U.S. FOOD & DRUG ADMINISTRATION

Salmonella qPCR Protocol

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Introduction

This FDA-developed quantitative PCR (qPCR) method is applicable for the confirmation of pure cultural isolates as *Salmonella* subspecies and for the rapid screening of *Salmonella* in food and environmental swab samples using the Applied Biosystems™ (ABI) 7500 Fast Real-Time PCR System. The method targets the *Salmonella* invasion gene (*invA*), which has been shown to be involved in the internalization of *S.* Typhimurium in mammalian epithelial cells (*1–4*). This gene is unique to *Salmonella* (*3,5*), and its DNA sequence is highly conserved among *Salmonella* spp. (*2,4,6*). The method uses custom-designed primers and a TaqMan® probe to amplify a 262-bp fragment of the *Salmonella*-specific *invA* gene with stringent specificity (*7,8,9*) and includes a custom-designed internal amplification control (*9*) to identify false-negative results caused by PCR inhibitors commonly found in food matrices.

This qPCR method has been demonstrated to be an effective and rapid screening tool for a broad range of sample types, including fruits, fresh leafy green vegetables, and herbs (blackberry, blueberry, raspberry, strawberry, baby spinach, cabbage, iceberg lettuce, romaine lettuce, spring mix, basil, cilantro, culantro, parsley, dill, oregano, watercress, sprouts, cantaloupe, and whole onion); low-moisture foods (almond, almond butter, chia seed powder, dried cereal, dried egg noodle, infant formula, peanut butter, pine nuts, soy formula, walnuts, all-purpose flour, cake mixes, cookies, egg powder, infant cereal, whey-based infant formula, nonfat dehydrated milk, cocoa powder, and collagen hydrolysate); seafoods (frozen fish, frozen shrimp, raw oysters, and smoked fish); whole shell eggs, liquid egg; raw milk, soft cheese, ice cream; spices (crushed red pepper, ground basil, ground black pepper, ground cumin, ground white pepper, paprika, and red chili flakes); environmental surfaces (plastic, stainless steel, ceramic tile, rubber, and cast iron); sprout spent irrigation water (SSIW), recycled washing water from different food manufacturers and multiple animal feeds (chick feed, premium alfalfa pellets, wheat bran, and whole oats). These matrices were prepared using either a soak or blend procedure across a series of Single-Laboratory Validation (SLV) studies (*7,8,9,10*). The method was further shown to be reproducible, sensitive, and specific in two Multi-Laboratory Validation (MLV) studies (*11*). The Limit of Detection at 50% (LOD_{50}), the concentration at which 50% of tests yield a positive result, was 0.811 CFU/25 g for the qPCR method and 0.837 CFU/25 g for the BAM culture method in the MLV baby spinach study (*11*), and 0.75 CFU/25 g for both the qPCR and culture methods in the MLV frozen fish study *(12).*

The qPCR assembly and data analysis procedures described in this protocol are specific to the ABI 7500 Fast Real-Time PCR System. Use of alternative platforms or protocols must first be validated in accordance with FDA microbiological methods validation guidelines (https://www.fda.gov/media/83812/download) or other internationally recognized validation guidelines, such as AOAC International's Appendix J: Guidelines for validation of microbiological methods for food and environmental

surfaces (http://www.eoma.aoac.org/app_j.pdf) or ISO 16140-2:2016 Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method (www.iso.org).

A. Equipment and Materials

1. ABI 7500 Fast Real-time PCR System (Software version 1.4, 2.04. 2.3) (ThermoFisher Scientific, Waltham, MA)
2. MicroAmp™ Fast Optical 96-Well Reaction Plates (ThermoFisher Scientific Cat# 4346906)
3. MicroAmp™ Optical Adhesive Film for 7500 Fast Plates (ThermoFisher Scientific Cat# 4311971)
4. Appropriate ABI 7500 Fast Plate Holder (for 96-well tray or 8-well strip tubes)
5. Microcentrifuge tubes (0.5 to 2.0 mL)
6. Sterile black microcentrifuge tubes (1.5 mL)
7. Aluminum foil pouch
8. Silica gel packets 10 gram (Fisher Scientific Cat# 50-930-892)
9. Pipettes (1-1000 µL volume)
10. Pipette tips (0.2 to 1000 µL volume) (aerosol resistant tips)
11. Serological pipettor (1-1000 µL)
12. QIAprep Spin Miniprep Kit (QIAgen Cat# 27104)
13. Qubit dsDNA BR Assay Kit (ThermoFisher Scientific Cat# 32850 or 32853)
14. Vortex Mixer
15. Water bath or heat block capable of maintaining 100°C

B. Media and Reagents

1. Sterile DNase/RNase-free molecular biology-grade water
2. InstaGene™ matrix (Bio-Rad Cat# 7326030)
3. PrepManTM Ultra reagent (ThermoFisher Scientific Cat# 4318930)
4. Applied Biosystems™ TaqMan Fast Advance Master Mix (ThermoFisher Scientific Cat# 4444556)
5. *Salmonella* primers and probes listed in **Table 1** are specific to real-time PCR platforms being used.
    a. **Primers** – Prepare a 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, Integrated DNA Technologies [IDT], or equivalent) by rehydrating with sterile molecular biology-grade water to appropriate concentrations. Store at -20°C

to -70°C non-frost-free freezer. Note: Frost-free freezers cycle between warm and cold temperatures, which can degrade primer solutions.

b. **Probes** – Prepare a10 µM working solution of each probe listed **in Table 1**. Dual hybridization probes should be purchased as HPLC-purified and labeled as indicated in **Table 1**. Stock (100 µM) and working solutions can be prepared from commercially synthesized probes (Biosearch Technologies, Integrated DNA Technologies [IDT], or equivalent) by rehydrating with sterile molecular biology-grade water to appropriate concentrations. Working solutions should be aliquoted in small volumes, stored frozen at 20°C to -70°C, and protected from light until use to prevent fluorophore degradation.

c. Exogenous Internal Amplification Control (IAC)

IAC is a synthetic 100 bp sequence:

5'-AGTTGCAGTGTAACCGTCATGTACCAGTAATCTGCGTCGCACGTGTGCACCTAGTCTAATCACTTATGACTCAGATAACTTAACAGCAGAGTCTCGTCGA-3'.

