Confirmation of *Salmonella* isolates by real-time quantitative PCR (qPCR)

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 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. 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.
2. MicroAmp™ Fast Optical 96-Well Reaction Plates (ThermoFisher Scientific # 4346906)
3. MicroAmp™ Optical Adhesive Film for 7500 Fast Plates (ThermoFisher Scientific # 4311971)
4. MicroAmp™ Fast Reaction 8-Tube Strips (ThermoFisher Scientific # 4358293)
5. MicroAmp™ Optical 8-Cap Strips (ThermoFisher Scientific # 4323032)
6. Appropriate ABI 7500 Fast Plate Holder (specific for 96-well tray or 8-strip well tubes)
7. Ice bucket and ice
8. Microcentrifuge tubes (0.5 to 2.0 mL)
9. Pipettes (1-1000 µL volume)
10. Pipette tips (0.2 to 1000 µL volume) (aerosol resistant tips)
11. QIAprep Spin Miniprep Kit (QIAgen # 27104)
12. Qubit dsDNA BR Assay Kit (ThermoFisher Scientific # 32850 or 32853)
13. Sterile gloves
14. Vortex Mixer
15. Water bath or heat block capable of maintaining 100°C

B. Media and Reagents

1. Sterile molecular grade water
2. Applied Biosystems™ TaqMan Fast Advance Master Mix (ThermoFisher Scientific # 4444556)
3. *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 (50 µg/mL) or ampicillin (100 µg/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 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 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 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 the 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 ABI 7500 Fast (Table 4)

Table 4. 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 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).

   Amplification Plot Setting:

   i. Data: Delta Rn vs. Cycle
   ii. Detector: All
   iii. Line Color: "Well color" or "Detector Color"

   Analysis Setting:

