W-2 WATER PROCESSING V.1

REDI-NET Consortium[1]

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VERSION 1

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Protocol status: Working
We use this protocol and it's working

DISCLAIMER

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ABSTRACT

This protocol details about water processing.

GUIDELINES

A

AOBJECTIVE

To outline steps for properly collecting sediment samples from waterholes to evaluate the risk of zoonotic disease transmission by the detection of pathogens from environmental DNA (eDNA).

SUMMARY/SCOPE

The overarching aim of the *REDI-NET* is to develop a collaborative laboratory network between domestic and international partnering institutions to address disease surveillance needs in order to effectively detect, predict and contain potentially emergent zoonosis. This SOP provides guidance on the protocol for the correct sediment sampling at CONUS/OCONUS sites to evaluate and predict the risk of zoonotic disease transmission.

RESPONSIBLE PERSON

Principal Investigator, Study Coordinator, Entomology Component Lead, Managers

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Note

NOTE: All study procedures must be conducted in compliance with national and local policies for prevention and control of COVID-19 infection.

MAINTENANCE OF EQUIPMENT

BEFORE EACH COLLECTION

1. Clean corer and scoops
2. Freeze and clean ice packs
3. Clean cool-boxes

Fully charge all equipment (e.g., GPS unit, tablets/phone). Make sure the tablet has enough free-space for field sampling pictures.

AFTER EACH COLLECTION

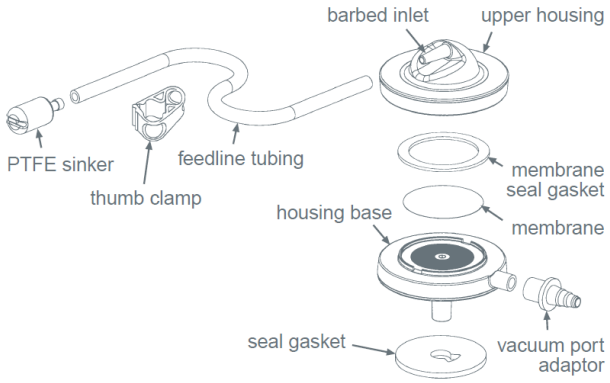
1. Clean all equipment thoroughly between sampling sites, including boots, cooler box (inside and outside), etc.
2. Store sterile equipment separate from used equipment and samples.

QUALITY CONTROL

This SOP is reviewed by the applicable supervisor annually or as required in order to maintain its relevance.

APPENDICES

APPENDIX 1: SolVAC Filter Holder Compartments



Specifications

Materials of Construction
Upper Housing, Housing Base: Polypropylene

Feedline Tubing: Ultra chemical-resistant Tygon*, 4.8 mm (3/16 in.) ID

Thumb Clamp: Celcon* plastic

Feedline Sinker: PTFE

Vacuum Port Adaptor, Membrane Seal Gasket, and Seal Gasket: Polyethylene

Effective Filtration Area
10.2 cm^2

Filter Size
Accepts 47 mm filter

Inlet/Outlet Connections
Tapered inlet accepts 3.2 - 6.4 mm (1/8 - 1/4 in.) ID tubing; outlet seals to bottles with openings 17.8 - 48.3 mm (0.7 - 1.9 in.) OD

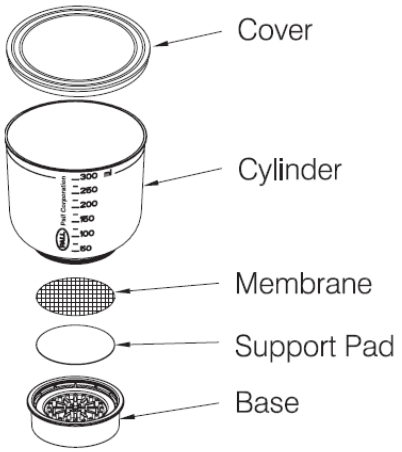
Vacuum Port Adaptor
4.8 - 7.9 mm (3/16 - 5/16 in.) tapered hose barb