The IAC plasmid pCR2.1-InC (Plasmid #83959) is available through Addgene at https://www.addgene.org/83959/. The host strain containing pCR2.1-InC plasmid (4029 bp) can be cultured in Lysogeny broth (LB) or Brain Heart Infusion (BHI) broth supplemented with kanamycin (50 µg/mL) or ampicillin (100 µg/mL) at 37°C for 16 hours.

The plasmid can be extracted from overnight culture using QIAprep Spin Miniprep Kit, and quantified with Qubit dsDNA BR Assay Kit, with an expected yield of 5 to 10 µg per preparation at a final concentration of 100-200 ng/µl in 50 µl of eluent.

Table 1. Primer/probe sequences for ABI 7500 Fast platforms

Primers*	**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*			
*Sal*1631PFAM		20	FAM-TGGAAGCGCTCGCATTGTGG-BHQ
IAC30PCy5		20	Cy5-ATCTGCGTCGCACGTGTGCA-BHQ

* 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. **Positive PCR Control:** Use a template prepared from *Salmonella* Typhimurium or any other serovar from *Salmonella enterica subsp. enterica*.
2. **Negative PCR Control:** Include a no-template control (NTC), using DNase/Rnase-free water, in every run

D. Sample Preparation and Preenrichment

Follow the BAM Chapter 5: *Salmonella* for sample preparation and preenrichment. A Whirl-Pak bag with filter is recommended for sample preenrichment. **Do not shake the sample bag after pre-enrichment**. Use a serological pipettor, transfer a 1 mL aliquot of preenriched cultures from the upper portion of the container into a 1.5 mL safe-lock microcentrifuge tube for DNA extraction. **Do not use non-serological pipettors.**

E. DNA Template Preparation from Preenriched Culture

1. Spin-boil Method

The spin-boil method is suitable for food samples prepared using soak sample preparation; however, for foods with high fat content, such as whole milk, powdered infant formula or nuts etc., use the modified spin-boil method in Section E(2) or the automatic DNA extraction methods in Section E(3).

(1) **Do not shake the sample bag after preenrichment.** Using a serological pipettor, transfer a 1 mL aliquot of the preenriched culture from the upper portion of the filtered side of the sample bag into a 1.5 mL safe-lock microcentrifuge tube.

(2) Centrifuge at 15,000 × g (RCF) for 5 minutes.

(3) Carefully remove and discard the supernatant, taking care not to disturb the pellet.

Optional wash step to remove PCR inhibitors: Resuspend the pellet in 1,000 µL of sterile DNase/RNase-free water by vertexing, ensuring the pellet is fully suspended. Centrifuge at 15,000 × g (RCF) for 5 minutes. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

(4) Resuspend the pellet in 200 µL sterile DNase/RNase-free water by vertexing, ensuring the pellet is fully suspended.

(5) Heat the suspension to 100°C in a heating block or boiling water bath for 10 min, then cool on ice for 5 minutes.

(6) Centrifuge at 15,000 × g (RCF) for 5 min.

(7) Carefully remove and retain the supernatant as the DNA template. The DNA template may be stored at −20°C or below for future use.

Note: Preenrichment broth residues in the DNA extract may cause qPCR IAC failure, indicated by a late C_T value or an undetermined result. If inhibition is suspected, repeat the qPCR using one of the following approaches:

- Dilution: Mix 5 µL of DNA extract with 45 µL DNase/RNase-free water (1:10) in a new microfuge tube.
- Purification: Use a commercially available DNA purification kit to purify the DNA extract, following the kit manufacturer's instructions.

2. Modified Spin-boil Method

The modified spin-boil method utilizes either InstaGene™ matrix or PrepMan™ Ultra reagent to bind or inactivate PCR inhibitors present in the sample, thereby improving the quality of the DNA extract. The method is suitable for food samples prepared by soaking or blending.

(1) **Do not shake the sample bag after preenrichment.** Using a serological pipettor, transfer a 1 mL aliquot of the preenriched culture from the upper portion of the filtered side of the sample bag into a 1.5 mL safe-lock microcentrifuge tube.

(2) Centrifuge at 900 × g (RCF) for 1 minute to remove large particles, then transfer 500 µL supernatant to a new microcentrifuge tube.

(3) Centrifuge 15,000 × g (RCF) for 5 minutes.

(4) Carefully remove and discard the supernatant, taking care not to disturb the pellet.

(5) Thoroughly vortex the InstaGene™ matrix or PrepMan™ Ultra reagent before use. Using a 1,000 µL pipette tip, add 100 µL of InstaGene™ matrix to the pellet and vortex to mix well, ensuring the pellet is fully suspended.

(6) Heat the suspension to 100°C in a heating block or boiling water bath for 10 minutes, vortex for 10 seconds, then centrifuge at 15,000 × g (RCF) for 5 minutes.

(7) Transfer 50 µL of the supernatant to a new microcentrifuge tube and retain as the DNA template. The DNA template may be stored at −20°C or below for future use.

(8) To prepare a 1:5 dilution for qPCR, transfer 10 µL of each DNA template into 40 µL of DNase/RNase-free water in a new microcentrifuge tube and mix well.

Note: Preenrichment broth residues in the DNA extract may cause qPCR IAC failure, indicated by a late C_T value or an undetermined result. If inhibition is suspected, repeat the qPCR using one of the following approaches:

- Dilution: Mix 5 µL of DNA extract with 45 µL DNase/RNase-free water (1:10) in a new microfuge tube.
- Purification: Use a commercially available DNA purification kit to purify the DNA extract, following the kit manufacturer's instructions.

3. Automated DNA Extraction for All Food Sample Preparations

The following instruments and commercial kits have been validated to be used for high throughput DNA extraction from preenriched food cultures:

(1) Maxwell® RSC 48 System with Maxwell® RSC Cultured Cells DNA kit: See Appendix 1.

(2) KingFisher Apex system / KingFisher Flex system / MagMax™ Express-96 Magnetic Particle Processor with BioSprint® 96 One-For-All Vet Kit: See Appendix 2.

(3) KingFisher Flex system / MagMax™ Express-96 Magnetic Particle Processor with PrepSEQ™ Nucleic Acid Extraction Kit for Food and Environmental Testing: See Appendix 3.

F. DNA Template Preparation from Pure Culture

1. Spin-boil Method

(1) Transfer 250 µL of overnight culture grown in BHI broth at 35 ± 2°C into a 1.5 mL microcentrifuge tube or suspend a single colony from a freshly prepared 24-hour BHI or non-selective plate in 250 µL of DNase/RNase-free sterile water in a 1.5 mL microcentrifuge tube.

(2) Centrifuge 10,000 × g for 5 minutes.

