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Ultrafiltration Methods for Concentrating and Detecting Salmonella Typhi and Salmonella Paratyphi A in Wastewater Samples

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ABSTRACT

Tangential flow ultrafiltration is used to concentrate large volumes of environmental water samples (*e.g.*, wastewater, wastewater-impacted surface waters, etc.) for detection of *S. Typhi* and other microorganisms, using molecular methods. This method is applicable for sites with high flow rates, as a large volume is required. Results are quantifiable when enrichment is not used.

This method was developed at Emory University, and it has been previously described in the following articles (Liu P et al, *J Microbiol Methods* 2012; Liu P et al, *J Appl Micobiology* 2013; Amin N et al, *Intern J Hygene Environ Health* 2020).

A 40-L grab sample is collected from the source water using a bucket. The sample is brought to the lab and concentrated through a tangential flow ultrafilter. *S.* Typhi and S. Paratyphi A are captured on the filter by size exclusion, and eluted in ~500 ml volume of eluant buffer. The eluate is further concentrated by polyethylene (PEG) precipitation. The final pellet is extracted directly for molecular testing.

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Ultrafiltration Preparation

1

1. Prepare workbench for ultra-filtration
    a. Wipe down with 10% bleach and 70% ethanol
    b. Place bench protector paper on bench to cover work area
2. Prepare pressure gauge
    a. Wipe down with 10% bleach and 70% ethanol
    b. Wrap Teflon tape around disinfected threaded end of gauge
3. Prepare Stand
    a. Wipe down stand and metal fittings with 10% bleach and 70% ethanol
4. Prepare stock solution (only if necessary and on a monthly basis)
5. Gather necessary materials for ultra-filtration including appropriate amount of tubing and filter
6. Clean out the carboys
    a. Add a 10% bleach solution (100ml of bleach with 900ml of water) to each carboy
    b. Quick rinse with the solution and then with regular water
    c. Add a tiny drop of Lysol to the carboy and add water
    d. Rinse the carboys enough times to eliminate all the suds
7. Gather necessary materials to be autoclaved
    a. (3) 500 ml nalgene bottles- 1 lid with three openings, 2 with regular lids
    b. (1) 250 ml glass tube
    c. (1) 500 ml glass tube
    d. (2) Polyethylene Glycol (PEG) bottles with lids (It is recommended to autoclave additional ones just in case, especially if you plan to run more than one sample; Make sure the lid of the PEG bottles has two parts!)
    e. (1) 250 ml glass bottle with lid
    f. (1) 500 ml glass bottle filled with DI water
    g. (1) 500 ml glass bottle filled with Elution Solution (label)
    h. (1) 250 glass bottle filled with Backwash Solution (label)
    i. Autoclave bag with ultra-filtration pieces (see below)
    j. (1) spinners placed in a small autoclave bag
    k. (x) carboys with lids (No. depends on the sample side)
    l. (1) small plastic bottle (for field collection of turbidity/chlorine)

*special instructions:

- Tin foil needs to be placed over all lids, all openings for the glass tubes, and on the lids for the carboys
- The PEG lids should be wrapped in tin foil separately from the bottles
- All the lids, especially the ones for the liquid solution, should be loose before placing into the autoclave. Loosening the lid with the liquid solutions is important, because otherwise the bottle explodes in the autoclave.
- Every item should have autoclave tape on it

- The autoclave should be set to either liquid 1 or gravity 1 depending on what materials are being autoclaved (i.e. liquid setting always for liquid reagents)

8. The autoclaving process should take about 1 ½ hrs.
9. After the carboys are autoclaved place 2 g of Sodium Polyphosphate (NaPP) and 0.63 g of Sodium Thiosulphate (Na Thio) into each one
10. After everything is autoclaved assemble ultra-filtration apparatus

Ultra-filtration pieces

- (1) metal 3-way valve
- (1) plastic 3-way valve
- (1) plastic adapter
- (1) adjustable pinch clamp
- (1) tube clamp
- (2) metal hose clamps
- (1) snap 4
- (1) snap 6

Tubing for Ultrafiltration Setup

- (1x) L/S 17 – variable length (this is the tube which gets placed into the sample/ carboy)
- (1x) L/S 17 – 8"
- (3x) L/S 17 – 3"
- (1x) L/S 17 – 14"
- (1x) L/S 36 – 9" (cannot be autoclaved)
- (1x) L/S 24 – 4"
- (1x) L/S 17 – 6"
- For backwash: (1x) L/S 24 – 22"
- Tubing on the flow meter is already on the flow meter and cannot be autoclaved!

