

APPENDIX N1

OHIO DISTRICT MICROBIOLOGY LABORATORY INHIBITOR REMOVAL PROCEDURE

WARNING: *Do not process samples in areas where RT-PCR products have been handled! Do not process samples after working in rooms where RT-PCR products have been handled!*

Typically, a set of five water samples and a negative process control are processed. Fewer samples can be processed, but each run must include a negative process control.

STEP 1. Preliminary procedures (for the day before processing samples)

- a. Spray or wipe the inside of biological safety cabinet or clean area used for sample processing with 10% bleach. If available, turn on the UV light and wait about 15 to 30 minutes before proceeding.

Prepare 10% bleach by adding 100 mL of household bleach to 850 mL of dH₂O. Adjust to pH 6-7 with 1 M HCl and bring to 1 L with dH₂O. Store at room temperature for up to seven days.

- b. Open a box of SW32Ti centrifuge tubes (Beckman Cat. No. 344058) in the area used for sample processing and place 6 in a rack. Fill each tube with PBS containing 0.2% BSA. Cover with parafilm. Fill 6 microcon-100 filter units and 6 microcon-100 microcentrifuge tubes completely with PBS containing 0.2% BSA. Place the filter units in microcentrifuge tubes without PBS containing 0.2% BSA and cap before removing from the hood/area. Soak the centrifuge tubes and microcon units overnight at 4°C.

Prepare PBS containing 0.2% BSA by adding 4 mL of 5% crystalline BSA to 96 mL of phosphate buffered saline (PBS; e.g., Sigma Cat. No. D-5652). Store PBS containing 0.2% BSA at 4°C.

Prepare 5% BSA by dissolving 5 g of crystalline albumin (United States Biochemical Cat. No. 10856) in 100 mL of dH₂O. Sterilize by passing the solution through a 0.2 µ sterilizing filter (e.g., Sigma Cat. No. F-9768). Store 5% BSA at 4°C.

- c. Soak SW32Ti rotor buckets (Beckman Cat. No. 369647) in 0.525% bleach (pH 6-7) for 10 minutes. Dechlorinate with sterile 0.05% thiosulfate. Rinse the buckets with sterile dH₂O and dry.

Step 2. Sample concentration by ultracentrifugation

Turn on centrifuge and put in rotor with vacuum to cool it down to 10°C.

a. Spray or wipe the biological safety cabinet or clean area used for sample processing with 0.525% bleach (pH 6-7). If available, turn on the UV light and wait about 15 to 30 minutes before proceeding.

b. Thaw 5 water sample celite concentrates and place on ice. Place 40 mL of sterile dH₂O to be used as a negative process control into a sterile 50 mL centrifuge tube. Add 80 µl of 5% crystalline BSA to each of the six 40 mL samples (final BSA concentration of 0.01%). Mix by turning. Record the sample numbers on the **Inhibitor Removal Processing Form** (see Appendix 3).

Step 3. Discard the PBS solution in the ultracentrifuge tubes into sink and tap to get liquid out. Label each tube with the appropriate sample number. Add 30 mL of sample.

Step 4. Underlay each sample with 5 mL of 30% sucrose. Add 2 additional milliliters of each sample to the appropriate tubes. Place tubes in SW32Ti buckets. Make sure tubes are dry.

Prepare 30% sucrose by dissolving 300 g of sucrose in 700 g of 20 mM tris, 1 M NaCl, 1 mM EDTA, 5 mM EGTA, pH 8.0. Sterilize by autoclaving. Cool and add 20 mL of 5% crystalline BSA. Store at 4 °C.

Prepare 20 mM tris, 1 M NaCl, 1 mM EDTA, 5 mM EGTA, pH 8.0 by dissolving 2.42 g of trizma base, 58.44 g of NaCl, 0.37 g of disodium ethylenediaminetetraacetic acid, dihydrate and 1.9 g of ethylene glycol-bis(β-aminoethyl ether in 950 mL of dH₂O. Adjust the pH to 8.0 with 1 M HCl and bring to 1000 mL with dH₂O. Sterilize by autoclaving and store at room temperature. Do not autoclave sucrose longer than 15 minutes – will caramelize.

Step 5. Balance the buckets using sterile PBS. Centrifuge at 27,000 rpm for 4.5 hours at 10°C with the brake on.