(3) Remove and discard the supernatant, then fully resuspend the pellet in 250 µL of DNase/RNase-free sterile water.

(4) Heat the suspension to 100°C in a heating block or boiling water bath for 20 minutes.

(5) Immediately cool on ice for 5 minutes.

(6) Centrifuge at 10,000 × g for 5 minutes. Carefully remove and retain the supernatant as the DNA template. The DNA template may be stored at −20°C or below for future use.

2. Automated DNA Extraction

All automated DNA extraction methods listed in Section E(3) can also be applied to pure culture isolates by following the respective kit manufacturer's instructions.

G. Preparation of qPCR *Salmonella* Master Mix

1. Preparation of Dehydrated qPCR *Salmonella* Master Mix

(1) Mix all components listed in **Table 2** in a 1.5 mL microcentrifuge tube by vortexing at top speed, then centrifuge briefly.

(2) Aliquot 5 μL Master Mix solution into 195 μL PCR-grade water to prepare a 2.5X master mix. Mix 5 μL of 2.5X master mix with 3 μL PCR-grade water, 10 μL TaqMan Fast Advance Master Mix, and 2 μL of DNA template from *Salmonella* and negative controls and run on the ABI 7500 Fast system for a quality assurance (QA) evaluation.

Table 2. Preparation of 100 tubes of dehydrated qPCR *Salmonella* Master Mix (100X)

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

***Prepare a fresh IAC DNA template working solution from the concentrated stock solution.**

(3) When both QA evaluation runs from *Salmonella* and negative controls yield satisfactory results, as indicated by an IAC C_T value of 28 ± 1 (see Figure 1), dispense 10 μL Master Mix solution per tube to sterile 1.5 mL black microcentrifuge tubes.

(4) Dry the qPCR *Salmonella* Master Mix solution with tube lids open in a vacuum chamber for 2 to 3 days, shielding the vacuum chamber completely from light.

(5) Select one vacuum-dried qPCR *Salmonella* Master mix tube each from the top and middle shelves and perform a QA run with *Salmonella* and negative controls on the ABI 7500 Fast system (see Section H for reaction assembly).

(6) When both evaluation runs from *Salmonella* and negative controls yield satisfactory results, the vacuum-dried qPCR *Salmonella* Master Mix can be stored under either of the following conditions:

- In a sealed aluminum foil porch with a silica gel packet, in a desiccator at room temperature, for up to 2 years.
- At -20ºC in a freezer or at refrigerator for up to 2 years.

After 2 years of storage, a QArun must be performed to confirm that the dehydrated Master Mix produces a normal amplification curve, as shown in Figure 1, before use.

(7) Each tube of qPCR *Salmonella* Master Mix is sufficient for fifty 20 μL reactions on ABI 7500 Fast systems.

2. Preparation of Wet qPCR *Salmonella* Master Mix

(1) Mix all components listed in **Table 3** in a 1.5 mL black microcentrifuge tube by vortexing at top speed, then centrifuge briefly.

(2) Dilute to 1X or 2.5X as needed for reaction set up (see Section H for reaction assembly).

(3) Perform a QA run for every batch of wet master mix on the ABI 7500 Fast system to confirm that the IAC C_T value is 28 ± 1.

(4) Shield the wet qPCR *Salmonella* master mix from light at all times. Store at 4°C for up to one week for immediate use, or store at -20 °C for up to 2 years. After 2 years of storage, a QA run must be performed to confirm that the Master Mix produces a normal amplification curve, as shown in Figure 1, before use.

(5) Each tube of qPCR *Salmonella* Master Mix is sufficient for fifty 20 μL reactions on ABI 7500 Fast systems

Table 3. Preparation of <u>1 tube</u> of Wet 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 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.

H. Real-time PCR Assembly for ABI 7500 Fast

1. qPCR Amplification Reaction Components

Table 4. qPCR Amplification Reaction Components

Volume (µL) /reaction	Component
8.0	2.5X working solution of qPCR *Salmonella* Master Mix (2.5X MM)*
10.0	TaqMan Fast Advance Master Mix
2.0	Template (sample or control)

*To prepare 2.5X MM from dehydrated Master Mix: Resuspend one tube of dehydrated Salmonella Master Mix in 400 µL of PCR-grade water.
*To prepare 2.5X MM from wet Master Mix: Add 300 µL of PCR-grade water to one tube of wet qPCR Salmonella Master Mix (10X).
Note: Each 400 µl tube of 2.5X qPCR *Salmonella* master mix requires 500 µl of TaqMan Fast Advance Master Mix for a total of 50 reactions.

2. Instructions for Preparing qPCR Reaction Master Mix (RMM) from Dehydrated *Salmonella* Master Mix

The dehydrated *Salmonella* Master Mix must be kept in the sealed aluminum foil pouch at room temperature in a desiccator until used. The *Salmonella* Master Mix **DOES NOT** contain Taq® polymerase, dNTPs, $MgCl_2$ and buffer. **The addition of the Applied Biosystems™ TaqMan Fast Advance Master Mix is required.**

Table 5. RMM from Dehydrated *Salmonella* Master Mix

Reagents	Amount
Dehydratred qPCR *Salmonella* Master Mix	1 tube (50 Reactions)
DNase-free, RNase-free Water	400 µL
ABI TaqMan Fast Advanced qPCR Master Mix	500 µL
Total	900 µL

On the day of PCR run:

1. Remove one dehydrated qPCR Salmonella Master Mix black tube from storage.
2. Add 400 µL of DNase/RNase-free water, vortex, and allow to stand for 10 minutes for complete dissolution.

3. Add 500 µL of TaqMan Fast Advanced qPCR Master Mix, vortex briefly, and centrifuge briefly to collect contents.
4. Label the tube "ABI RMM" (Reaction Master Mix) and record the date.
5. One prepared "ABI RMM" tube is sufficient for 50 reactions.
6. The prepared ABI RMM may be stored at 4°C for up to 1 week.
7. Aliquot 18 µL of the qPCR Reaction Mix into each well of a 96-well plate. If duplicate wells are required for each sample, aliquot accordingly. Add 2 µL of sample or control DNA template to each reaction well.

3. Instructions for Preparing qPCR Reaction Mix from Wet qPCR *Salmonella* Master Mix

The wet *Salmonella* Master Mix must be stored protected from light at 4°C for up to one week for immediate use, or at -20 °C for up to 2 years. After 2 years of storage, a QArun must be performed to confirm that the Master Mix produces a normal amplification curve, as shown in Figure 1, before use.

