United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health Science
Laboratory QA Staff
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Laboratory Guidebook
Notice of Change

Chapter **new**, revised**,** or archived: MLG 5C Appendix 5.00

Title: PCR Platform Instructions for the real-time PCR detection of Shiga toxin gene and H7 gene in *E. coli* O157:H7

Effective Date: 02/04/2019

Description and purpose of change(s):

This procedure provides instructions for using the ABI® 7500 FAST PCR Platform to detect the fliCH7 gene encoding the H7 antigen of *E. coli* (including O157:H7) migrating the assay from the SmartCycler® to the ABI® 7500 FAST system. Details include analysis of the data and interpretations for the control results.

Procedure Outline

A5 5C.1 Introduction

This procedure provides instructions for using the ABI® 7500 FAST PCR Platform to detect the fliCH7 gene encoding the H7 antigen of *E. coli* (including O157:H7) migrating the assay from the SmartCycler® to the ABI® 7500 FAST system. The real-time PCR assay discerns the shiga toxin (*stx*), intimin (*eae*), fliCH7 and 16S genes of *E. coli* in a single assay. This assay can be used to confirm these targets in *E. coli* O157:H7 strains. Details include analysis of the data and interpretations for the control results.

A5 5C.2 Safety Precautions

E. coli O157:H7 is categorized as a Biosafety Level 2 human pathogen with a low infectious dose (ingestion of 100 cells can cause disease). CDC guidelines for manipulating Biosafety Level 2 pathogens should be followed whenever live cultures of *E. coli* O157 are used. The use of gloves and eye protection is recommended for all post enrichment viable culture work. Work surfaces should be disinfected prior to and immediately after use. A Class II laminar flow biosafety cabinet is recommended for activities in which infectious aerosols or splashes may be created. The Safety Data Sheets (SDS) must 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.

A5 5C.3 Equipment, Reagents, Media and Test Kits

A5 5C.3.1 Equipment

a. Incubator (35 ± 2°C)
b. Heating block (99 ± 2°C) or Thermocycler for DNA template preparation
c. Mini Vortex mixer
d. Freezer (-20 ± 2°C)
e. Clean bench or hood
f. Applied Biosystems® ABI® 7500 FAST PCR platform
g. Applied Biosystems® 7500 FAST MicroAMP™ Fast Optical 96-well Reaction Plate with Barcode (Part# 4346906)
h. Applied Biosystems® 7500 FAST MicroAMP™ Splash Free 96-Well Base (Part# 4312063)
i. Applied Biosystems® 7500 FAST Optical Adhesive Film (Part# 4311971)

A5 5C.3.2 Media, Reagents and Cultures

a. Sterile, certified RNase-free, DNase-free molecular grade water (Teknova), or equivalent
b. 1X Tris-EDTA (TE) Buffer (Teknova Catalog# T0224, or equivalent provider)
c. Applied Biosystems® TaqMan® Environmental Master Mix 2.0 (Part# 4396838)
d. Primers and Probes (IDT Technologies, Coralville, IA)
e. Tryptic soy agar with 5% sheep blood (SBA) plates, (Remel, Lenexa, KS)
f. *E. coli* 43894: O157: H7+, stx+, eae+ or equivalent as the positive control

A5 5C.3.3 Primer and Probe Sequences

When receiving lyophilized primers and probes, reconstitute the probe to a stock solution using 1X TE buffer. Working concentrations should be prepared by diluting stocks to the appropriate working concentration. Stocks and working solutions should be stored in the -20°C freezer.

fliC$_{H7}$
Forward Primer 1068F: 5' TAC CAT CGC AAA AGC AAC TCC 3'
Reverse Primer 1314R: 5' GTC GGC AAC GTT AGT GAT ACC 3'
Probe fliCh7 247: 5'-6-Cy3/ CGG CTG CCG CGA CAT CTT CAA T-IBRQ 3'

stx
Forward Primer: 5'-TTTGTYACTGTSACAGCWGAAGCYTTACS-3'
Reverse Primer: 5'-CCCCAGTTCARWGTRAGRTCMACDTC-3'
Probe stx1: 5'-6-FAM/ZEN/CTGGATGATCTCAGTGGGCGTTCTTATGTAA-IBFQ-3'
Probe stx2: 5'-6-FAM/ZEN/TCGTCAGGCACTGTCTGAAACTGCTCC-IBFQ-3'

eae
Forward Primer: 5'-CATTGATCAGGATTTTTCTGGTGATA-3'
Reverse Primer: 5'-CTCATGCGGAAATAGCCGTTM-3'
Probe: 5'-MAX/ATAGTCTCGCCAGTATTCGCCACCAATACC-IBFQ-3'

16S rRNA Internal Control
Forward Primer: 5' CCT CTT GCC ATC GGA TGT G-3'
Reverse Primer: 5' GGC TGG TCA TCC TCT CAG ACC-3'
Probe: 5' TYE665/GTG GGG TAA CGG CTC ACC TAG GCG AC-IBRQ-3'

A5 5C.4 Procedures

A5 5C.4.1 Sample preparation

Note: A template from a 16-24 h isolate on SBA should be used for the PCR assay but the template can be prepared from a selective medium, if desired.