Step 6. Immediately after the rotor stops, remove the centrifuge tubes and aspirate off the supernatant as quickly as possible in the sample preparation area. Place the tubes upside down on paper towels to drain. Tap to remove excess liquid. After the tubes have drained, place them upright into a rack and add 100 µl of sterile PBS containing 0.2% BSA onto each pellet.

Step 7. Using a variable pipetter (e.g., Rainin Instrument Cat. No. P-200) set at about 110 µl and a pipette tip with an aerosol barrier (e.g., USA/Scientific Plastics Cat. No. 1010-8810), thoroughly scrape the pellet from the process control sample to remove it from the centrifuge tube. Draw the liquid back and forth to dissolve the pellet. Wash the sides of the curved area of the centrifuge tube with the dissolved material and then transfer it to a labeled sterile 1.5 mL microcentrifuge tube placed on ice.

There should not be a visible pellet in the centrifuge tube containing the negative process control.

Step 8. Wash the centrifuge tube with another 100 µl of PBS containing 0.2% BSA and combine the second wash with the first.

Step 9. Repeat steps 7-8 with the remaining five samples.

Step 10. Prepare a solvent mix by mixing 0.1 mL of 0.01% dithiozone, 0.9 mL of 0.01 M 8-hydroxyquinoline, 1 mL butanol, 0.25 mL methanol and 0.25 mL trichloroethane. (Dark blue solution – make fresh every time)

Prepare 0.01% dithiozone by dissolving 0.01 g of diphenyl thiocarbazon (Fisher Cat. No. D90) in 100 mL of chloroform. Prepare 0.01 M 8-hydroxyquinoline (Fisher Cat. No. 0261) by dissolving 0.1452 g of 8-hydroxyquinoline in 100 mL of chloroform. Store 0.01% dithiozone and 0.01 M 8-hydroxyquinoline at 4 °C for up to one month.

Step 11. Using a P-1000 pipetter, add 200 µl of solvent mix to each microcentrifuge tube containing dissolved water sample pellets. Vortex for 30 seconds at room temperature. Let sit for 15 seconds. Vortex for an additional 30 seconds. Let sit for 30 seconds. Centrifuge at about 14,000 ×g (14,000 rpm) in the microcentrifuge (e.g, Eppendorf Model 5402) at 4°C for 5 minutes. Record the solvent color, interphase characteristics and any abnormal observations on the **Inhibitor Removal Processing Form** (Appendix 3).

Step 12. Pour off and discard the PBS solution from the microcon units. Using a P-200 pipetter set at 100µl, carefully transfer the aqueous layer from each sample (tilt tube) to a PBS containing 0.2% BSA-treated microcon-100 filter unit labeled with the sample number. Cap and centrifuge the filter units at 3,000 ×g (6,000 rpm) in a microcentrifuge for 30 minutes at 4°C. Add 80 µl of PBS containing 0.2% BSA and centrifuge again at 3,000 ×g for 20 minutes.

Step 13. Add 10 µl of PBS containing 0.2% BSA. Place the housing in a clean microcentrifuge tube, cap and vortex for 15 seconds. Invert all housings into the microcentrifuge tubes treated with PBS containing 0.2% BSA. Centrifuge at 1,000 ×g (3,500 rpm) for 3 minutes at 4°C.

Step 14. Discard the microcon filter housings. Measure the volume of the concentrated sample. Bring to 60 µl with PBS containing 0.2% BSA and record the volume. Store samples on ice or at 4°C until assayed, but freeze at -70°C if they cannot be analyzed on the day of processing.

INHIBITOR REMOVAL PROCESSING FORM

Date of Sample Processing:

Record Sample Numbers and Description from Step Procedure:

Centrifuge Tube	Sample Numbers*	Date	Description/Comments
1.	dH O 2		
2.			
3.			
4.			
5.			
6.			

Record Solvent Color and Interphase Characteristics from Step 11 of the Inhibitor Removal Procedure:

	Sample Number	Solvent Color	Interphase
1.			
2.			
3.			
4.			
5.			
6.			

Record Sample Volume from Step 14 of the Inhibitor Removal Procedure:

	Sample Number	Volume
1.		
2.		
3.		
4.		
5.		
6.		

Comments: