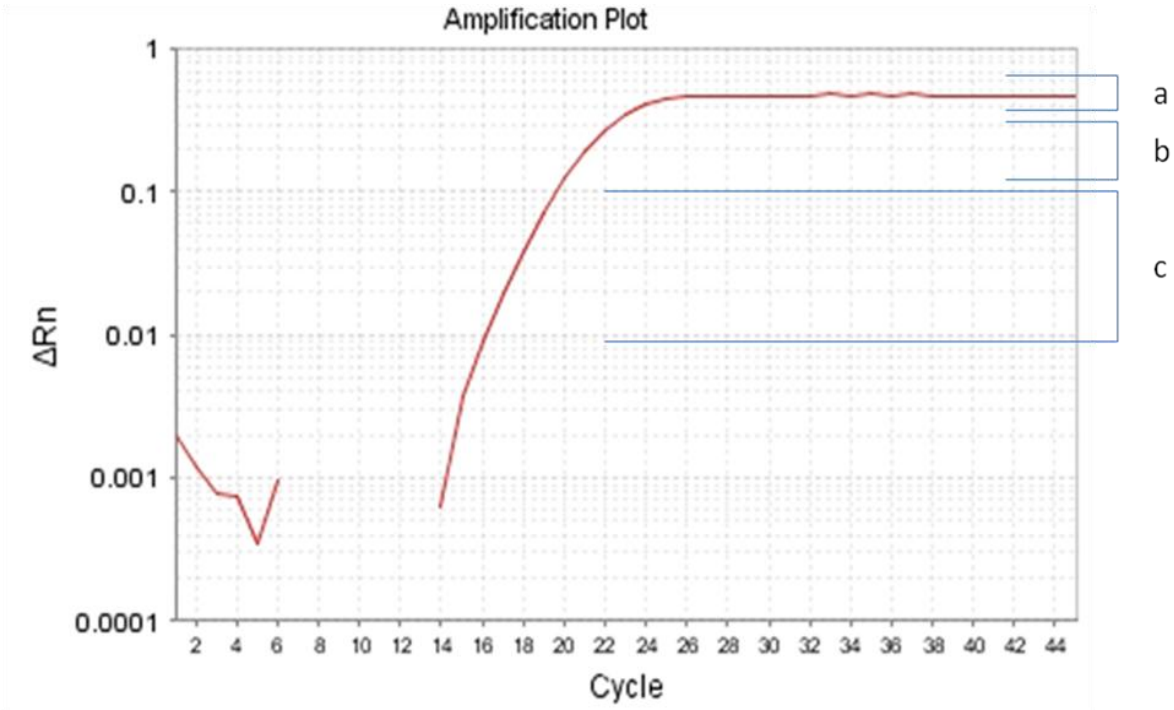
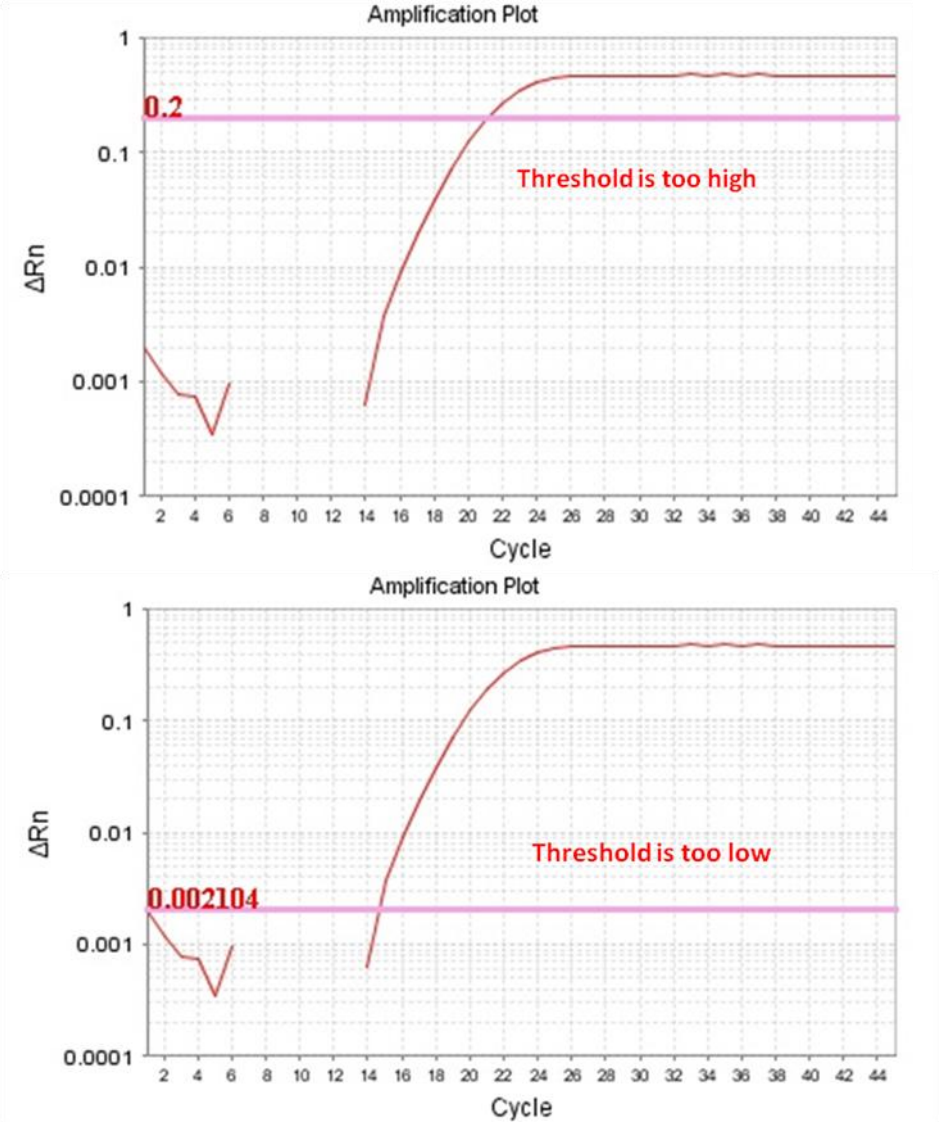