Stock Solution (prepare once a month) *All Stock Solutions Must Also Be Autoclaved*

- NaPP stock – 1g NaPP dissolved in 100 ml DI H2O
- Tween 80 stock – 1 ml Tween 80 dissolved in 100 ml DI H2O
- Antifoam A stock – 1 ml Antifoam A dissolved in 100 ml DI H2O

Elution Solution (for 1 run)

- 440 ml DI water
- 50 ml 10X PBS
- 5 ml NaPP stock
- 5 ml Tween 80 stock
- 500 ul Antifoam A stock

Backwash Solution (1 run)

- 220 ml DI water
- 25 ml 10X PBS
- 2.5 ml NaPP stock
- 1.25 ml Tween 80 (original Tween 80 not the stock solution)
- 250 ul Antifoam A stock

What is needed for Sample Collection

- A clean Cooler (For a 1 L Raw Influent sample; no cooler is needed for the carboys. However, the carboys need to be transported back from field after collection)
- Autoclaved carboys with the added 2g of NaPP and 0.63g of Na Thio in each
- The number of autoclaved carboys needed depends on the sample collection side
- For the Raw influent a 1L bottle is needed
- Disinfected Ice Packs

Blocking

- Make a 200-250 ml 5% solution of Bovine Calf Serum with 1XPBS.
- Pipette the solution slowly through one of the side ports of the filter, making sure the solution makes its way through the entire filter.
- Cap all ends of the filter and rotate the filter overnight.

2

Priming the filter

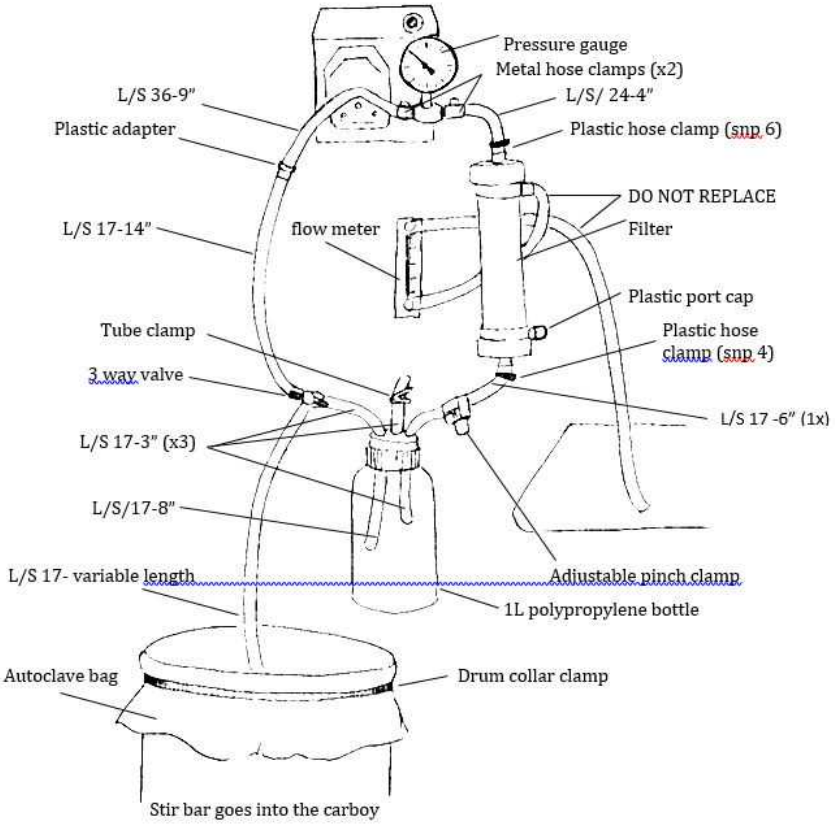
1. Prime the filter with 500 ml sterilized DI water (dial should be set to ~700, 3-way valve should be set to: ┤) Turn the switch of the pump to the right.
2. Once the filter is primed, stop the pump and transfer intake tube to the sample
3. Run ultrafiltration, with sample flowing clockwise and pumps at full speed (~700 on dial, flow @ 1100, pressure not over 14 psi)
4. Start the sample with the 3-way valve at ┤
5. Once the sample is flowing correctly, switch the 3-way valve to recirculating/all-way: ┬
6. Continue to run the sample on recirculating until the sample is gone (try to keep the water filled around half way of the nalgene bottle)
7. Then concentrate the sample in the nalgene bottle to 100-150 ml: the 3-way valve should be set to ┴
8. Stop the pump. Unscrew the nalgene bottle.
9. Measure the volume of the retentate by placing the liquid into the 250 ml glass tube.

