

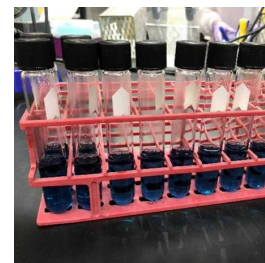
Feb 02, 2023

Version 2

Salmonella detection with spin column extraction and 7500-FAST enrichment PCR V.2



Version 1 is forked from [Extraction of bacterial DNA using MagMAX™ CORE Nucleic Acid Purification Kit on KingFisher™ Flex Instrument](#)



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We use this protocol and it's working

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Disclaimer

Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

Abstract

This procedure is used to test enrichment broth from environmental or animal specimens using Qiagen spin columns and the MicroSEQ Salmonella spp. Detection Kit.

The RVS enrichment broth should be prepared in advance to allow for quality control. The rest of the method is performed over two days:

Day 1 - sample setup and enrichment

Day 2 - DNA purification and real-time PCR



Guidelines

Wipe down sample tubes and gloves with 10% bleach to minimize cross-contamination.

Materials

Qiagen DNeasy Blood & Tissue extraction kit

MicroSEQ™ Salmonella Spp. PCR kit (Thermo/ABI cat# 4403930) contains 96 reactions pre-loaded into tube strips in a lyophilized format. A negative PCR setup control is also provided.

Safety warnings

- ⚠ Follow biosafety procedures consistent with your institution.



Preparation of RVS broth and Quality Check

- 1 This broth is used for the enrichment of specimens for *Salmonella* spp. isolation. The Rappaport Vassiliadis (RVS) media has an expiration date of **6 months** from the preparation date.

Determine batch size:

	A	B	C	D	E	F	G
	RO water	0.5 L	1 L	2 L	3 L	4 L	5 L
	RVS broth powder	13.55 g	27.11 g	54.22 g	81.33 g	108.44 g	135.55 g

RVS powder (Rappaport Vassalidius) can be obtained from HIMEDIA (#M1491)

- 2 Add half the amount of RO water needed for the batch and a sterile stir bar to a sterile flask of appropriate size
- 3 Place the flask on a stir plate and start rotating the stir bar to "stir" the water
- 4 Add the entire amount of RVS powder to the flask while stirring
- 5 Add the remaining amount of water to the flask
- 6 Heat the flask until the powder is completely dissolved
- 7 Dispense **10 ml** of the broth from the flask into sterile 20 × 125 mm screw-capped tubes using sterile tubing and the Doselt. Loosely cap each tube.
- 8 Autoclave the tubes of broth for **15 minutes** on liquid cycle @ 121°C



- 9 Allow to cool to RT and then tighten caps.
- 10 Label racks of tubes as "RVS Broth", date made, initials,& expiration date (months)
- 11 Perform QC using the fields below according to the manufacturer's directions
- 12 Store RVS Broth @ 2-8°C protected from light

13 **Quality Control Procedure:RVS Broth**

RVS Broth/BP inoculated:Initials:Date:

BP examined:Initials:Date:

BP : ☐growth ☐no growth

BG/XLD inoculated:Initials:Date:

BG/XLD Subculture plates examined: Initials:Date:

Positive Control plates:

BG: ☐typical colonies ☐atypical colonies ☐no growth ☐mixed growth

XLD:☐typical colonies ☐atypical colonies ☐no growth ☐mixed growth

Final Results(check one) Pass ☐ Fail/Batch Discarded ☐

Date:

Initials:

Sample processing and enrichment

- 14 Thoroughly disinfect a biosafety cabinet (BSC) using a disinfectant spray. Place a piece of absorbent bench paper in the BSC after cleaning. Allow the BSC to run for several minutes before placing items in it.
- 15 Obtain the following and place into the running BSC:
 - Prepared aliquots of RVS (9ml per sample) to accommodate the total number of samples being tested plus one negative enrichment setup control. Positive controls

are optional for this exercise. Retrieve additional aliquots of RVS if you will be adding positive controls.

- Racks to hold 15 ml tubes
- Lab towels soaked with 10% bleach contained within a plastic holder.
- Small biohazard bag (to collect waste)
- Labeled samples (organized in appropriate sized rack)
- Optional: one positive control culture each for *Salmonella* Typhimurium and *Salmonella* Dublin grown on blood agar
- Sterile 10 μ L loop (optional if using an in-house positive culture control)

- 16 For the Vet-LIRN Salmonella Interlaboratory Comparison Exercise, samples to be tested will be liquid suspensions of bovine intestinal scrapings in PBS. The test sample tubes contain 1ml of sample in a 15 ml tube.