Maximum Vacuum
64 cm Hg (25 in. Hg) at 25 °C

Operating Temperature
Ambient; not to exceed 38 °C (100 °F); not autoclavable

Chemical Compatibility
500 mL of each of the following HPLC-grade solvents—water, acetonitrile, methanol, tetrahydrofuran, hexane, and NMP—were filtered through a new SolVac™ filter holder. Three-milliliter aliquots from each filtrate were tested for extractable materials under common HPLC conditions. None of the chromatograms exhibited any trace of extractables leached from the SolVac filter holder to the final filtrate. For the complete technical data, go online to www.pall.com/lab and see, "Chemical Compatibility Guide and Life Expectancy for the SolVac Filter Holder."

300 mL MicroFunnel standard and ST filter funnels



Part Number	Description
4815	0.45 µm GN-6, gridded
4812	0.45 µm GN-6, gridded*

APPENDIX 4: Measuring Spoon for 0.1 mm Beating Beads

The spoon (Next Advance, MSP01-RNA) is used for 0.1 mm beating beads measurement. The step is described on 6.1.7 the preparation before sample homogenization. One spoon equals to 100 uL.

APPENDIX 6: Expected Outcomes

Sample	Amount	Sample condition	Elution volume	DNA conc. (ng/ul)	RNA conc. (ng/ul)
Water	750 ml	Half of the membrane	75	<0.025 - 20	<0.01 - 20

MATERIALS

EQUIPMENT AND MATERIALS

Note

NOTE*: If product number is listed, please ensure use of this or equivalent product.*

	A	B
	Equipment	**Mfg / Product #**
	KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head or KingFisher™ Duo Prime Magnetic Particle Processor	ThermoFisher, 5400630 or ThermoFisher, 5400110
	Bullet Blender 24 Gold	Next Advance, BB24-AU
	Adjustable micropipettes	Locally sourced
	Multi-channel micropipettes	Locally sourced
	Sartorius Microsart™ Maxi Vacuum Pumps	Fisher Scientific, 14-555-788 or equivalent
	SolVac Filter Holder	PALL, 4020
	Vortex	Locally sourced
	Glass storage bottles, autoclavable	Fisher Scientific, 12-141-322 or equivalent
	Tube centrifuge	Locally sourced
	Plate centrifuge	Locally sourced
	Qubit 4 Fluorometer	ThermoFisher, Q33238
	Mini Block Heater	VWR, 10818-597 or locally sourced
	Autoclave	Locally sourced
	Stir bar	Locally sourced
	RT2 Basic Hotplate Stirrer	ThermoFisher, 88880004 or locally sourced

	A	B	C
	Material	**Description**	**Mfg / Product #**
	Water Samples	Collected using SOP W-1; stored in 250 ml sterile bottle at 4°C for processing the same day or at -80°C or -20°C for long-term storage	From REDI-NET Program
	Sterile 1x PBS as negative control	180 mL, sterile.	Thermo Fisher, 10010023
	Sterile 1x PBS spike-in positive control	180 ml sterile 1x PBS, spike in 37.5 µl of ZymoBIOMICS Microbial Community Standard and 100 µl HIV standard, 100 µl EBV standard.	Thermo Fisher, 10010023

	A	B	C
	ZymoBIOMICS Microbial Community Standard Material	For positive controls	Zymo Research, D6300
	AcroMetrix HIV-1 Controls	For TNA extraction positive control; BSL-2	ThermoFisher, CLS430320-12EA
	Human gammaherpesvirus (EBV) positive control	For TNA extraction positive control	NMRC made
	Easy Grip Polystyrene Storage Bottles	1 L, *(consumable)*	Corning, 430518 or equivalent
	Easy Grip Polystyrene Storage Bottles	250 ml	Corning, 430281 or equivalent
	Beaker	1 L	Fisher Scientific, 02-591-32 or equivalent
	Fisher brand Sterile Sampling Bags with Flat-Wire Closures	Clear, 42 oz/1190 ml, 3.5 mil thickness *(consumable)*	Fisher Scientific, 14-955-188 or equivalent
	Versapor® Acrylic Copolymer 5000 Membrane Disc Filters	47 mm diameter, 5 µm pore *(consumable)*	PALL, 60178
	MicroFunnel™ST filter funnel with GN-6 Membrane	300 mL, 0.45 µm pore *(consumable)*	PALL, 4812
	Cardinal Health™ Medi-Vac™ Guardian™ Suction Canisters	3 liter *(consumable)*	Fisher Scientific, 19-162-321 or equivalent
	Gauze	Sterile, 4 x 4 inches, 8-ply	Fisher Scientific, 10-000-684 or equivalent
	Coffee filter paper with compatible funnel	10-15 cm diameter	Locally Sourced
	IndiMag Pathogen Kit	w/o plastics, 384 reactions	Indical Bioscience, SP947257
	Buffer ATL	200 mL, Tissue Lysis Buffer	Qiagen, 19076
	Reagent DX	1 mL, Antifoaming Reagent	Qiagen, 19088
	Measuring Spoon 100µL	RNase Free, pack of 10	Next Advance, MSP01-RNA
	Clear RINO brand microcentrifuge tubes	1.5 mL, screw-cap	Next Advance, TUBE1R5-S
	Stainless Steel UFO Beads	3.5 mm, RNase free	Next Advance, SSUFO35-RNA
	Zirconium oxidase beads	0.1 mm, 400 g	Fisher Scientific, 50-154-2950
	KingFisher™ Deepwell 96 Plate	*(consumable)*	ThermoFisher, 95040450
	KingFisher™ 96 KF microplate	KingFisher Flex ONLY *(consumable)*	ThermoFisher, 97002540
	KingFisher™ 96 tip comb for DW Magnets	KingFisher Flex ONLY *(consumable)*	ThermoFisher, 97002534

	A	B	C
	KingFisher™ Duo Prime 12-tip comb	KingFisher Duo Prime ONLY *(consumable)*	ThermoFisher, 97003500
	Elution Strip	KingFisher Duo Prime ONLY *(consumable)*	ThermoFisher, 97003520
	KingFisher™ Duo Cap for Elution Strip	KingFisher Duo Prime ONLY *(consumable)*	ThermoFisher, 97003540
	MicroAmp™ Clear Adhesive Film	KingFisher *(consumable)*	ThermoFisher, 4306311
	Nonstick, RNase-Free Microfuge Tubes	1.5 mL *(consumable)*	ThermoFisher, AM12450
	Nonstick, RNase-Free Microfuge Tubes	2.0 mL *(consumable)*	ThermoFisher, AM12475
	RNaseZap™ RNase Decontamination Solution	To remove RNase from working area *(consumable)*	ThermoFisher, AM9780
	PEG-8000	Poly(ethylene glycol) BioUltra, 8,000 (*consumable*)	Millipore Sigma, 89510-1KG-F
	VacuCap 90 Vacuum Filtration Devices	0.2 µm, 90 mm, gamma-irradiated *(consumable)*	PALL, TA4622
	NaCl	For PEG-8000 buffer preparation	Sigma Aldrich, S9888-1KG
	Qubit™ 1X dsDNA HS Assay Kit	*(consumable)*	ThermoFisher, Q33230
	Qubit™ RNA HS Assay Kit	*(consumable)*	ThermoFisher, Q32852
	Qubit Assay tubes	For Qubit DNA/RNA measurement (*consumable*)	Thermo Fisher, Q32856
	Ethanol	100%, molecular biology grade *(consumable)*	Locally Sourced
	Isopropanol	100%, molecular biology grade *(consumable)*	Locally Sourced
	Forceps	Stainless, sterile *(consumable)*	PALL, 51147 or equivalent
	Tubing	Plastic *(consumable)*	Locally sourced
	Razor blades	*(consumable)*	Fisher Scientific, 12-640 or equivalent
	Petri dishes	60 mm disposable *(consumable)*	Fisher Scientific, FB0875713A or equivalent
	Wire racks	*(consumable)*	Fisher Scientific, FB147916A or equivalent
	Excelta Medical-Grade Scissors	For filter membrane cutting	Fisher Scientific, 17-456-005 or equivalent
	Parafilm M	Used for petri dish sealing (*consumable*)	Fisher Scientific, 13-374-12 or equivalent
	Kimwipes	To dry material	Locally sourced

	A	B	C
	Conical centrifuge tubes	50 mL (*consumable*)	Fisher Scientific, 14-432-22 or equivalent
	DNA/RNA Shield Reagent	50 ml	Zymo Research, R1100-50
	Conical centrifuge tubes	15 mL *(consumable)*	Fisher Scientific, 14-959-53A or equivalent
	Nuclease-free water	*(consumable)*	Locally sourced
	Dry ice	To maintain cold chain during sample handling *(consumable)*	Locally sourced
	Data Sheets	REDI-NET DCS SP-1 Sample Processing Form	REDI-NET Data Portal

Equipment

KingFisher™ Flex Purification System, KingFisher with 96 Deep-well Head

NAME

Purification System

TYPE

Thermo Scientific™

BRAND

5400630

SKU

https://www.thermofisher.com/order/catalog/product/5400630

LINK

Equipment

Bullet Blender 24 Gold (1.5 mL snap and screw cap tubes, 4°C cooling)

NAME

Blender

TYPE

Next Advance

BRAND

BB24-AU

SKU

https://www.nextadvance.com/product/bullet-blender-24-gold/

LINK

Equipment

Sartorius Microsart™ Maxi Vacuum Pumps	NAME
Vacuum Pumps	TYPE
Fisher Scientific	BRAND
14-555-788	SKU
https://www.fishersci.com/shop/products/sartorius-microsart-maxi-vacuum-pumps/14555788	LINK

Equipment

Qubit Fluorometer	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU
https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238	LINK

Equipment

Mini Block Heater, Greiner Bio-One	NAME
Mini Block Heater	TYPE
VWR	BRAND
10818-597	SKU
https://us.vwr.com/store/product/20823794/mini-block-heater-greiner-bio-one	LINK

Equipment	
RT2 Basic Hotplate Stirrer	NAME
Hotplate Stirrer	TYPE
Thermo Scientific™	BRAND
88880004	SKU
https://www.thermofisher.com/order/catalog/product/88880004	LINK

DWK Life Sciences Kimble™ KIMAX™ Storage/Media Bottles **Fisher Scientific Catalog #12-141-322**

PBS buffer **Thermo Fisher Scientific Catalog #10010023**

ZymoBIOMICS Microbial Community Standard **Zymo Research Catalog #D6300**

Corning® 1L Easy Grip Polystyrene Storage Bottles with 45 mm Caps **Corning Catalog #430518**

250ml Storage Bottle - Disposable With Plug Seal Cap Sterile 45mm neck **Corning Catalog #430281**

Fisherbrand™ Low-Form Polypropylene Beakers **Fisher Scientific Catalog #02-591-32**

Fisherbrand™ Sterile Sampling Bags with Flat-Wire Closures **Fisher Scientific Catalog #14-955-188**

Versapor™ acrylic copolymer 5000 membrane disc filters 5 µm - 47 mm **Pall Corporation Catalog #60178**

MicroFunnel ST Disposable filter funnels **Pall Corporation Catalog #4812**

Cardinal Health™ Medi-Vac™ Guardian™ Suction Canisters **Fisher Scientific Catalog #19-162-321**

Stoelting™ Sterile Gauze **Fisher Scientific Catalog #10-000-684**

IndiMag Pathogen Kit w/o plastics (384 reactions) **INDICAL BIOSCIENCE Catalog #SP947257**

Buffer AL, Lysis buffer **Qiagen Catalog #19076**

Reagent DX **Qiagen Catalog #19088**

Measuring Spoon 100 uL RNase Free pack of 10 **Next Advance Catalog #MSP01-RNA**

Sterile Microcentrifuge Tube 1.5 mL (RINO®) 500/case **Next Advance Catalog #TUBE1R5-S**