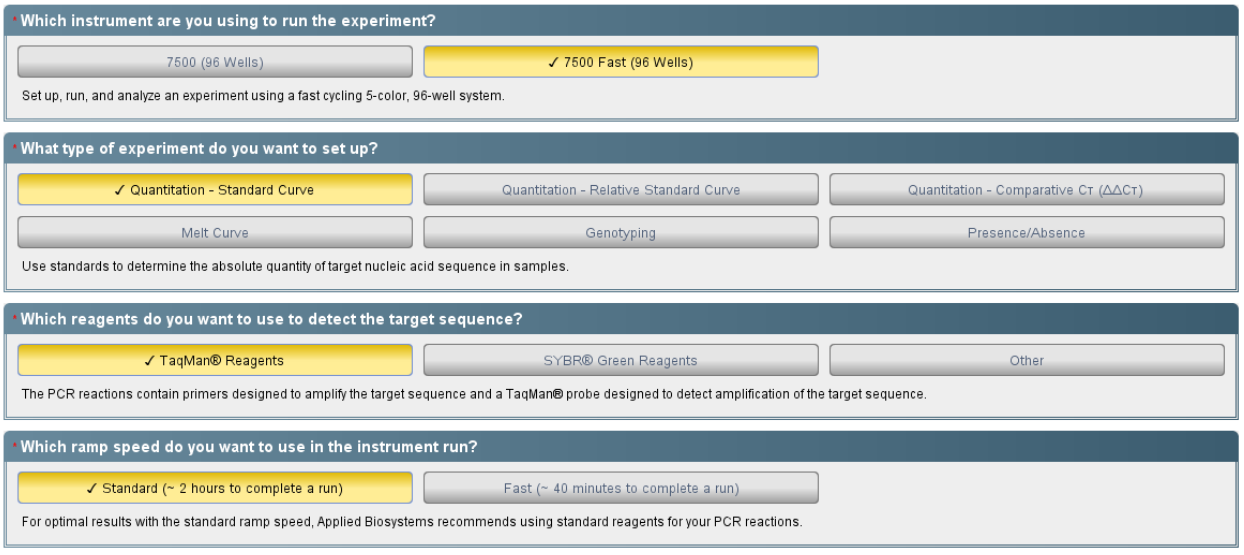
a. For each sample and the control (H7 and *stx* positive *E. coli*), streak a SBA plate to obtain isolated colonies and incubate at 35 ± 2°C for 16-24 hrs.
b. For each sample and control, dispense 50 ± 1µl of sterile molecular grade water into a labeled sterile micro centrifuge tube (for use on heating block) or thin-walled PCR tube (for use on thermocycler).
c. Transfer one isolated colony from Step 1 into each labeled tube. One tube will have no colony (only water) as a blank.
d. Heat the tubes in a thermocycler or heating block for 10 minutes at 99 ± 2°C. These samples will be templates for PCR.

e. Keep tubes at 2-8°C until ready to use.

A5 5C.4.2 ABI® 7500 FAST PCR Platform Instructions

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

e. Enter the name of the file according to the laboratory-naming scheme (e.g. MF12345).
f. 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.



g. Click on the plate set-up tab on the left

h. Click on "Define Targets and Samples" tab. Enter the targets for the assays listed in Table 1.

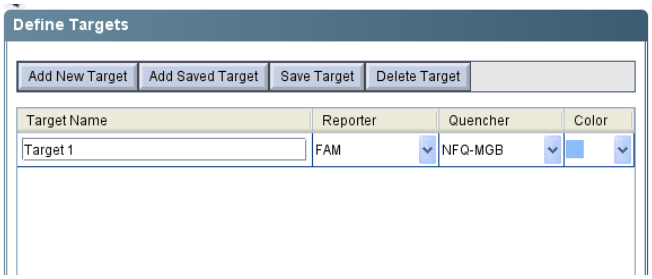
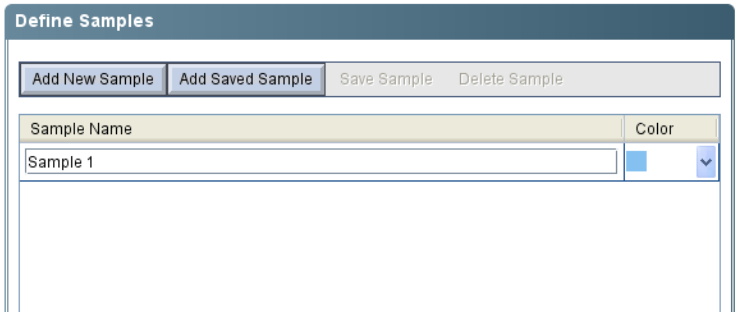