   i. Manual C_T
   ii. Threshold: **0.05** (w/ROX)
   iii. Manual Baseline

   Start (cycle): 3

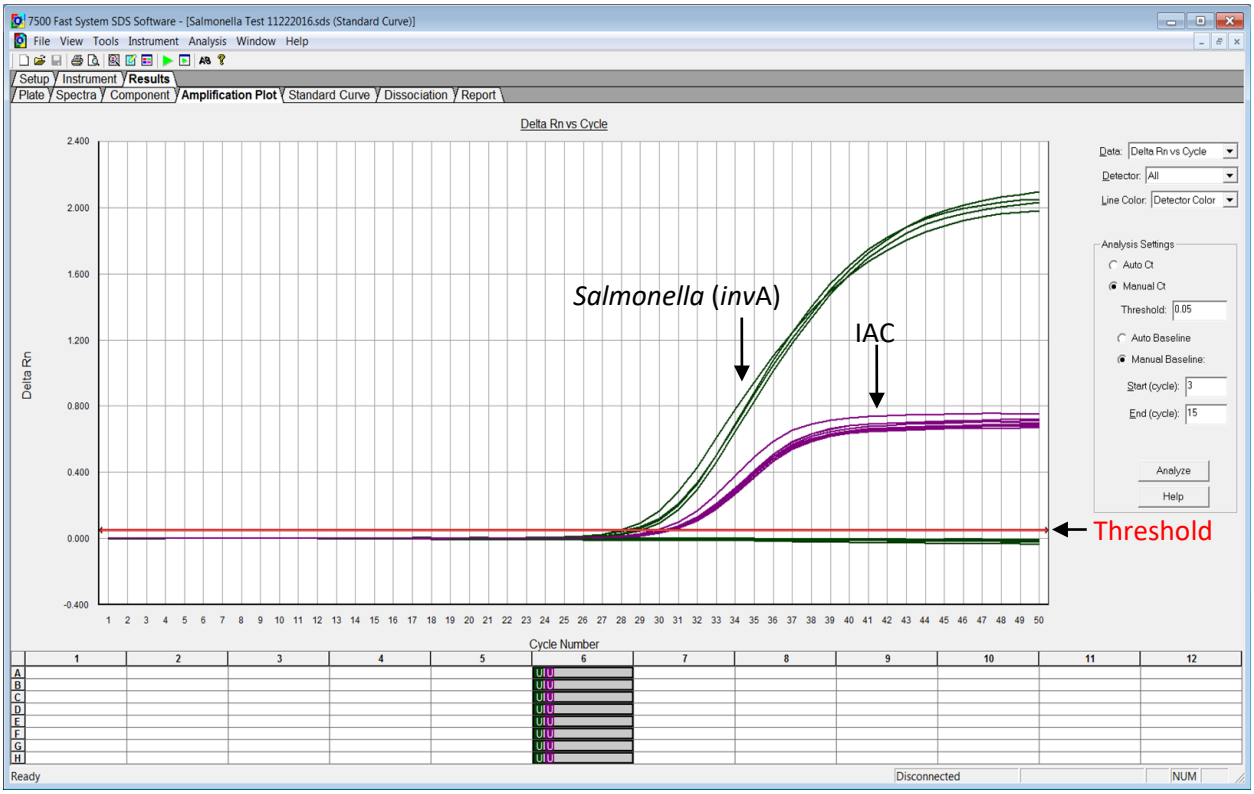
   End (cycle): 15

m. Interpretation of result

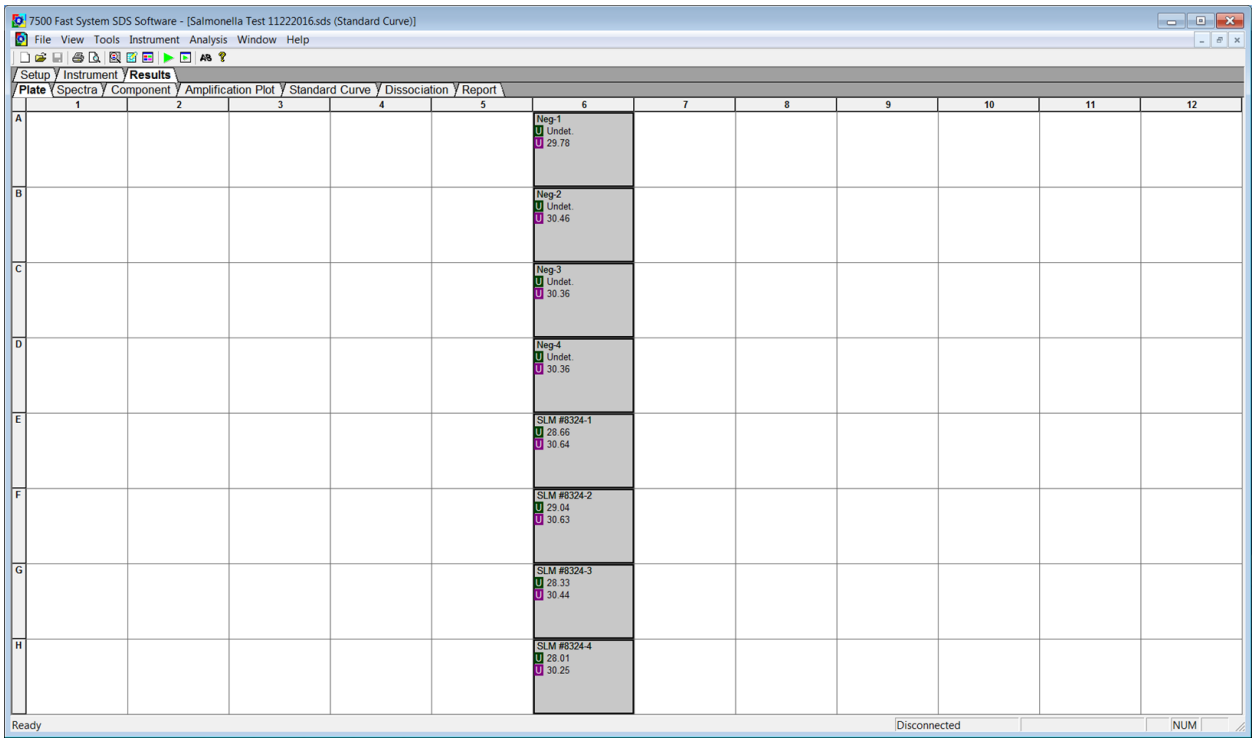
   If both *Salmonella* target (*invA* gene) in the FAM channel and IAC in the Cy5 channels are positive with Ct values, the isolate is a *Salmonell*a spp. If only the Cy5 (IAC) channel is positive with a Ct value and undetected (undetermined) in FAM channel, the isolate is a non-*Salmonella* spp. For each sample, check if it has a normal amplification plot as shown in Figure 1A.

Figure 1. Example of results output from ABI 7500 Fast

1A. View of *Salmonella* amplification plot. The PCR cycle number is shown on the x-axis, and the magnitude of normalized fluorescence (ΔRn) generated at each cycle is shown on the y-aixs.



1B. View of plate layout of qPCR results from *Salmonella* (ATCC 8324) and negative controls (water). Each well displays sample name and Ct values of *Salmonella* target and IAC.



1C. View of report of qPCR results from *Salmonella* (ATCC 8324) and negative controls (water).This report lists all the information related to each well.