The *Salmonella* Master Mix **DOES NOT** contain Taq® polymerase, dNTPs, $MgCl_2$ and buffer. The addition of the Applied Biosystems™ TaqMan Fast Advance Master Mix is required.

Table 6. RMM for Wet *Salmonella* Master Mix

Reagents	Amount
Wet qPCR *Salmonella* Master Mix	1 tube (50 Reactions)
DNase-free, RNase-free Water	300 µL
ABI TaqMan Fast Advanced qPCR Master Mix	500 µL
Total	900 µL

On the day of PCR run:

1. Remove one dehydrated qPCR Salmonella Master Mix black tube from storage.
2. Add 400 µL of DNase/RNase-free water, vortex, and allow to stand for 10 minutes for complete dissolution.
3. Add 500 µL of TaqMan Fast Advanced qPCR Master Mix, vortex briefly, and centrifuge briefly to collect contents.
4. Label the tube "ABI RMM" (Reaction Master Mix) and record the date.
5. One prepared "ABI RMM" tube is sufficient for 50 reactions.
6. The prepared ABI RMM may be stored at 4°C for up to 1 week.

7. Aliquot 18 µL of the qPCR Reaction Mix into each well of a 96-well plate. If duplicate wells are required for each sample, aliquot accordingly. Add 2 µL of sample or control DNA template to each reaction well

I. Real-Time PCR run set up on ABI 7500 Fast

1. qPCR Running Protocol

Table 7. PCR Running Protocol – 2 steps

First Hold stage Activation of UDG	Second Hold stage Initial Activation	Cycling stage Each of 50 Cycles	
50°C, 2 min	95°C, 5 min	95°C, 3 sec	60°C, 30 sec

2. qPCR Result Analysis Setup

Table 8. qPCR Result Analysis Setup

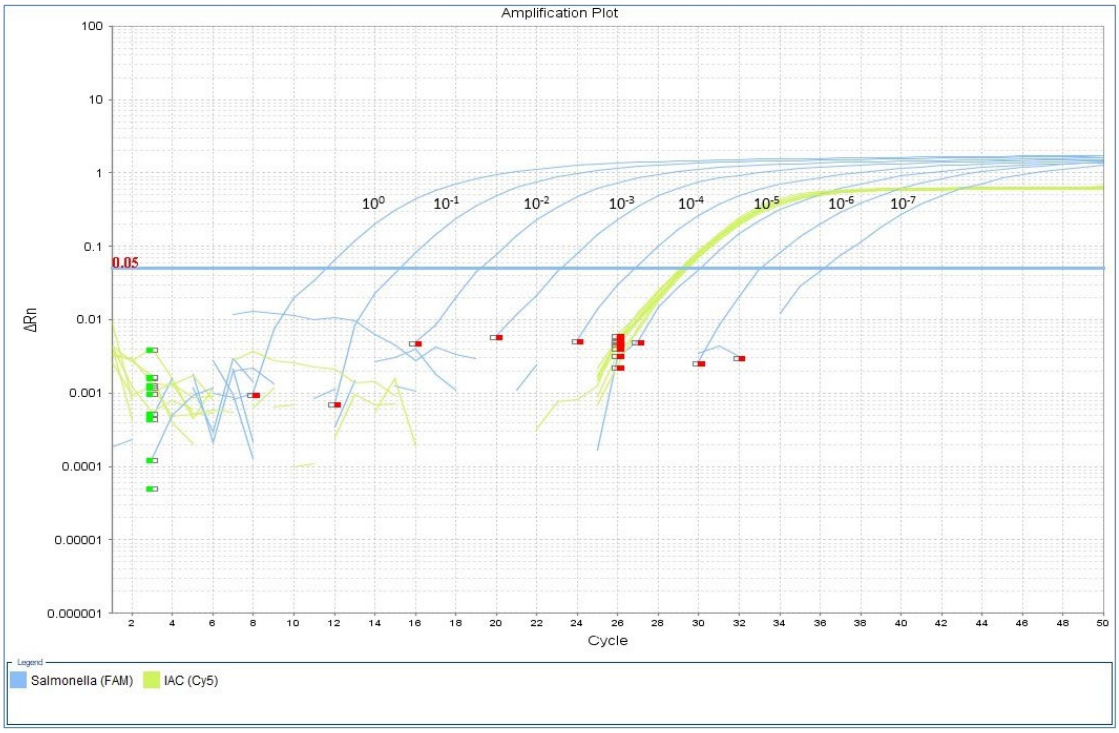
Amplification Plot Setting	Analysis Setting
1. Data: Delta Rn vs. Cycle 2. Detector: All 3. Line Color: "Well color" or "Detector Color"	1. Manual C_T 2. Threshold: 0.05 (w/ROX) 3. Manual Baseline Start (cycle): 3 End (cycle): 15

3. qPCR Result Interpretation

PCR results are considered valid only when the IAC amplification is not inhibited and the IAC displays a normal amplification curve. Fluorescence signals are analyzed using ABI 7500 software by manually setting the threshold at 0.05, with the baseline starting at cycle 3 and ending at cycle 15. The threshold cycle (C_T value) is recorded for each PCR reaction for both the target and the IAC.

A C_T value of less than 45 cycles is considered a *Salmonella*-positive result. If IAC inhibition is observed but the tested sample is *Salmonella* positive, the amplification curve should be examined carefully. The sample may be considered *Salmonella* positive only if the amplification curve displays the same baseline, exponential, and plateau phases as the positive control, as shown in Figure 1. If the IAC is inhibited, the qPCR reaction should be repeated using diluted DNA to reduce inhibition from the DNA extract.

Figure 1. *Salmonella* amplification plot (log scale) from a 10-fold serial dilution of *Salmonella* pure culture on the ABI 7500 Fast system. 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-axis.



4. qPCR Run Setup on ABI 7500 Fast System (V.1.4, 1.5, and 2.3)

Version 1.4 and 1.5

(1) Turn on the computer and the ABI 7500 Fast instrument.

(2) Launch the ABI 7500 Fast System Software.

(3) Create a new run using the New Document Wizard.

(4) Highlight all wells and select "Detector Manager" under the "Tools" menu.

(5) Shift-select Salmonella (FAM) and IAC (Cy5), then click "Add to Plate Document."

(6) Under "View," open "Well Inspector" and mark both "Salmonella" and "IAC." Select ROX as the Passive Reference.