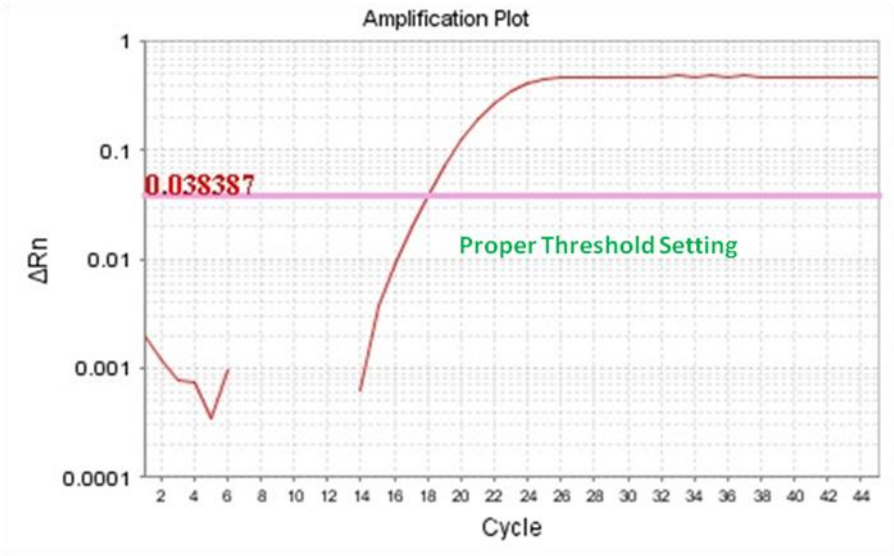