Elute filter

10. Elution
    a. Reduce pump setting to 2000 ml/min (604 on dial). Turn 3-way value to ┤
    b. Begin pump and let run until solution is circulating through entire system (~half the bottle of solution)
    c. Turn off pump and wait for at least 5 minutes
    d. Turn pump back on with the same setting as before (┤) until the elution solution is gone.
    e. Concentrate elute solution to 100 ml by turning the 3-way valve to ┴
    f. Stop pump
    g. Place the elute solution into the same 250 ml solution and record volume

Backwash

11. Backflush filter
    a. Disconnect end of 4 inch L/S 24 tube segment from pressure gauge metal fitting—leave connected to top of filter
    b. Disconnect end of 6 inch L/S 17 tube segment from nalgene bottle—leave 6 inch L/S 17 connected to bottom of filter. Place third nalgene bottle under the tube segment.
    c. Carefully empty the water from the filter into the sink
    d. Disconnect entire system that includes 3 way connecter (disconnected from the 14 inch L/S 17 segment), other tubing, and nalgene lid and place aside.
    e. Place the end of the 14 inch L/S 17 tube (disconnected from the 3-way connecter) into the backwash solution (new intake tube)
    f. Attach a new 18 inch L/S 36 tube segment from the pressure gauge out to the LOWER filter port.
    g. Clamp 4 inch L/S 24 attached to the top of the filter with the tube clamp
    h. Place 6 inch L/S 17 tube segment into the third nalgene bottle
    i. Set pump to 56 (80 ml/min), set 3-way value to ┤ and fill the filter with water (top opening and the side opening of the filter should be opened; stop the pump before water comes out of the open port)
    j. Carefully remove the filter from the clamps, hold horizontally over the sink
    k. Move the 18 inch L/S 36 from the lower port to the upper port of the filter and cap the lower port
    l. Leave 3-way value on ┤
    m. Run the pump at 219 (650 ml/min) and collect 200 mL of backflush product
12. Combine the backflush product and the combined retentate/concentrate solution into the 500 ml glass tube and record total volume.
13. Pipette the sample into the PEG bottles. Make sure the sample is spread out evenly between the two bottles)
14. Write the amount (mL) on a label on the bottle



Ultrafiltration Cleanup

3

1. Disassemble ultra-filtration apparatuses
    a. Discard specified tubing and ultra-filter in biohazard waste (if the tubing is not reusable)
        i. If reusable tubing was used, disassemble specified tubing and prepare for autoclaving process
    b. Place all ultra-filtration parts (plus magnetic stir bars) into a clean glass beaker then add bleach and water to achieve 10% bleach concentration. Let sit until rest of clean-up is done
    c. Wipe down pressure gauge with 10% bleach and 70% ethanol
2. Wipe down everything else (ultra-filtration stand, metal fittings, bench, and magnetic stir plate with bleach and ethanol)
3. Place all the parts used for ultrafiltration on the 'dirty' cart to be washed

PEG Precipitation

4 **PURPOSE:**

Polyethylene Glycol (PEG) is used as a precipitant to precipitate microorganisms from concentrated water sample. This protocol serves to concentrate microorganisms from liquid phase and concentrate microorganisms and other dirt into a pellet.

MATERIALS:

- Bovine Serum Albumin (BSA)
- Polyethylene Glycol (PEG-8000)
- Sodium Chloride (NaCl)
- 250 ml centrifuge bottles
- Scale

PROCEDURE:

1. Measure water sample and place it in a glass bottle with cap.
2. Weigh 1% BSA (1 gram per 100 ml), 12% PEG 8000 (12 grams per 100 ml), and 0.9 M of NaCl.
3. Shake the tubes until the solids are dissolved.
4. Place the bottle in the cold room with a magnetic stir bar and stir slowly overnight.
5. Before centrifuging the bottles, make sure the sample bottles have the same weight.
6. Put the bottles across from each other and make sure that you always have an even number of bottles in the centrifuge and they are balanced.
7. Centrifuge the sample at 10,000 rpm for 30 minutes at 4°C.
8. Discard the supernatant from each tube and scoop each pellet from the bottle into the PowerWater DNA Bead Tubes, provided in the Qiagen kits, followed by DNA extraction.

DNA Extraction

5 **Material**

- Qiagen DNeasy® PowerWater® Kit (QIAGEN, 14900-50-NF)
- 10-20 ml 70% ethanol
- Vortexer
- 1 ml and 200 ul pipettes
- 1 ml and 200 ul tips
- Discarding beaker
- Marker
- Microcentrifuge with speed up to 13,000 rpm

