Confirmation of *Salmonella* isolates by Real-time Polymerase Chain Reaction (PCR)

This method is applicable to the confirmation of pure cultural isolates as *Salmonella* spp. Real-time PCR assembly and data analysis protocols are described below for two instrument platforms: SmartCycler II and Applied Biosystems 7500 Fast Real-time PCR thermocycler. Use of other platforms and protocols must first be validated per FDA microbiological methods validation guidelines (https://www.fda.gov/downloads/ScienceResearch/FieldScience/UCM298730.pdf) or other internationally recognized validation guidelines such as AOAC International's Appendix J (http://www.eoma.aoac.org/app_j.pdf) or the International Organization for Standardization's 16140 (www.iso.org).

A. Equipment and Materials

1. SmartCycler II PCR thermolcycler (Cepheid, Sunnyvale, CA) capable of performing cycling parameters described below and simultaneous real-time sequence detection for FAM, Texas Red, and Cy5 dyes.
2. ABI 7500 Fast Real-time PCR thermocycler (SDS version 1.4) (ThermoFisher Scientific, Waltham, MA), capable of performing cycling parameters described below and simultaneous real-time sequence detection for FAM, Texas Red, and Cy5 dyes.
3. SmartCycler PCR reaction tubes (minimum reaction volume of 25 µl; Cepheid # 900-0003) and racks (Cepheid # 900-0087) compatible with PCR thermocycler
4. MicroAmp™ Fast Optical 96-Well Reaction Plates (ThermoFisher Scientific # 4346906)
5. MicroAmp™ Optical Adhesive Film for 7500 Fast Plates (ThermoFisher Scientific # 4311971)
6. MicroAmp™ Fast Reaction 8-Tube Strips (ThermoFisher Scientific # 4358293)
7. MicroAmp™ Optical 8-Cap Strips (ThermoFisher Scientific # 4323032)
8. Appropriate ABI 7500 Fast Plate Holder (specific for 96-well tray or 8-strip well tubes)
9. Ice bucket and ice
10. Microcentrifuge tubes (0.5 to 2.0 mL)
11. Pipets (1-1000 µL volume)
12. Pippete tips (0.2 to 1000 µL volume) (aerosol resistant tips)
13. QIAprep Spin Miniprep Kit (QIAgen # 27104)
14. Qubit dsDNA BR Assay Kit (ThermoFisher Scientific # 32850 or 32853)
15. Sterile gloves
16. Vortex Mixer
17. Water bath or heat block capable of maintaining 100°C

B. Media and Reagents

1. Sterile Molecular Grade water
2. illustra™ PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare # 27955801)
3. Applied Biosystems™ TaqMan Fast Advance Master Mix (ThermoFisher Scientific # 4444556)
4. *Salmonella* primers and probes listed in Table 1 are specific to real-time PCR platforms being used.
   a. Primers - 10 µM working solution of each primer listed in Table 1. Stock (1000 µM) and working solutions can be prepared from commercially synthesized primers with basic desalt purification

(Biosearch Technologies or equivalent) by rehydrating with sterile molecular biology grade water to appropriate concentrations. Store at -20°C to -70°C non-frost-free freezer.

b. Probes - 10 µM working solution of each probe listed in Table 1. Dual hybridization probes should be purchased as Dual HPLC-purified and labeled as indicated in Table 1. Stock (100 µM) and working solutions can be prepared from commercially synthesized probes (Biosearch Technologies or equivalent) by rehydrating with sterile molecular biology grade water to appropriate concentrations. Working solutions should be aliquoted in small amounts and stored frozen (-20 to -70°C) and away from light until use to avoid fluorophore degradation.

c. Exogenous Internal Amplification Control (IAC)

IAC is a synthetic 100 bp sequence: 5'-
AGTTGCAGTGTAACCGTCATGTACCAGTAATCTGCGTCGCACGTGTGCACCTAGTCTA
ATCACTTATGACTCAGATAACTTAACAGCAGAGTCTCGTCGA.