Note

Important! The entire sample will be used; do not transfer the sample out of this tube at this time.

- 17 Place the first sample in a tube rack and loosen the cap. Clean your entire gloved hand with the 10% bleach towel.
- 18 Chose one 9ml RVS aliquot and pour the entire volume into the first sample tube. Use care to avoid back splashing. Recap the tube. Discard the empty aliquot tube in the biohazard waste.
- 19 Clean your entire gloved hand with the 10% bleach towel. Place the inoculated tube, which now has both sample and RVS broth, back into the original rack. Clean your gloves again.
- 20 Repeat steps 16-19 with the remaining samples.
- 21 Reserve one aliquot of RVS broth and label as negative setup enrichment control: this tube will remain un-inoculated. *Note! After over-night incubation, the negative setup enrichment control will be extracted and tested by PCR along with the samples.*
- 22 Optional: Inoculate one positive control using a sterile loop.
- 23 Move the rack(s) to a 40-44°C incubator, with shaking preferred. Incubate overnight, at least 18-24 hours.



DNA Extraction

- 24 Add 200 μ L ethanol (200 proof) to each tube and mix thoroughly by vortexing followed by a brief pulse spin.
- 25 Transfer 1 mL of culture to a labelled microcentrifuge tube and centrifuge at 5,000 g for 10 minutes and remove supernatant.

Note

This step is high risk for cross-contamination. Clean gloves with 10% bleach in between opening and closing each tube. Continue to take extreme care to avoid cross-contamination throughout the extraction procedure.

- 26 Add 180 μ L Buffer ATL to the tube and resuspend the pellet. Add 20 μ L Proteinase K to the tube, then vortex.

If the ATL buffer solution is cloudy and has precipitate formation, be sure to heat the solution at 50 - 55°C until solution is clear before adding to culture.

- 27 Incubate at 56°C for a minimum of 1 hour or up to overnight, vortexing periodically.
- 28 Optional: Add 4 μ L of RNase A to each tube, vortex and then incubate at RT for 3-5 minutes.
- 29 Add 200 μ L Buffer AL to the tube.
- 30 Pipet the mixture from step 14 into a labelled DNeasy Mini spin column. Centrifuge at 6,000xg for 1 minute. Discard flow-through and the collection tube.
- 31 Add 500 μ L Buffer AW1, and centrifuge for 1 minute at 6,000xg (8,000rpm). Discard the flow-through and the collection tube.
- 32 Transfer the spin column to a new collection tube.



- 33 Centrifuge for 1 minute at 18,000-20,000 x g (14,000 rpm) to dry.
- 34 Add 500 µL Buffer AW1, and centrifuge for 1 minute at 6,000xg (8,000rpm). Discard the flow-through and the collection tube.
- 35 Add 500 µL Buffer AW2, and centrifuge for 1 minute at 6,000xg (8,000rpm). Discard the flow-through and the collection tube.
- 36 Transfer the spin column to a new collection tube.
- 37 Centrifuge for 1 minute at 18,000-20,000 x g (14,000 rpm) to dry.
- 38 Place the spin column in a clean, labelled 1.5 mL microcentrifuge tube. Pipet 100 µL Buffer EB directly onto the DNeasy membrane.
- 39 Incubate at room temperature for 1 minute and then centrifuge for 1 minute at 18,000-20,000 x g (14,000 rpm) to elute.
- 40 Discard spin column and store DNA sample at 4°C for up to 48 hours. For long term storage (>48 hours), store DNA sample at -20°C.

PCR Setup

- 41 Program the ABI 7500-FAST machine with the following settings (use fast mode):

	A	B	C	D
	c	Reps	Temperature	Duration (sec)
	Stage 1	1	95° C	20
	Stage 2	40	95° C	03
			60° C	30

- 42 Set up reactions at ROOM TEMPERATURE (do not use cold blocks or ice). Chilled nucleic acid and lyophilized components will make resuspension of the reaction mixture difficult.

Slowly remove the rounded caps on the tube strips, one row at a time, and discard them.

- 43 Add 30 μ l of sample nucleic acid elution to the PCR tube containing the lyophilized master mix. Wait 3-5 seconds and then resuspend the reagent pellet by pipetting up and down 2-3 times.

Important! It is critical to wait 3-5 seconds to avoid aspirating part of the still lyophilized PCR master mix into the pipette tip.

- 44 Add 30 μ l of the negative amplification control (supplied with the kit) to a PCR tube containing the lyophilized master mix. Wait for 3-5 seconds and then resuspend the reagent pellet by pipetting up and down 2-3 times.