Table 1. *Salmonella* confirmatory PCR targets

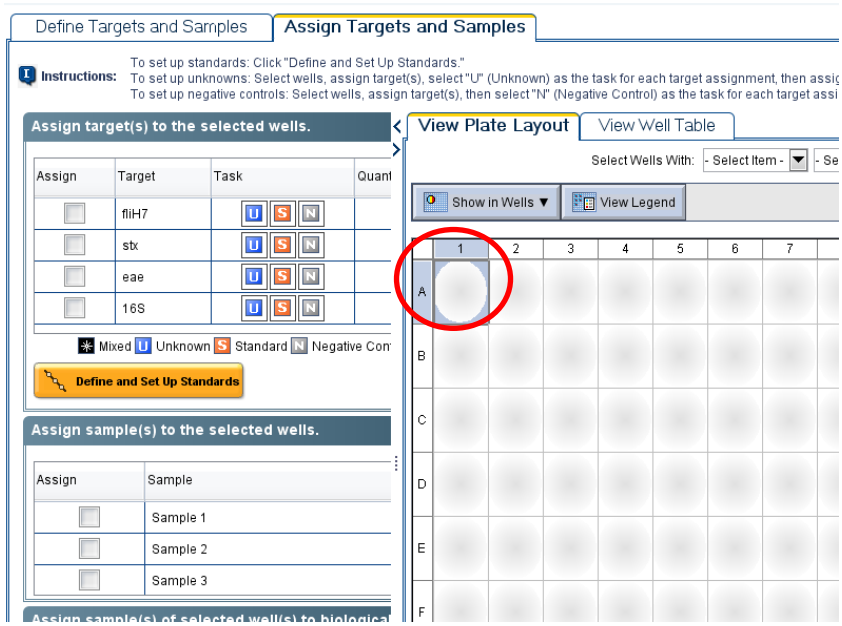
Target	Dye	Quencher
fliCH7	Cy3	None
stx	FAM	None
Eae	Joe	None
16S	Cy5	None

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

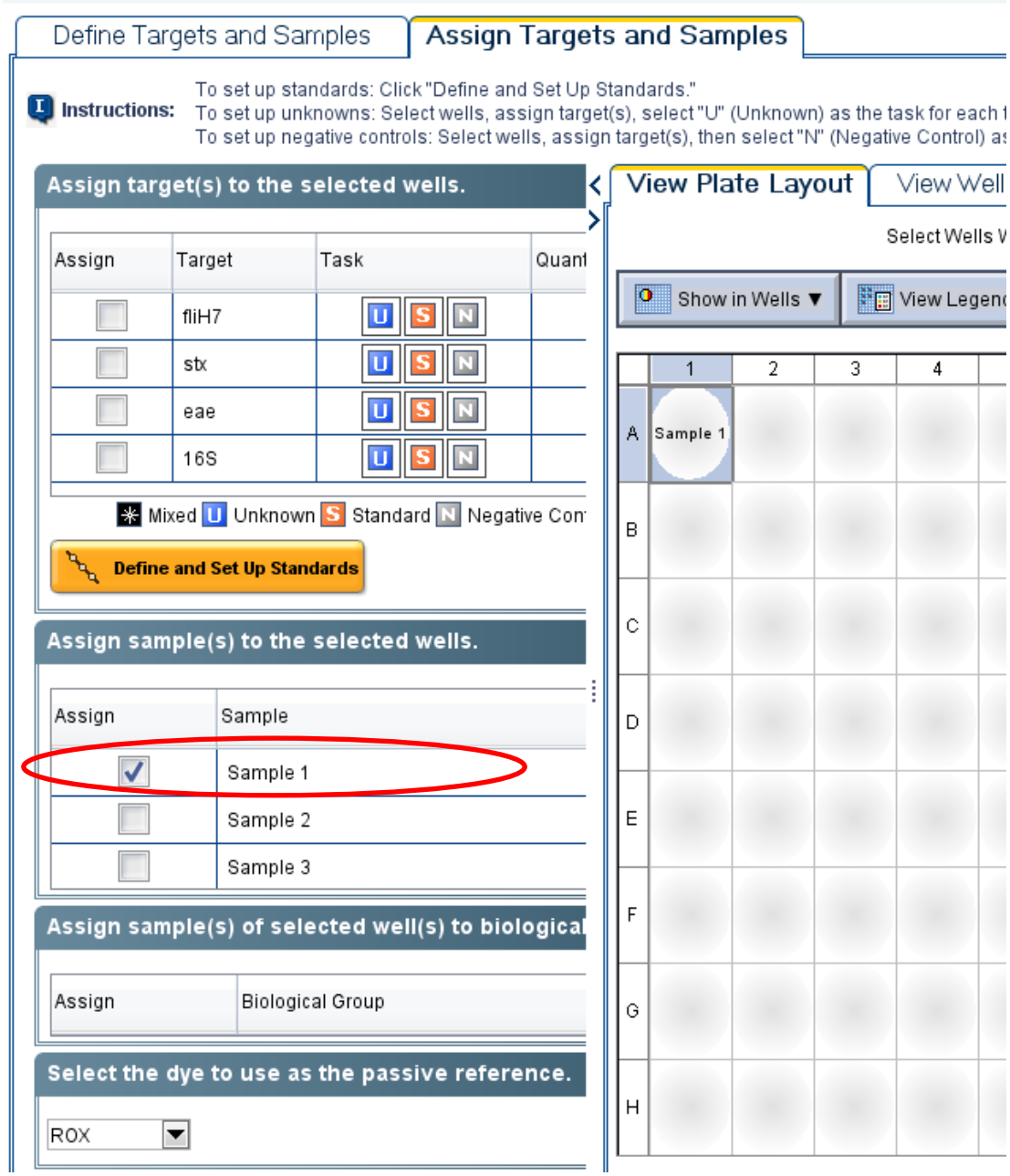


i. After you change the sample name (or do this after the run) and choose any color changes (optional) you want, click on the "Assign Target and Samples" tab.

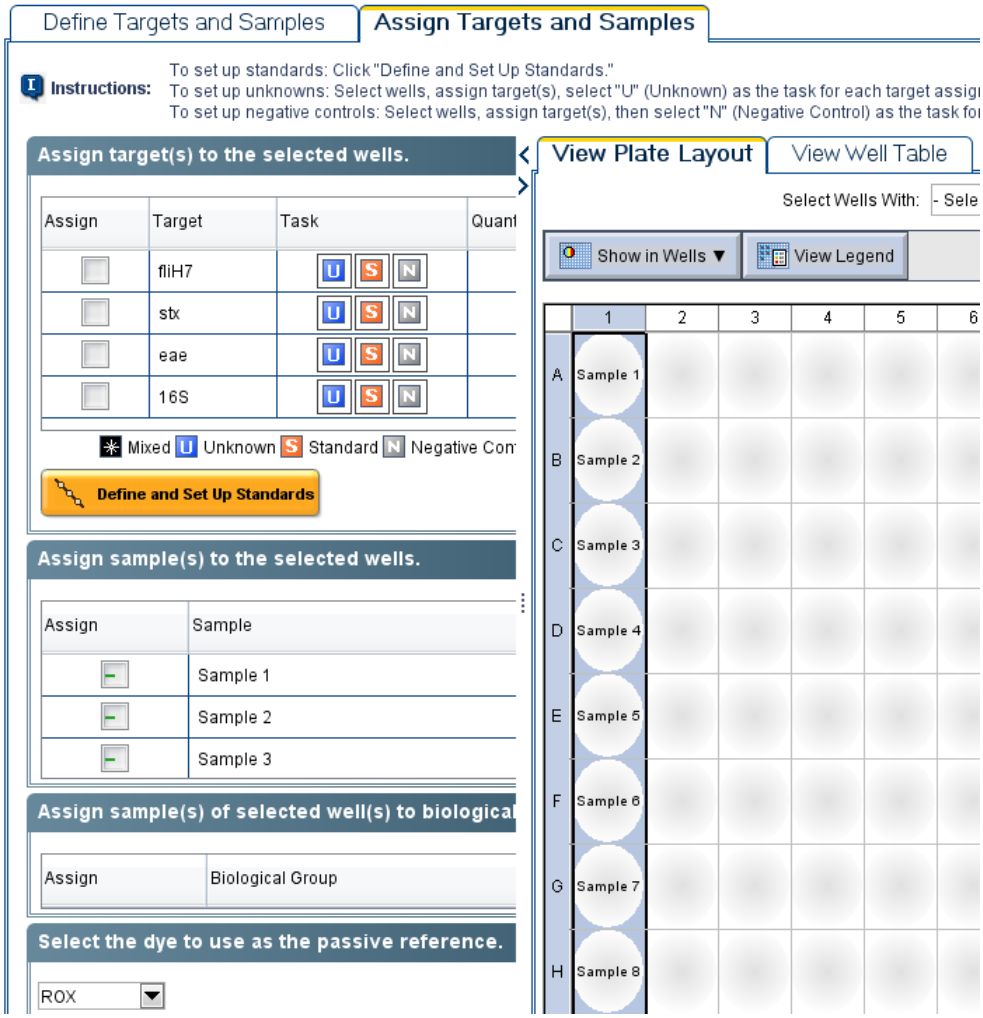
Highlight the well that you want to place the sample.