IAC plasmid pCR2.1-InC (Plasmid #83959) is available through Addgene at https://www.addgene.org/83959. Host strain containing pCR2.1-InC plasmid can be cultured in LB (Lysogeny broth) or BHI (Brain Heart Infusion) broth containing either kanamycin (50µg/mL) or ampicillin (100µg/mL) at 37°C for 16 hours.

The pCR2.1-InC plasmid can be extracted from overnight culture by using QIAprep Spin Miniprep Kit. The plasmid concentration can be quantitated with Qubit dsDNA BR Assay Kit.

Table 1. Primer/probe sequences for use on SmartCycler II and ABI 7500 Fast platforms

Primers[1]	**Gen Bank #**	**Bases**	**5' → 3' Sequence**
*Sal*1598 F	U43273	20	AACGTGTTTCCGTGCGTAAT
*Sal*1859 R	U43273	20	TCCATCAAATTAGCGGAGGC
IAC F		22	AGTTGCAGTGTAACCGTCATGT
IAC R		22	TCGACGAGACTCTGCTGTTAAG
Probes[1]			
*Sal*1631PFAM		20	FAM-TGGAAGCGCTCGCATTGTGG-BHQ
IAC30PCy5		20	Cy5-ATCTGCGTCGCACGTGTGCA-BHQ

[1]Primer/Probe name composed of target (*Sal* = *Salmonella* species targeting *invA* gene, IAC = Internal Amplification Control), 5' base position of oligonucleotide in the respective gene sequence specified in column 3 and forward primer (F), reverse primer (R) or probe (P).

C. PCR Controls

1. For a positive PCR control, include a template prepared from *Salmonella enterica*, such as ATCC 8324, *Salmonella* Gaminara.
2. Always include a no template (water) negative control in every run.

D. DNA Template Preparation

1. Transfer 250 µL of overnight culture in BHI broth at 35±2°C to a 1.5 mL microcentrifuge tube; or suspend a colony from a 24 h fresh prepared BHI plate in 250 µL sterile water in a 1.5 mL microcentrifuge tube.
2. Centrifuge 10,000 × g for 5 min.
3. Remove the supernatant and completely resuspend pellet in 250 µL sterile water.
4. Boil templates for 20 min.
5. Immediately cool down on ice for 5 min.
6. Centrifuge at 10,000 × g for 5 min. Remove and save the supernatant as DNA template (This may be frozen, minimum -20°C, for future PCR tests).

E. Preparation of qPCR *Salmonella* Master Mix

1. Preparation of dehydrated qPCR *Salmonella* Master Mix

a. Mix all components in Table 2 in 1.5 mL microcentrifuge tube by vortex at top speed and centrifuge briefly.

b. Aliquot 5 µL Master Mix solution for an evaluation run with *Salmonella* and negative controls on SmartCycler II, and a second 5 µL aliquot for evaluation run on ABI 7500 Fast (see F for Reaction assembly).

c. When both evaluation runs were satisfactory, dispense 10 µL Master Mix solution per tube to sterile 1.5 mL black microcentrifuge tubes.

d. Dry the qPCR *Salmonella* Master Mix solution with tube lids open in a vacuum chamber for 2 to 3 days and shield the vacuum chamber completely from light.

e. The vacuum dried qPCR *Salmonella* Master Mix can be stored in aluminum porch with silica gel at ambient for 2 years.

f. Each tube of qPCR *Salmonella* Master Mix can carry out forty 25 µL reactions on SmartCycler II or fifty 20 µL reactions on ABI 7500 Fast systems.

Table 2. Recipe for **100 tubes** of qPCR *Salmonella* Master Mix

Component	**Volume (µL)**
Primer *Sal*1598 F (1000 µM Solution)	16.0
Primer *Sal*1859 R (1000 µM Solution)	16.0
Primer IAC F (1000 µM Solution)	6.7
Primer IAC R (1000 µM Solution)	6.7
Probe *Sal*1631PFAM (100 µM Solution)	200.0
Probe IAC30PCy5 (100 µM Solution)	250.0
IAC DNA template (0.75 pg/µL)	25.0
20% sucrose (0.22 µM filter-sterilized)	130.0

PCR grade water	349.6
Total	1000.0

2. Preparation of wet qPCR *Salmonella* Master Mix

a. Mix all components in Table 3 in 1.5 mL black microcentrifuge tube by vortex at top speed and centrifuge briefly.

b. Dilute to 1X or 2.5X for reaction set up accordingly (see F for Reaction assembly).

c. Perform a QA run for every batch of wet master mix on SmartCycler II and ABI 7500 Fast.

d. Shield the wet qPCR *Salmonella* master mix from light and stored at 4°C up to two months.