(7) Under "Instrument," select the 2-step PCR protocol as described in Section I(1).

(8) Save the newly created file as a template in .sdt format (e.g., "Salmonella_Template.sdt"). This template file can be used for future .sds run files with all preset parameters.

(9) Save the file again in .sds format as a run file with a distinct name (e.g., "Salmonella_Test.sds").

(10) Assign sample names to the corresponding wells and save the file.

(11) Load the samples and initiate the run under "Instrument." If the "Start" button is not active, close and reopen the "Salmonella_Test.sds" file to reinitialize the ABI 7500 Fast system, then click "Start."

(12) After the run completes successfully, analyze, view, and report results as described in Section I(2).

(13) Interpret results as described in Section I(3).

Version 2.3

(1) Turn on the computer and the ABI 7500 Fast instrument.

(2) Launch the ABI 7500 Fast System Software v2.3.

(3) Click "New Experiment" to create and configure a new experiment.

(4) Click "Experiment Properties" and complete the following:

- Enter the experiment name.
- Select 7500 Fast (96 wells) as the instrument type.
- Select Fast (~40 minutes to complete run) as the run mode.

(5) Click "Plate Setup" and complete the following:

- Under the Define Targets and Samples tab:
  - Click "Define Targets" and define the first target as *Salmonella* (Reporter: FAM; Quencher: None). Add a second target as IAC (Reporter: Cy5; Quencher: None).
  - Click "Define Samples" and add all sample names.
- Under the Assign Targets and Samples tab:
  - Select the wells corresponding to samples and controls, highlight these wells, and under "Assign Target(s) to Selected Well," check both *Salmonella* and IAC.
  - Highlight each well individually and assign the corresponding sample name under "Assign Sample(s) to Selected Well."
  - Under "Select the dye to use as the Passive Reference," select ROX.

(6) Click "Run Method" and complete the following:

- Under Graphical View, set the reaction volume per well to 20 µL.
- Configure the run method as described in Section I(1).

(7) Click the Save icon and save the file as a template in .edt format (e.g., "Salmonella_Template.edt") for use as a template for future .eds run files with all preset parameters.

(8) Click the Save icon again and save the file in .eds format as a run file with a distinct name (e.g., "Salmonella_Test.eds").

(9) Load the sample plate into the ABI 7500 Fast system.

(10) Click the green START RUN button in the upper right corner.

(11) After the run completes successfully, analyze, view, and report results as described in Section I(2).

(12) Interpret results as described in Section I(3).

J. Confirmation

Presumptive positive results should be confirmed by following BAM Chapter 5 Section E-F for the isolation, identification, and confirmation (https://www.fda.gov/media/172194/download?attachment).

K. References

1. Galan, J. E., Curtiss III, R., 1989. Cloning and molecular characterization of genes whose products allow *S. typhimurium* to penetrate tissue culture cells. *Proc. Natl. Acad. Sci. USA* 86, 6383–6387.
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Appendix: Automated DNA Template Preparation Protocols

Appendix 1. DNA Extraction from Preenrichment Culture Using Maxwell® RSC Instrument

Purpose/Objective

This protocol describes the procedure for obtaining purified DNA from food preenrichment culture for up to 48 samples at a time using the Promega Maxwell® RSC (Rapid Sample Concentrator) automated instrument. The Maxwell® RSC DNA or RNA extraction methods use cartridges prefilled with purification reagents and paramagnetic particles. After sample addition, the instrument moves the paramagnetic particles and associated nucleic acids through multiple purification steps, ultimately yielding highly pure RNA or DNA in 30–100µl.

Because the Maxwell® RSC functions as a magnetic particle mover rather than a liquid handler, it offers several advantages over other automated systems, including minimal risk of cross-contamination, no liquid handling or splashing during sample processing, and fewer disruptions to downstream workflows due to the absence of clogs and reduced instrument downtime. This instrument has been validated with the Maxwell® RSC Cultured Cells DNA Kit for rapid, automated purification of bacterial DNA from a broad range of food samples, providing a fast, high-throughput DNA extraction method for the FDA qPCR method.

Materials

1. Maxwell® RSC automated instrument
2. Maxwell® RSC Cultured Cells DNA kit (Promega Cat# AS1620), which includes:
    - Prefilled Reagent Cartridge (48 Preps)
    - Plunger Pack (48 Plungers)
    - 50 Elution Tubes (0.5ml)
    - Elution Buffer (20 mL)
3. Pipettors and pipette tips for sample transfer into prefilled reagent cartridges
4. Microcentrifuge tubes (1.5 or 2.0 mL)
5. Serological pipettor
6. Pipettor aid

Sample Collection

1. Do not shake the food sample bag after preenrichment. Using a serological pipettor, transfer a 1 mL aliquot of preenrichment culture from the upper portion of the filtered side of the sample bag into a 1.5 mL microcentrifuge tube.
2. Store the collected samples tubes at 4˚C until the prefilled reagent cartridges are set up in the cartridge rack.
3. Transfer 400 µL of preenrichment cultures from 1.5 mL microcentrifuge tube to well #1 of the cartridge.

Instrument Operation

1. Confirm that the Maxwell® RSC-16 or RSC-48 instrument and the attached tablet are powered on. If not, turn both on and allow the instrument to initialize.
    - **RSC-48:** Turn on the rocker switch located at the back left near the power cord (first), then press the push button on the front right side (second).
    - **RSC-16:** Turn on the rocker switch located at the back left near the power cord.
    - **Tablet:** Press the push button at the top left to power on. Once the tablet is on, open the Maxwell® RSC software and wait for the instrument self-test to complete.
2. From the **Home** screen, select **Start** to begin the extraction method setup.
3. On the **Methods** screen, select the method from the list that matches the kit name or catalog number. Alternatively, scan the barcode on the Maxwell® RSC reagent kit box at the location labeled **Scan Here**.
4. On the **Cartridge Setup** screen *(displayed for RSC-16 only)*: touch cartridge positions to select or deselect positions for the current extraction run. Enter any required sample tracking information (the default setting requires no information, including sample ID).
5. After all required information has been entered, touch **Proceed** to display the **Door** prompt. Press **OK** to open the Maxwell® RSC instrument door.
6. Remove the appropriate deck tray(s):
    - For 1–24 samples: remove the front tray only.
    - For 25–48 samples: remove both trays.
7. Confirm that sample preprocessing is complete. If preprocessing is required, refer to the Technical Manual for the specific Maxwell® RSC reagent kit.
8. Change gloves before handling Maxwell® RSC cartridges, plungers, and elution tubes.