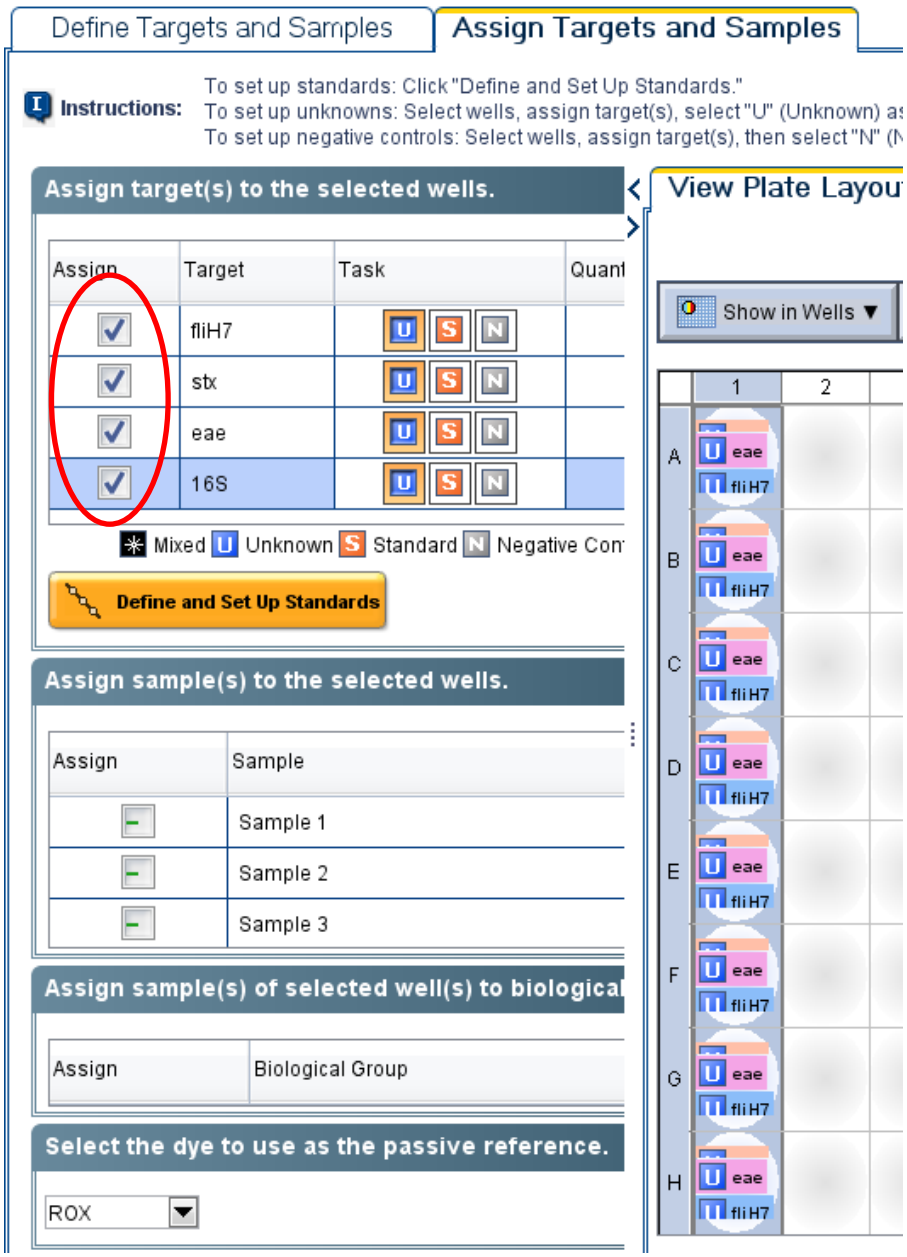
Select the sample from the assign samples box by checking the box. Repeat until all samples are placed in the correct locations.