Table 3. Recipe for **1 tube** of qPCR *Salmonella* Master Mix (10X)

Component	**Volume (µL)**
Primer *Sal*1598 F (10 µM Solution)*	16.0
Primer *Sal*1859 R (10 µM Solution)*	16.0
Primer IAC F (10 µM Solution)*	6.7
Primer IAC R (10 µM Solution)*	6.7
Probe *Sal*1631PFAM (10 µM Solution)**	20.0
Probe IAC30PCy5 (10 µM Solution)**	25.0
IAC plasmid DNA template (0.075 pg/µl)***	2.5
20% sucrose (0.22 µM filter-sterilized)	1.3
PCR grade water	5.8
Total	100.0

* 10 µM primer working solutions were used.

** 10 µM probe working solutions were used.

*** 10-fold diluted IAC template was used.

F. Real-Time PCR assembly

1. Reaction assembly for SmartCycler II (Table 4)

Table 4. SmartCycler II Amplification Reaction Components

Volume (µL) **/rxn**	**Component**
22.5	1X working solution qPCR *Salmonella* Master Mix (1X MM) [1]
1.5 beads	illustra PuReTaq Ready-To-Go PCR beads
2.5	Template (Sample or control)

[1] Resuspend one tube of dehydrated *Salmonella* Master Mix in 900 µL of PCR grade water to make 1X MM.

[1]Add 800 µL of PCR grade water to 1 tube of wet qPCR *Salmonella* master mix to make 1X MM.

Note: Each 900 µL of 1X qPCR *Salmonella* master mix tube will need 60 PCR beads for total 40 reactions.

2. Reaction Assembly for ABI 7500 Fast (Table 5)

Table 5. ABI 7500 Fast Amplification Reaction Components

Volume (µL) /rxn	**Component**
8.0	2.5X working solution of qPCR *Salmonella* Master Mix (2.5X MM) [2]
10.0	TaqMan Fast Advance Master Mix
2.0	Template (Sample or control)

[2] Resuspend one tube of dehydrated *Salmonella* Master Mix in 400 µl of PCR grade water to make 2.5X MM.

[2]Add 300 µl of PCR grade water to 1 tube of wet qPCR *Salmonella* master mix (10X) to make 2.5X MM.

Note: Each 400 µl of 2.5X qPCR *Salmonella* master mix tube will need 500 µl TaqMan Fast Advance Master Mix for total 50 reactions.

G. Real-Time PCR run

1. Run on SmartCycler II

a. Create run on SmartCycler II.

Give each run a unique run name, select Dye set FCTC25, select 2-step PCR protocol as described below and assign appropriate sites on SmartCycler block.

Initial Activation	Each of 50 Cycles	
95°C, 180 sec	95°C, 15 sec	60°C, 30 sec
Optics Off	Optics Off	Optics On

b. Qualitative analysis setting

On SmartCycler II Instrument, set the following analysis settings for FAM, and Cy5 channels. Update analysis settings if they are changed before recording results.

Usage	Assay
Background subtraction	ON
Background Min. Cycle	5
Background Max. Cycle	40
Curve Analysis	Primary
Threshold Setting	Manual
Manual Threshold Fluorescence Units	20.0
Auto Min Cycle	5

Auto Max Cycle	10
Valid Min Cycle	3
Valid Max. Cycle	60
Boxcar Avg. Cycles	0

c. Report of Result

i. Primary fluorescence curves that cross the threshold will be recorded as "POS".

ii. The cycle the sample crossed the threshold will be displayed in the Results Table view.

iii. Results can also be viewed graphically.

iv. A report can be generated or a screen capture of the results table view can be used to record data (see Figure 1 for an example).

d. Interpretation of Results

If *inv*A gene target in the FAM and IAC in the Cy5 channels are POS, the isolate is a *Salmonella* spp. If only the Cy5 channel is POS, the isolate is a *non-Salmonella* spp.