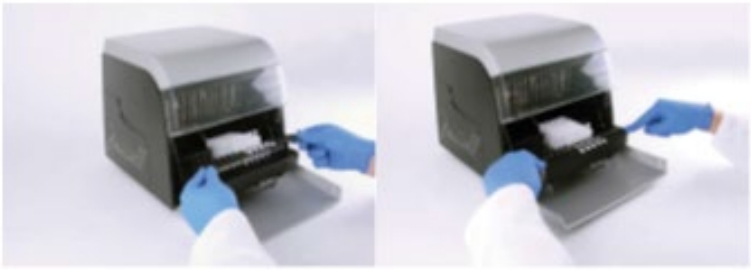
9. Place each cartridge (one per sample) in the deck tray(s) with the printed plastic side facing away from the elution position (i.e., facing away from the numbered side of the tray).
10. Press down firmly on each cartridge to snap it into place at both ends. An audible click should be heard.

Note: If processing fewer than 24 samples (RSC-48) or fewer than 16 samples (RSC-16), center the cartridges on the deck tray.

11. Carefully peel back the seal so that all plastic is removed from the top of the cartridge. Ensure that all sealing tape and any residual adhesive are fully removed before placing cartridges in the instrument.
12. Transfer the cultured cell sample to well #1 of each cartridge. Thoroughly mix the sample into the lysis buffer by pipetting up and down at least 10 times. Well #1 is the well closest to the printed side of the cartridge and furthest from the elution tube. Change pipette tips between samples.
13. Place one plunger into well #8 of each cartridge. Well #8 is the well closest to the elution tube.
14. Place an empty elution tube into the elution tube position for each cartridge in the deck tray(s). Elution tubes may be labeled now or upon completion of the extraction run.
15. Add 100 µL of Elution Buffer to the bottom of each elution tube. Note that the final elution volume will be less than 100 µL. Leave the elution tube lids open.

    Note: Use only the 0.5 mL Elution Tubes provided with the kit. Other tube types may be incompatible with the Maxwell® RSC instrument.

16. When placing the deck tray(s) into the instrument, hold the tray by the sides to avoid dislodging cartridges. Ensure that the deck tray is oriented with the elution tubes closest to the instrument door.



17. Angle the back of the deck tray downward and slide it into the instrument so that the back of the tray rests against the back of the instrument platform.
18. Press down on the front of the deck tray to firmly seat it on the instrument platform. If the tray does not fit properly, verify that it is in the correct orientation.
19. Confirm that the deck tray is level and fully seated on the instrument platform.
20. Review the **Extraction Checklist** screen displayed on the tablet. Confirm that all deck tray and instrument preparation steps (steps 5–18) have been completed as indicated.
21. After confirming all checklist items, touch **Start** to begin the purification run.
22. Monitor the **Running** screen. When the method completes successfully, the **Current Step** field will display **Completed**.
23. Touch **Open Door** to open the Maxwell® RSC instrument door.
24. Close the caps of all elution tubes.
25. Verify that all cartridges have a plunger seated in well #8. Remove the deck tray by gripping it firmly at the elution tube position, lifting upward, and pulling the tray out. Note that the deck tray may be warm after a completed run; exercise caution during removal. If any cartridges are missing plungers, remove them by running the **Clean Up** method.
26. The purified DNA is present in the eluate. Confirm that all elution tubes are labeled appropriately or label them at this time. After purification, resin may adhere to the inner walls of the elution tubes; this is normal and will not affect downstream assay performance. Residual particles may be removed by centrifuging the elution tube or placing it on a magnet and transferring the supernatant to a clean tube (not provided).
27. Remove the used cartridges and plungers from the Maxwell® RSC deck tray and dispose of them in a sharps biohazard container.
28. Sanitize the deck tray with 70% ethanol and return it to the instrument. Sanitize the instrument using the **Sanitization** function accessible from the **Home** screen.

Appendix 2. DNA Extraction from Preenrichment Culture Using KingFisher Apex, KingFisher Flex with BioSprint® 96 One-For-All Vet Kit

Purpose/Objective

This protocol describes the procedure for obtaining purified DNA from food preenrichment cultures for up to 96 samples at a time using the KingFisher Apex system, KingFisher Flex system, or MagMax™ Express-96 Magnetic Particle Processor with the BioSprint® 96 One-For-All Vet Kit. The BioSprint® 96 One-For-All Vet Kit uses magnetic-particle technology for rapid purification of nucleic acids. This kit has been validated for rapid, automated purification of bacterial DNA from a broad range of food samples on the KingFisher Apex, KingFisher Flex, and MagMax automated instruments, providing a fast, high-throughput DNA extraction method for the FDA qPCR method.

Materials and Reagent

1. KingFisher Flex system or KingFisher Apex system
2. Plastics for KingFisher **Apex** system:
    - 96-well Deep-well (DW) Plate (Fisher Scientific Cat# 14387349)
    - 96-well DW Tip Comb (Fisher Scientific Cat# 14387354)
    - 96-well DW Tip Comb& Plate (Fisher Scientific Cat# 14387358)
3. Plastics for KingFisher **Flex** system and MagMax:
    - 96-well Deep-well (DW) Plate (Fisher Scientific Cat# 22387031).
    - 96-well DW Tip Comb (Fisher Scientific Cat# 22387029);
    - 96-well DW Tip Comb& Plate (Fisher Scientific Cat# FER97002820)
4. BioSprint 96 One-For-All Vet Kit (INDICAL BIOSCIENCE Cat# SP947057).
    - The kit includes plasticware and all reagents except Ethanol and Isopropanol, which are needed for DNA extraction.
    - **Do not use** plasticware provided in the kit on KingFisher Apex and KingFisher Flex.
5. Pipettors and pipette tips
6. Microcentrifuge tubes (1.5 or 2.0 mL)
7. Serological pipettor (1-1000 mL)
8. Vortex mixer
9. Graduated cylinder
10. Ethanol (200 proof)

11. Isopropanol

Notes:

- Using the ThermoFisher 96-well DW plates and tip comb for the DNA extraction on KingFisher Apex and Flex.
- Ethanol and isopropanol are not included in the kit and must be obtained separately.