Procedure

1) Add 1.0ml of PW1 to the PowerWater DNA Bead Tube.
2) Vortex at maximum speed for 5 minutes.
3) Centrifuge at 1,500 rpm for 2 minutes.
4) Transfer supernatant to a clean 2ml collection tube using a 1 ml pipette tip, avoiding the beads.
5) Centrifuge at 13,000 g for 1 minute at room temperature.
6) Avoiding the pellet, transfer the supernatant to a clean 2 ml collection tube.
7) Add 200 µl of Solution IRS and vortex briefly to mix. Incubate at 4°C for 5 minutes.
8) Centrifuge at 13,000 g for 1 minute.
9) Avoiding the pellet, transfer the supernatant to a clean 2 ml collection tube.
10) Add 650 µl of solution PW3 and vortex briefly to mix.
11) Load 650 µl of solution into the MB Spin Column. Centrifuge at 13,000 g for 1 minute.
12) Discard flow through and repeat until all of the solution has been processed.
13) Place the MB Spin Column Filter into a clean 2 ml collection tube.
14) Add 650 µl of solution PW4 (Shake before use). Centrifuge at 13,000 g for 1 minute.
15) Discard flow through, add 650 µl of Ethanol and centrifuge at 13,000 g for 1 minute.
16) Discard flow through and centrifuge at 13,000 g for 2 minutes.
17) Add 100 µl of Solution EB to the center of the white filter membrane.
18) Centrifuge at 13,000 g for 1 minute.
19) Discard the membrane and transfer the solution to any subsequent white membrane filters to concentrate the solution with the DNA.
20) Once all filters have been spun with solution discard the membrane and the DNA is ready for downstream applications.

6 Equipment and Materials:

- iQ powermix (Bio Rad, cat#1725849)
- S. typhi primer Forward (10 µM)
- S. typhi primer Reverse (10 µM)
- S. typhi Probe (10 µM)
- S. Paratyphi A primer Forward (10 µM)
- S. Paratyphi A primer Reverse (10 µM)
- S. Paratyphi A Probe (10 µM)
- 96-well plates (Bio-rad, cat# HSP9601)
- Microseal B Adhesive seals for PCR plates (Bio-rad, cat# MSB1001)
- Bio-rad Real-Time PCR (qPCR) machine

Procedures:

1) Spray surface of hood and pipettes with 70% ethanol, then wipe off
2) Thaw the reagents from the Bio-Rad iQ powermix, primers, and probes at room temperature

3) Place all the reagents on ice.

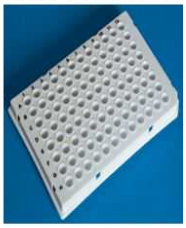
4) Prepare the following mixture under a clean hood:

Reagent	S. Typhi Volume (ul)	S.Paratyphi (ul)
Bio-Rad iQ Multiplex Powermix	12.5	12.5
S. Typhi forward primer (10 µM)	1.0	
S. Typhi reverse primer (10 µM)	1.0	
S. Typhi probe (10 µM)	0.5	
S. Paratyphi forward primer (10 µM)	-	1.0
S. Paratyphi reverse primer (10 µM)	-	1.0
S. Paratyphi probe (10 µM)	-	0.5
Molecular Water	5.0	5.0
Total	20.0	20.0

5) Calculate how much the reagents needed in total (according to the number of the wells needed for RT-PCR).
6) Aliquot 20 µl of the mixture created to each well of the PCR plate
a. Dispose of used pipet tips and plastic cup into trash
7) Move the PCR plate into a new area
8) Spray surface of the new area and pipettes with 10% bleach, then wipe off

9) Spray surface of the new area and pipettes with 70% bleach, then wipe off
10) Add 5 µl of DNA into the PCR plate

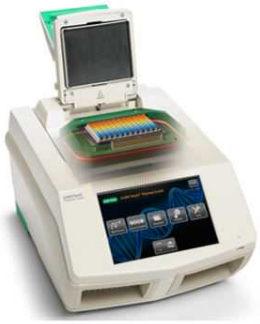
- For the PCR negative control, add 5 µl of molecular grade water
- For the Extraction negative control, add 5 µl of extraction material
- For the DNA extraction positive control, add 5 µl of DNA control



11) Dispose of used pipet tips and plastic cup into trash
12) Place samples in Bio-Rad Detection System with the following program:

95°C	15 mins	
95°C	30 secs	} 40 cycles
60°C	30 secs	
72°C	30 secs	

13) Place the 96-well plate into the Bio-rad PCR machine. Remember to correctly select the probe/fluorescence to FAM and start the cycles.



14) Results are interpreted using computer software available with Real-Time PCR machines by monitoring increase in fluorescence throughout the amplification cycles and reported as Ct value. The Ct value is the number of amplification cycles required to detect a fluorescent signal above a given threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample. In general, Ct values < 29 are considered strong positive reactions and are indicative of abundant target nucleic acid in the sample, while Ct values of 30 to35 are positive reactions indicative of moderate amounts of the target, and Ct values of 38 to 40 are considered weak reactions with little or no target nucleic acid in the sample.