2. Run Real-Time PCR on ABI 7500 Fast

a. Turn on the computer and ABI 7500 Fast.

b. Launch ABI 7500 Fast System Software v1.4.2.

c. Create run by using New Document Wizard.

d. Highlight all wells and select “Detector Manager” under “Tool”.

e. Shift select *Salmonella* (FAM) and IAC (Cy5) and click “Add to Plate Document.

f. Under “Well Inspector” in “View”, mark both “*Salmonella*” and “IAC”, and select “ROX” for Passive Reference.

g. Under “Instrument”, select 2-step PCR protocol as described below.

Activation of UDG	Initial Activation	Each of 50 Cycles	
50°C, 2 min	95°C, 5 min	95°C, 3 sec	60°C, 30 sec

h. Save the newly created file as template for *Salmonella* in **.sdt** format. The *Salmonella*_ template.**sdt** file can be used as template for future .sds run file with all preset parameters.

i. Save the file again in .**sds** format as a run file with a different name such as “*Salmonella*_Test.**sds**”.

j. Assign appropriate sites with sample names on corresponding wells and save the file.

k. Load the samples and start the run under “Instrument”. If the “Start” button under the “Instrument” is not highlighted, close and re-open the “*Salmonella*_Test.sds” file to initialize ABI 7500 Fast System. Start the run by clicking the “Start” button.

l. After the reaction completed successfully, the results can be analyzed, viewed and reported (see Figure 2 for an example).

Analysis setting:

i. Manual Ct

ii. Threshold: **0.05** (w/ROX)

iii. Manual Baseline

Start (cycle): 3

End (cycle): 15

m. Interpretation of Results

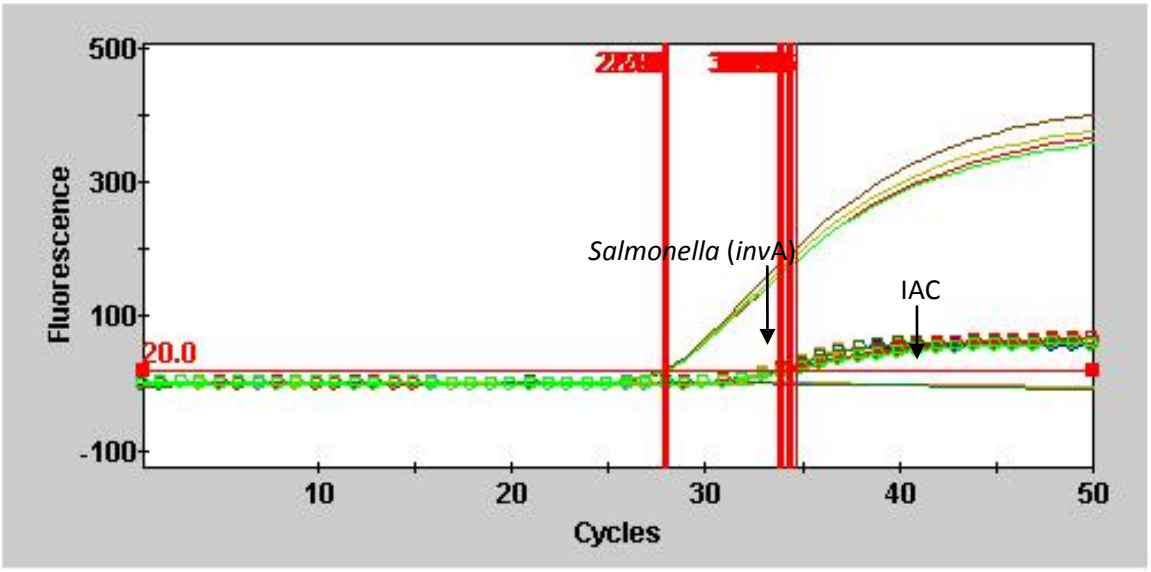
If both *Salmonella* target (invA gene) in the FAM channel and InC in the Cy5 channels are positive with Ct values, the isolate is a *Salmonell*a spp. If only the Cy5 (InC) channel is positive with a Ct value and undetected (undetermined) in FAM channel, the isolate is a non-*Salmonella* spp. For each sample, check if it has a normal amplification plot as shown in Figure 2.