Installation of the automated protocol "Combined Boil 3 Wash" on KingFisher Flex/MagMax:

1. Install BindIt 4.1Software on your computer: https://www.thermofisher.com/us/en/home/global/forms/life-science/download-bindit-software-kingfisher-instruments.html
2. Download and save the protocol "Combined Boil 3 Wash" in your computer.
3. Connect your computer to the KingFisher instrument via USB cable and select **Home > Connect** in the BindIt Software.
4. You may run the protocol directly from BindIt or transfer it to the instrument. To transfer the protocol, select **Home > Transfer** in the BindIt Software.
5. Select **Upload** and locate the saved protocol file. Click **Open**.
6. Select the folder (category) in which you want to store the protocol on the instrument.
7. The protocol will now be stored in the instrument memory.
8. Disconnect the instrument from BindIt and confirm using the keypad that the protocol is saved in the desired folder.

Note: If the protocol "Combined Boil 3 Wash" cannot be transferred to the instrument, you may create the protocol manually following the protocol steps below in BindIt 4.1 Software, then transfer it to the instrument using procedures described above.

Program the following steps in Bindlx 4.1 Software specific to the instrument

1. **The protocol contains 6 plate positions:**
    - Lysis – Position 1
    - Wash 1 – Position 2
    - Wash 2 – Position 3
    - Wash 3 – Position 4
    - Elution – Position 5
    - Rod Cover – Position 6
2. **Lysis Plate – 3 action steps:**
    - Heat: 15 minutes at 96°C with mixing at medium speed
    - Pause: Add Buffer RLT
    - Lysis/Bind: 5-minute bind with mixing at medium speed
3. **Wash 1 Plate** – 1 action step: 2-minute wash with mixing at medium speed
4. **Wash 2 Plate** – 1 action step: 2-minute wash with mixing at medium speed
5. **Wash 3 Plate** – 1 action step: 2-minute wash with mixing at medium speed
6. **Air-Dry step** (outside of Wash Plate 3): 9-minute drying time
7. **Elution Plate:** Release beads slowly with a release time of 5 seconds. Elute for 5 minutes using grind mix setting.
8. Return the magnets and rod cover to Wash Plate 3 when the run is complete.

Installation of the automated protocol "Combined Boil 3 Wash" on KingFisher Apex

1. Create the protocol "Combined Boil 3 Wash" using Bindlx software directly on the instrument.
2. Download the KingFisher Apex **User Guide** which includes Bindlx software instructions on pages 40-55: PDF.
3. Follow pages 40-55 of the User Guide to create the protocol according to protocol steps outlined above: PDF

Chemical lysis protocol for the isolation of DNA from Gram-negative bacteria

1. Open BioSpint 96 One-For-All Vet Kit and remove the following items:
    - 1 Bottle of Buffer AVE (Elution Buffer)
    - 1 Bottle of Buffer AW1 (Wash Buffer 1)
    - 1 Bottle of Buffer RPE (Wash Buffer 2 &3)

- 1 Bottle of Buffer RLT (Lysis Buffer)
- 1 Bottle of MagAttract® Suspension G

2. Prepare Buffer AW1 by adding 160 mL ethanol to the bottle and mixing thoroughly.
3. Prepare Buffer RPE by adding 220 mL ethanol to the bottle and mixing thoroughly.
4. Prepare Buffer RLT by thoroughly mixing 35 mL RLT, 35 mL isopropanol, and 3 mL of MagAttract® Suspension G (Vortex the Magnetic Particles for 5 seconds until fully suspended).
5. Label ThermoFisher 96-well DW plates for use with KingFisher Apex/Flex as follows:
   - Lysis
   - Wash 1
   - Wash 2
   - Wash 3
   - Elution
   - Tip Comb in microtiter 96-well DW plate (label not needed)
6. Sample collection:
   - Do not shake the food sample bag after preenrichment. Using a serological pipettor, transfer a 1 mL aliquot of preenrichment culture from the upper portion of the filtered side of the sample bag into a 1.5 mL microcentrifuge tube.
   - Store the collected samples tubes at 4˚C while 96 deep-well plates are being prepared.

     Note: If the sample produces a large pellet at this step (e.g., chocolate food samples), the following preclarification procedure is recommended:

     - Transfer 1 mL of sample into a 1.5-mL microcentrifuge tube.
     - Centrifuge for 1 minute at 900 x g.
     - Transfer the supernatant to a new 1.5-mL microcentrifuge tube without disturbing the pellet
     - Proceed to step 7.
7. Transfer 500 µL of preenrichment cultures from the1.5 mL microcentrifuge tube to the appropriate well of the KingFisher microtiter 96-well deep well plate.
8. Prepare the remaining plates as follows:
   - **Elution plate:** Add 100 µL of Elution Buffer (AVE) to each well corresponding to a sample well in the lysis plate.

- **Wash plate 1:** Add 700 µL of Wash Buffer 1 (AW1) to each well corresponding to a sample well in the lysis plate.
- **Wash plates 2 and 3:** Add 500 µL of Wash Buffer 2 & 3 (RPE) to each well corresponding to a sample well in the lysis plate.

9. Select run protocol **"Combined Boil 3 Wash"** from the KingFisher Apex/ Flex/MagMax Express magnetic particle processor. Press **Start.**
10. Load the plates as prompted by the instrument readout. Verify orientation A1-to-A1 orientation for each plate:
    - Tip Comb plate – press **Start**
    - Elution plate (100 µL Buffer AVE) – press **Start**
    - Wash plate 3 (500 µL Buffer RPE) – press **Start**
    - Wash plate 2 (500 µL Buffer RPE) – press **Start**
    - Wash plate 1 (700 µL Buffer AW1) – press **Start**
    - Lysis plate (500 µL sample) – press **Start**
11. After approximately 15–20 minutes, the instrument will prompt you to add 500 µL of **Binding Mix** (Buffer RLT combined with magnetic beads) to each well of the lysis plate. Vortex the RLT mixture immediately before adding.
12. Return the lysis plate to the instrument. Press **Start.**
13. After approximately 30 minutes, the instrument will prompt you to remove the plates. Retain the elution plate (containing the purified DNA template) and discard all other plates.
14. Transfer the eluate to sterile microcentrifuge tubes, or seal the 96-well elution plate. Label appropriately with "KF" and relevant sample information.
15. Store templates at −20°C for future PCR testing.