Define Targets and Samples
Assign Targets and Samples
Instructions:
To set up standards: Click "Define and Set Up Standards."
To set up unknowns: Select wells, assign target(s), select "U" (Unknown) as the task for each
To set up negative controls: Select wells, assign target(s), then select "N" (Negative Control) as
Assign target(s) to the selected wells.
Assign
Target
Task
Quant
fliH7
stx
eae
16S
Mixed
Unknown
Standard
Negative Con
Define and Set Up Standards
Assign sample(s) to the selected wells.
Assign
Sample
Sample 1
Sample 2
Sample 3
Assign sample(s) of selected well(s) to biological
Assign
Biological Group
Select the dye to use as the passive reference.
ROX
View Plate Layout
View Well
Select Wells
Show in Wells
View Legend
1
2
3
4
A
B
C
D
E
F
G
H
Sample 1

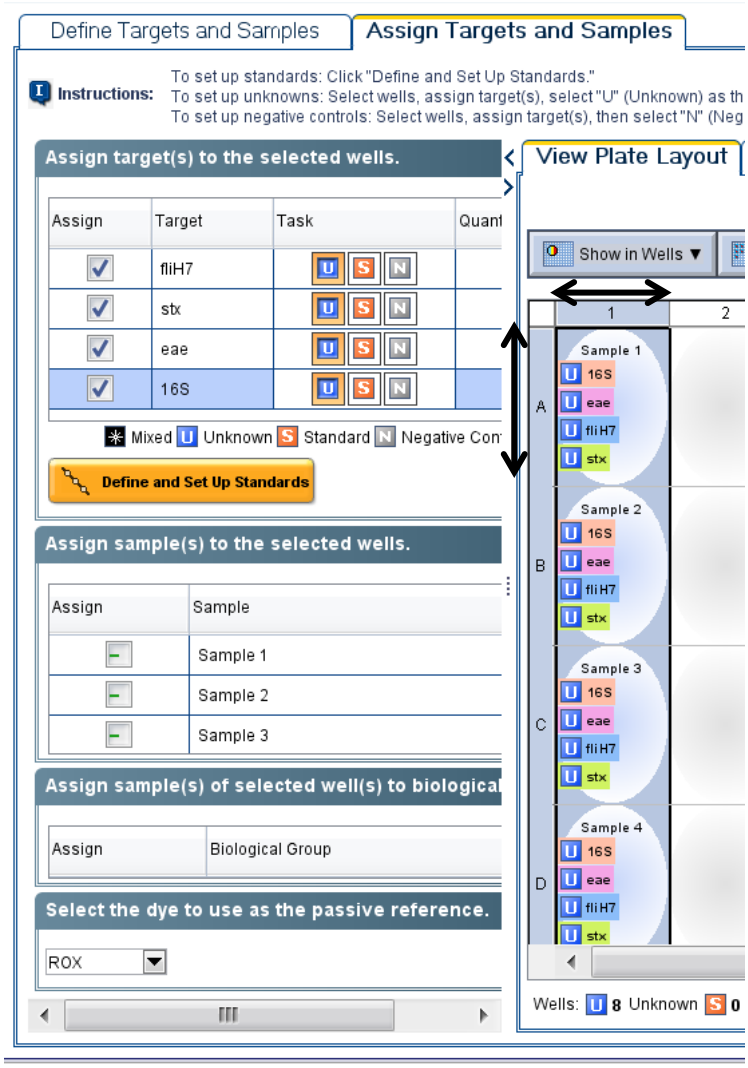
Highlight all wells that contain samples, hold down the CTRL button to add multiple wells.



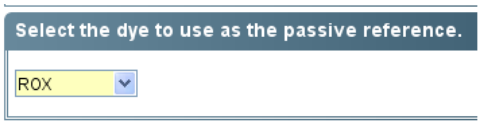
After you assign all of your samples to the wells, select the targets (fliH7, stx, eae and 16S) you want from the assign targets box. It will bring up 4 colors in each well box which is why it is best to put sample names before this step so that you can see them.


Define Targets and Samples
Assign Targets and Samples
Instructions:
To set up standards: Click "Define and Set Up Standards."
To set up unknowns: Select wells, assign target(s), select "U" (Unknown) as
To set up negative controls: Select wells, assign target(s), then select "N" (N
Assign target(s) to the selected wells.
View Plate Layou
Assign
Target
Task
Quant
fliH7
stx
eae
16S
Mixed
Unknown
Standard
Negative Con
Define and Set Up Standards
Show in Wells
1
2
A
B
C
D
E
F
G
H
eae
fliH7
Assign sample(s) to the selected wells.
Assign
Sample
Sample 1
Sample 2
Sample 3
Assign sample(s) of selected well(s) to biological
Assign
Biological Group
Select the dye to use as the passive reference.
ROX

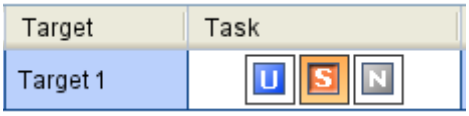
In order to see all of the targets and sample names in each well you may stretch the wells using the arrows. This will allow all the contents of the well to be seen.



j. Make sure the little box that is at the very bottom of the page says passive reference dye set to “ROX”.



k. **(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 that parameter should not require checking.



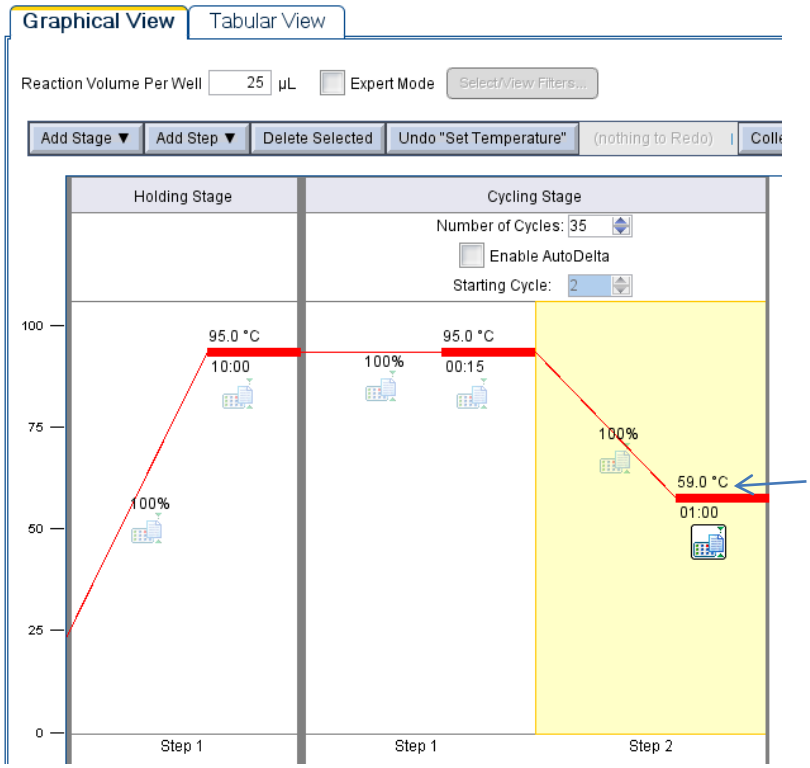
l. Click on the left tab to set-up run method.

Remove the first holding step by right clicking that box and deleting. Change the temperatures and cycles to the parameters defined in Table 2.

Table 2. PCR assay parameters

Number of Cycles	Temperature	Time
1 (Holding Stage)	95°C	10 minutes
35 (Cycling Stages)	95°C	15 seconds
	59°C	60 seconds

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.



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

Reaction Volume Per Well 25 µL

n. Prepare your PCR assay plate at this step.

A5 5C.4.3 Preparation of Master Mix

Perform this procedure in a designated pre-PCR area (clean bench or hood). Always wear gloves while working in this area. Keep the pipettors in this area only for PCR preparation. Keep all reagents and Master Mix at 2-8°C at all times.

Probes are light sensitive, so it is necessary to limit exposure to light as much as possible.

Use two controls for each assay:

- *E. coli* O157:H7, toxin positive
- No Template Control (NTC)– Molecular Grade water

a. Calculate the number of samples plus controls to be tested (plus at least one to compensate for pipettor error). Calculate the volume of each primer, probe, ABI® Environmental Mastermix and water to be added to make the master mix by following the chart below.

Reaction vol (µL)	25
Number of rxns	1

Component	µL
ABI® Mastermix	**12.50 µL x # of reactions**
primer 16S F (20uM working)	**0.20 µL x # of reactions**
primer 16S R (20uM working)	**0.20 µL x # of reactions**
fliCH7 F (100uM working)	**0.25 µL x # of reactions**
fliCH7 R (100uM working)	**0.25 µL x # of reactions**
Stx F (100uM working)	**0.31 µL x # of reactions**
Stx R (100uM working)	**0.31 µL x # of reactions**
Eae F (100um working)	**0.25 µL x # of reactions**
Eae R (100um working)	**0.25 µL x # of reactions**