- 45 Add 30 μ l of the positive amplification control to a PCR tube containing the lyophilized master mix. Wait for 3-5 seconds and then resuspend the reagent pellet by pipetting up and down 2-3 times.

- 46 Cap the tubes with the flat caps provided with the kit. The cap strip must be seated level as shown in the picture.

Place a small mark at one end of each of the strip caps in order to keep proper orientation and order of the tubes. Do not mark the sides of the tubes or place any marks over the main surface of the optical cap.



Proper seating of cap strip



Poor seating, strip is not horizontal
Lips of caps not secure inside of tubes

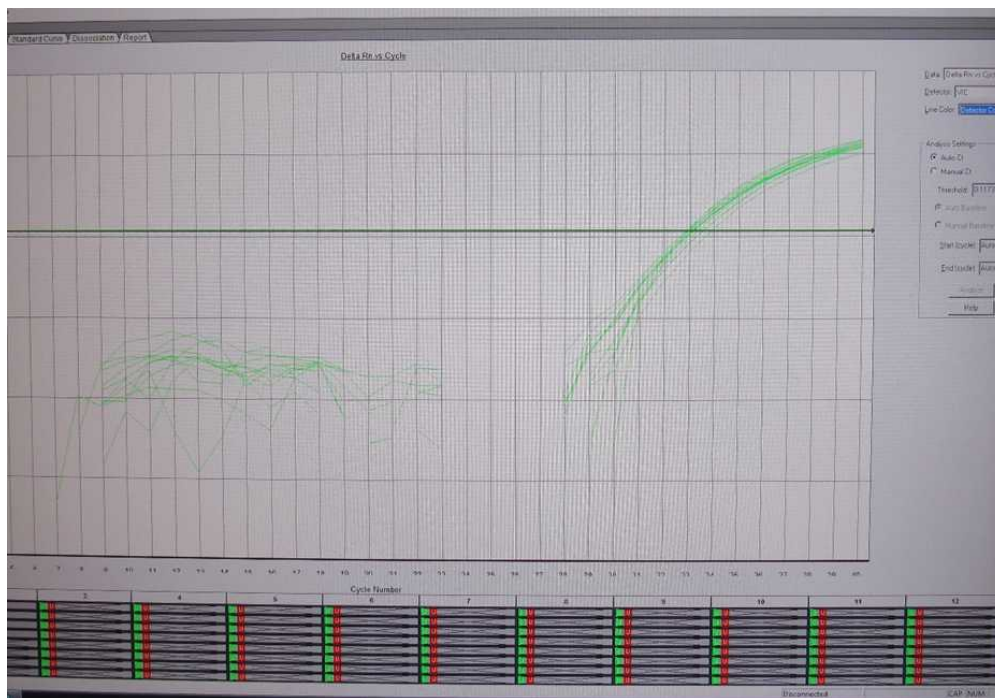
- 47 Pulse spin the strip tubes in order to collect liquid in the bottom of the tubes and displace any air bubbles.
- 48 Place Samples in ABI-7500 FAST thermocycler using the tube strip adapter. Balance the tubes on each side according to the manufacturer's instructions (blank tubes may be used for this purpose if necessary). If you do not have this adapter or are using a different machine, you can transfer the reactions into a plate that is compatible with your setup if needed.



Balance the tubes on each side of the 7500-FAST insert tray for tube strips

- 49 Start the run.

- 50 Following run, analyze the results using the automatic analysis setting. All samples should have a VIC (internal control) signal. If the VIC is >35 or undetermined, repeat the PCR using a 1:5 dilution of the sample in nuclease-free water and interpret based on the result that gives the lowest Ct for the VIC.



- 51 Save a copy of the original run file and export the data into an Excel file. Interpret the results according to the following guidelines:

	A	B	C	D
	FAMa	VICb	Interpretation	Action
	undetermined	detected	Not Detected	None – this result is final



	A	B	C	D
	< 35	disregard	Positive	Culture confirmation is optional
	≥ 35	detected	Suspect	Culture confirmation is optional
	undetermined	undetermined	Inconclusive	Culture confirmation is optional

^aFAM (6-carboxyfluorescein) is the most commonly used fluorescent dye attachment for oligonucleotides and is calibrated as part of the standard set of dyes for the ABI 7500-FAST machine. Its max excitation wavelength is 495 nm and emission wavelength is 520 nm

^bVIC is an ABI-proprietary dye that with excitation wavelength 488 nm/Emission Wavelength 552 nm that is also calibrated as part of the standard set of dyes for the ABI 7500-FAST machine.

52 Copy the results into the provided SecureSheet file.

53 Optional: proceed with culture confirmation for samples with a *Salmonella* (FAM) Ct detected