Appendix 3. DNA Extraction from Preenrichment Culture Using KingFisher Flex with PrepSEQ™ Nucleic Acid Extraction Kit

Purpose/Objective

This protocol describes the procedure for obtaining purified DNA from food preenrichment cultures for up to 96 samples at a time using the KingFisher Flex system or MagMax™ Express-96 Magnetic Particle Processor with the PrepSEQ™ Nucleic Acid Extraction Kit for food and environmental testing. The kit uses magnetic-particle technology for rapid purification of nucleic acids. This kit has been validated for rapid, automated purification of bacterial DNA from a broad range of food samples on the KingFisher Flex and MagMax automated instruments, providing a fast, high-throughput DNA extraction method for the FDA qPCR method.

Materials and Reagent

1. KingFisher Flex system,
2. Plastics for KingFisher **Flex** system:
    - 96 Deep-well (DW) Plate (Fisher Scientific Cat# 22387031).
    - 96 Deep-well Tip Comb (Fisher Scientific Cat# 22387029);
    - 96 Deep-well Tip Comb& Plate (Fisher Scientific Cat# FER97002820)
3. PrepSEQ™ Nucleic Acid Extraction Kit for food and environmental testing (300 preps; ThermoFisher Cat# 4428176). The kit provides all reagents except ethanol and isopropanol, which must be supplied separately.
4. Pipettors and pipette tips for sample transfer into 96-well DW plate
5. Microcentrifuge tubes (1.5 or 2.0 mL)
6. Serological pipettor (1-1000 mL)
7. Vortex mixer
8. Graduated cylinder
9. Ethanol (95%)
10. Isopropanol

Installation of the automated protocol “4412639PrepSEQ_Sal” on KingFisher Flex/MagMax:

1. Install BindIt 4.1Software on your computer: https://www.thermofisher.com/us/en/home/global/forms/life-science/download-bindit-software-kingfisher-instruments.html

2. Download and save the protocol " 4412639PrepSEQ_Sal" to your computer: https://www.thermofisher.com/order/catalog/product/4412639?SID=srch-hj-4412639
3. Connect your computer to the KingFisher instrument via USB cable and select **Home > Connect** in the BindIt Software.
4. You may run the protocol directly from BindIt or transfer it to the instrument. To transfer the protocol, select **Home > Transfer** in the BindIt Software.
5. Select **Upload** and locate the saved protocol file. Click **Open**.
6. Select the folder (category) in which you want to store the protocol on the instrument.
7. The protocol will now be stored in the instrument memory.
8. Disconnect the instrument from BindIt and confirm using the keypad that the protocol is saved in the desired folder.

   Note: If the protocol "4412639PrepSEQ_Sal" cannot be transferred to the instrument, you may create the protocol manually by following the Protocol steps on pages 5-6 of the kit insert in the BindIt 4.1 Software, then transfer it to the instrument using the procedures described above.

Chemical lysis protocol for the isolation of DNA from Gram-negative bacteria

1. Open PrepSEQ™ Nucleic Acid Extraction Kit and remove the following items:

   - 1 Bottle of Elution Buffer (25 mL)
   - 2 Bottles of Wash Buffer Concentrate (26 mL/bottle)
   - 1 Empty Bottle for Binding Solution (Isopropanol, not provided in the kit)
   - 2 Bottles of Lysis Buffer (50 mL/bottle)
   - 2 Tubes of Magnetic Particles (1.5 mL/tube)
   - 1 Bottle of Proteinase K (PK) Buffer (50 mL)
   - 1 Tube of Proteinase K (1.25 mL)

     Note: For information on storage of kit components, refer to the "Storage" section of the packaging insert.
2. Prepare Binding Solution by adding 30 mL of 100% isopropanol to the empty Binding Solution bottle. Label the bottle to indicate that isopropanol has been added.
3. Prepare Wash Buffer RPE by adding 74 mL of 95% ethanol to the Wash Buffer Concentrate bottle. Mix well and label the bottle to indicate that ethanol has been added.

4. Label KingFisher microtiter 96-well DW plates as follows:
    - Lysis
    - Wash 1
    - Wash 2
    - Elution
    - Tip Comb (no label required)
5. **Sample collection:**
    - Do not shake the food sample bag after preenrichment. Using a serological pipettor, transfer a 1 mL aliquot of preenrichment culture from the upper portion of the filtered side of the sample bag into a 1.5 mL microcentrifuge tube.
    - Centrifuge the tube for 3 minutes at 16,000 × g. Discard the supernatant without disturbing the pellet, removing the liquid as quickly as possible to prevent pellet dissipation.

      Note: If the sample produces a large pellet at this step (e.g., chocolate food samples), the following PrepSEQ™ preclarification procedure is recommended:
        - Transfer 1 mL of sample into a 1.5-mL microcentrifuge tube.
        - Centrifuge for 1 minute at 4000 x g.
        - Transfer the supernatant to a new 1.5-mL microcentrifuge tube without disturbing the pellet
        - Proceed to step 5b.
6. Add 300 µL of Lysis Buffer to the tube. Resuspend the pellet by pipetting up and down or by vortexing until fully resuspended. Perform a quick spin (5 seconds) to remove any Lysis Buffer from the tube lid.
7. Transfer the resuspended sample to the appropriate well of the KingFisher microtiter 96-well deep-well lysis plate.
8. Prepare the remaining plates as follows:
    - **Elution plate:** Add 140 µL of Elution Buffer to each well corresponding to a sample well in the lysis plate.
    - **Wash plates 1 and 2:** Add 300 µL of Wash Buffer to each well corresponding to a sample well in the lysis plate.
9. Select the protocol "**4412639PrepSEQ_Sal**" from the KingFisher Flex/MagMax Express magnetic particle processor. Press **Start.**

10. Load the plates as prompted by the instrument readout. Verify orientation A1 to A1 orientation for each plate:
    - Tip Comb plate – press **Start**.
    - Elution plate (140 µL Elution Buffer) – press **Start**
    - Wash plate 2 (300 µL Wash Buffer) – press **Start**
    - Wash plate 1 (300 µL Wash Buffer) – press **Start**
    - Lysis plate (sample in Lysis Buffer) – press **Start**
11. After approximately 18 minutes, the instrument will prompt you to dispense the **Binding Mix**:
    - Vortex the Magnetic Particles for 5 seconds until fully resuspended.
    - Add 30 µL of Magnetic Particles to each well. Swirl the plate to mix.
    - Add 180 µL of Binding Solution to each well. Swirl the plate to mix.
    - Return the lysis plate to the instrument. Press **Start.**
12. When sample preparation is complete, the message **"Enjoy your DNA"** will appear on the screen. Remove the elution plate (containing the purified DNA templates) and discard all other plates. Seal the elution plate and store at −20°C for future PCR testing.