Figure 1. Example of results output from Smart Cycler II

1A. Results table view for *Salmonella enterica*, ATCC 8324 and negative control.

Views	Site ID	Protocol	Sample ID	Sample Type	Notes	Status	FAM Std/Res	FAM Ct	Cy5 Std/Res	Cy5 Ct
Results Table	A1	Salmonella	Neg-1	UNKN		OK	NEG	0.00	POS	33.85
Analysis Settings	A2	Salmonella	Neg-2	UNKN		OK	NEG	0.00	POS	33.94
Protocols	A3	Salmonella	Neg-3	UNKN		OK	NEG	0.00	POS	33.73
NTM of Melt	A4	Salmonella	Neg-4	UNKN		OK	NEG	0.00	POS	33.90
Temperature	A5	Salmonella	SLM 8324-1	UNKN		OK	POS	27.88	POS	34.35
Intercalate	A6	Salmonella	SLM 8324-2	UNKN		OK	POS	27.80	POS	34.44
Melt	A7	Salmonella	SLM 8324-3	UNKN		OK	POS	28.07	POS	34.26
FAM	A8	Salmonella	SLM 8324-4	UNKN		OK	POS	27.95	POS	34.66
Cy3										
Texas Red										
Cy5										
Standard										
FAM-Cy5										

1B. Results: Graphical view for *Salmonella enterica* ATCC 8324 and negative control.



Site Legend

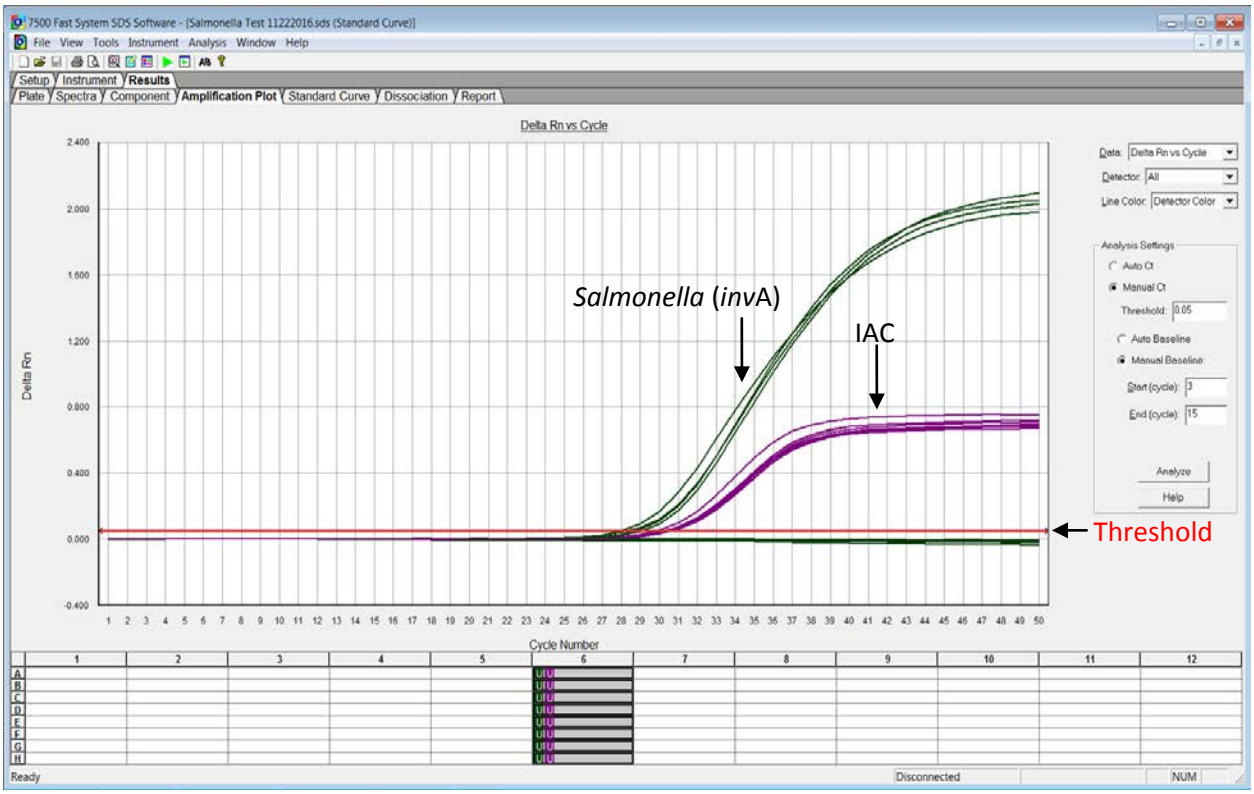
Site ID	Sample ID	FAM Ct	Cy5 Ct	Protocol
A1	Neg-1	0.00	33.85	Salmonella
A2	Neg-2	0.00	33.94	Salmonella
A3	Neg-3	0.00	33.73	Salmonella
A4	Neg-4	0.00	33.90	Salmonella
A5	SLM 8324-1	27.88	34.35	Salmonella
A6	SLM 8324-2	27.80	34.44	Salmonella
A7	SLM 8324-3	28.07	34.26	Salmonella
A8	SLM 8324-4	27.95	34.66	Salmonella

Data Type	Line Type
Primary Curve	———

Channel	Symbol
FAM	None
Cy5	▷ ▷ ▷

Figure 2. Example of results output from ABI 7500 Fast

2A. Amplification Plot *Salmonella* (FAM) and IAC (Cy5)



Amplification Plot

Data: Delta Rn vs. Cycle

Detector: All,

Line Color: "Well color" or "Detector Color"

Analysis Settings

Manual Ct

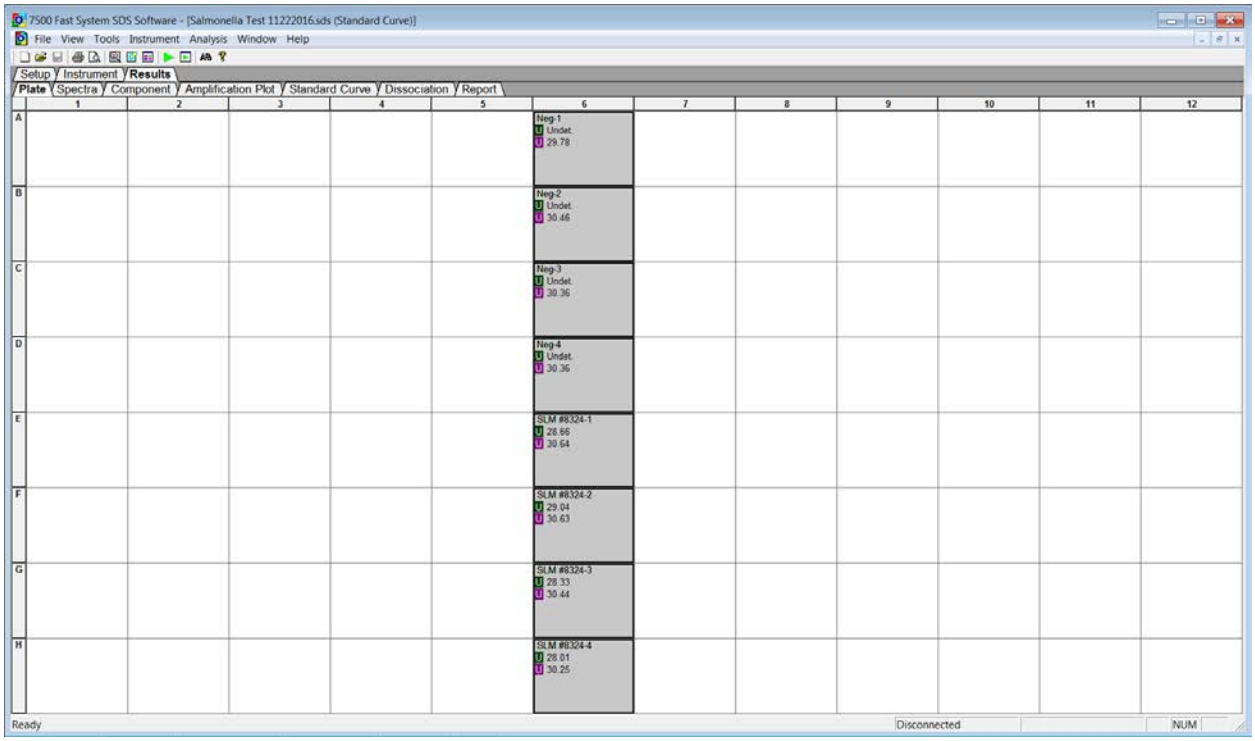
Threshold: **0.05** (w/ROX)