Figure 2. Threshold Positions




Amplification Plot
1
0.1
0.01
0.001
0.0001
ΔRn
0.038387
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

A5B.4 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 5B

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

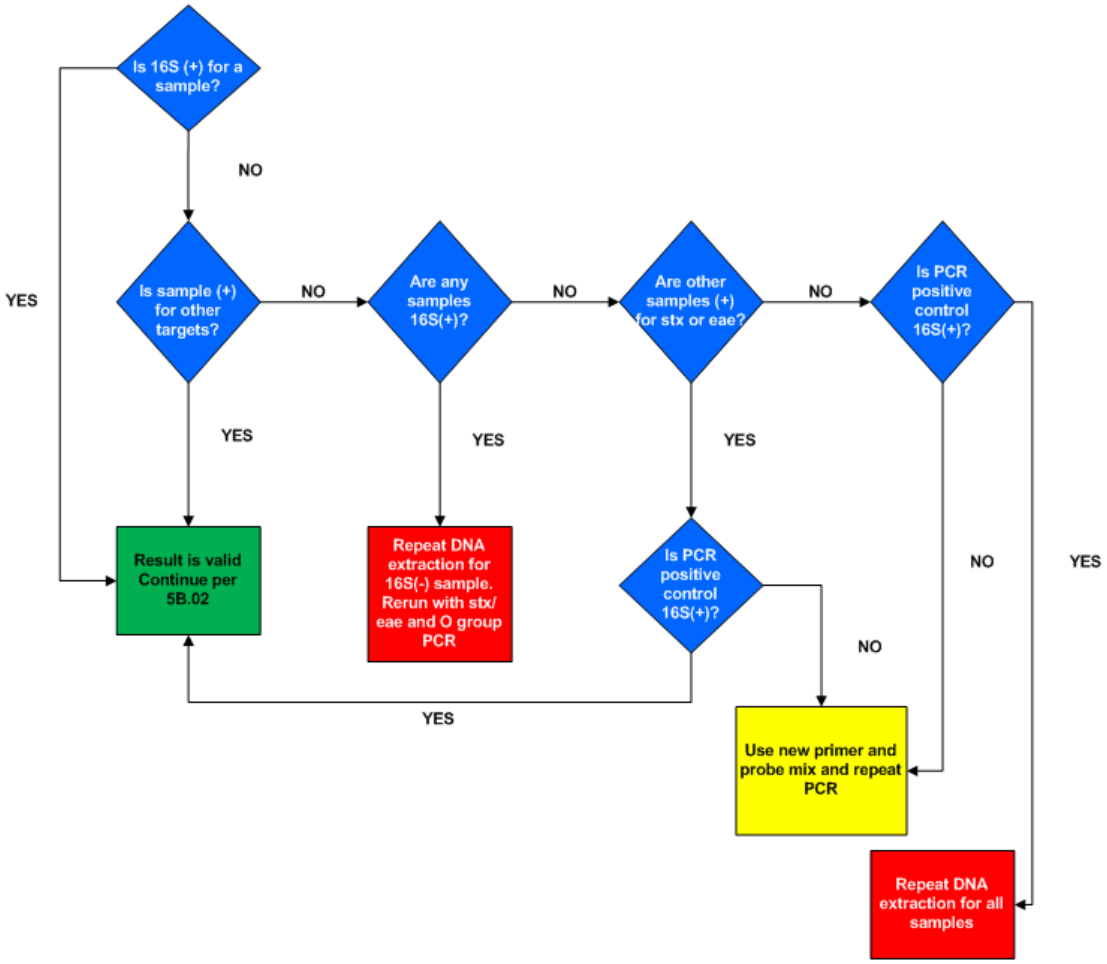


Figure 4. Interpretation of positive extraction control results.