16S P (5uM working)	**0.50 µL x # of reactions**
fliCH7 P (5uM working)	**1.0 µL x # of reactions**
Stx1 P (5uM working)	**1.25 µL x # of reactions**
Stx2 P (5uM working)	**1.25 µL x # of reactions**
Eae P (5uM working)	**1.0 µL x # of reactions**
dH2O	**0.475 µL x # of reactions**
Template DNA	**5uL**

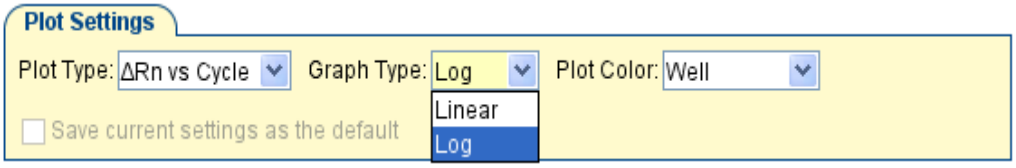
b. Add each reagent accordingly to a labeled microcentrifuge tube.
c. Mix by inversion or vortexing. Briefly centrifuge the mixture to remove any liquid from the lid.
d. Aliquot 20.0 µl of the master mix into the designated tubes or wells of the 96 well plate.
e. Add 5.0 µl of template to the appropriate tubes or wells.
f. Close the tubes.
g. Centrifuge the tubes or 96 well plate using a pulse spin setting. Ensure that the there are no bubbles present at the bottom of the wells.
h. 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.
i. 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 three columns of reactions (24 reactions), it is more economical to use the 96-well plates and seals.
j. Set the plate or strips into the holder and re-close using the indention on the right.
k. In the software interface, the temperature cycling graph should still be up. Click the large Green button labeled “Start Run”. Store the experiment run

data in a location accessible to the laboratory. If you do not want to make any changes to where the file is stored, click open and the machine will start running.

l. The start run button will turn from green to red and it will show the amount of time left and what step is running in the program.
m. Do not edit or click anything while it is running. There should be a graph showing all the raw data as the program proceeds.
n. After it is finished, take out your plate, close the door and turn off the machine.
o. 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.

A5 5C.4.4 Analyzing Data

a. To see the amplification curves, choose target: invA and/or 16S at the bottom left options panel. It starts by showing all three in the log phase. Change to linear phase.



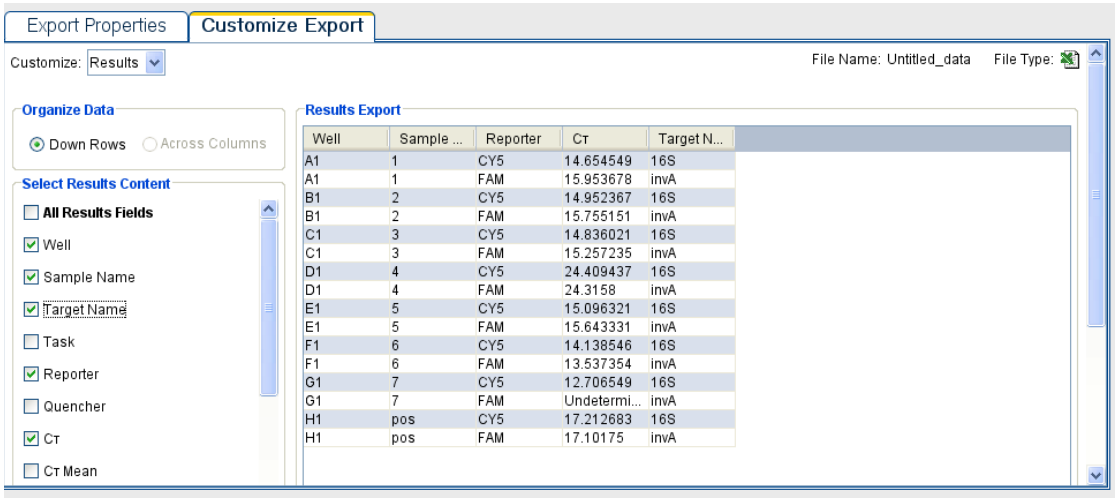
b. To see the threshold automatically determined by the software, check the show threshold box. Auto will be checked since the threshold has been set automatically. A thick line with the threshold for the selected target should come up. To see the threshold line and value, leave as is with all three and it will show three thresholds or click on a single target using the drop-down box.
c. 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 (e.g. sample name or location), click the green analyze button for the changes to take effect.

d. 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.

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

f. 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.



g. **(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.

h. Data can be analyzed directly from the ABI® 7500 FAST machine or from the exported file. A reported C_T value (e.g. 24.5 for fliH7) for a target indicates that the sample is positive for that target.