Manual Baseline

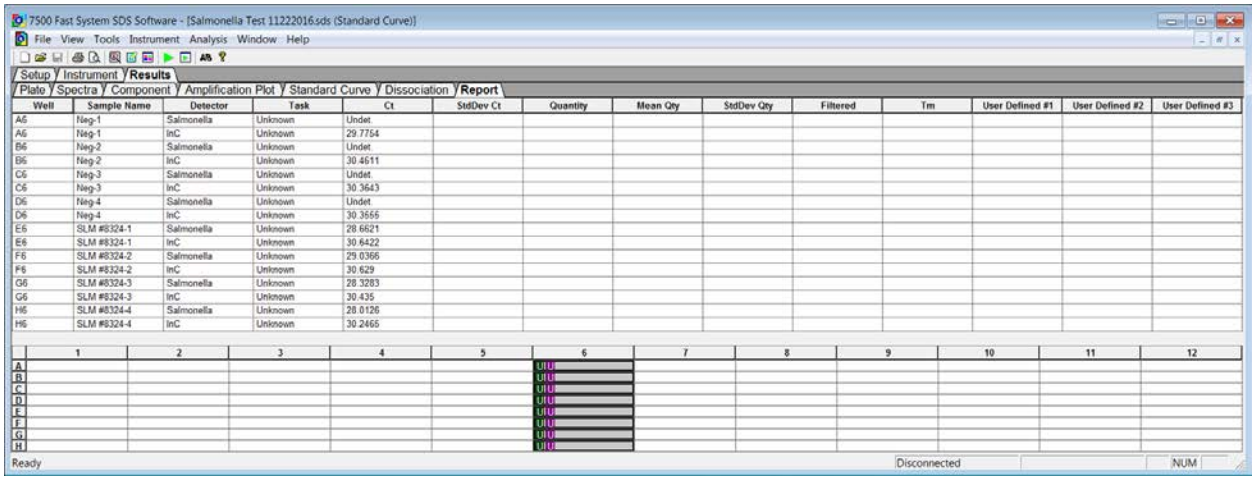
Start (cycle): 3

End (cycle): 15

2B. Plate



3C. Report



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm	User Defined #1	User Defined #2	User Defined #3
A6	Neg-1	Salmonella	Unknown	Undet.									
A6	Neg-1	InC	Unknown	29.7754									
B6	Neg-2	Salmonella	Unknown	Undet.									
B6	Neg-2	InC	Unknown	30.4611									
C6	Neg-3	Salmonella	Unknown	Undet.									
C6	Neg-3	InC	Unknown	30.3643									
D6	Neg-4	Salmonella	Unknown	Undet.									
D6	Neg-4	InC	Unknown	30.3666									
E6	SLM #8324-1	Salmonella	Unknown	28.6621									
E6	SLM #8324-1	InC	Unknown	30.6422									
F6	SLM #8324-2	Salmonella	Unknown	29.0366									
F6	SLM #8324-2	InC	Unknown	30.629									
G6	SLM #8324-3	Salmonella	Unknown	28.3283									
G6	SLM #8324-3	InC	Unknown	30.435									
H6	SLM #8324-4	Salmonella	Unknown	28.0126									
H6	SLM #8324-4	InC	Unknown	30.2465									

InC: internal amplification control (IAC)