Stainless Steel UFO Beads 3.5 mm RNase Free 10 mL **Next Advance Catalog #SSUFO35-RNA**

Bertin Corp 0.1mm Zirconium oxide beads (450g) (qty 500) **Fisher Scientific Catalog #50-154-2950**

KingFisher™ Plastics for 96 deep-well format **Thermo Fisher Scientific Catalog #95040450**

KingFisher™ Flex™ Systems Consumables, KingFisher 96 KF micropla (200µL) **Thermo Fisher Catalog #97002540**

KingFisher™ Flex™ Systems Consumables, KingFisher 96 tip comb fo DW magnets **Thermo Fisher Catalog #97002534**

KingFisher™ Duo and KingFisher™ Duo Prime Consumables, Elution strip **Thermo Fisher Catalog #97003520**

KingFisher™ Duo and KingFisher™ Duo Prime Consumables, KingFish Duo Cap for elution strip **Thermo Fisher Catalog #97003540**

MicroAmp Clear Adhesive Film **Applied Biosystems (ThermoFisher Scientific) Catalog #4306311**

Nonstick, RNase-free Microfuge Tubes, 1.5 mL **Thermo Fisher Catalog #AM12450**

Nonstick, RNase-free Microfuge Tubes, 2.0 mL **Thermo Fisher Catalog #AM12475**

RNaseZap™ RNase Decontamination Solution **Thermo Fisher Scientific Catalog #AM9780**

Polyethylenglycol (MW=8000) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #89510-1KG-F**

VacuCap 0.2 µm 90 mm gamma-irradiated (10/pkg) **Pall Corporation Catalog #TA4622**

Sodium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S9888**

Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q33230**

Qubit RNA HS (High Sensitivity) assay **Thermo Fisher Scientific Catalog #Q32852**

Qubit™ Assay Tubes **Invitrogen - Thermo Fisher Catalog #Q32856**

Forceps Stainless Steel with black Grips 1 **Pall Corporation Catalog #51147**

Razor blades **Fisher Scientific Catalog #12-640**

Fisherbrand™ Petri Dishes with Clear Lid **Fisher Scientific Catalog #FB0875713A**

Fisherbrand™ HDPE Coated Wire Racks **Fisher Scientific Catalog #FB147916A**

Excelta™ Medical-Grade Scissors **Fisher Scientific Catalog #17-456-005**

Bemis™ Parafilm™ M Laboratory Wrapping Film **Fisher Scientific Catalog #13-374-12**

Falcon 50mL Conical Centrifuge Tubes **Fisher Scientific Catalog #14-432-22**

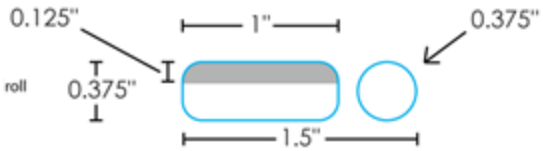
DNA/RNA Shield **Zymo Research Catalog #R1100-50**

Falcon™ 15mL Conical Centrifuge Tubes **Fisher Scientific Catalog #14-959-53A**

APPENDIX 7. Set-up instructions for barcode printing

A	B	C
Equipment / Material	**Description**	**Mfg / Product #**
Thermal Printer	Zebra ZD421T Desktop Dual Barcode Printer - 203 dpi	Uline, H-9581W
Thermal Transfer Ribbon	For use with Zebra thermal printer; Desktop thermal transfer ribbons - wax/resin, 4.33” x 244 (12/case)	Uline, S-18466
Cryo-labels	667 1.00" x 0.38" Cap & Wrap CryoLabel® w/0.375" Cap, Blanks, 1" Core Color bar breakdown: Grey - 31,24,25,0 Orange - 0,80,95,0 Blue - 85,50,0,0 Brown - 35,60,80,25 Yellow - pending from vendor	Electronic Imaging Materials, #335774-COLOR
Handheld scanner	To scan barcode	Zebra, LS2208-SR20001R-NA
123Scan Software	To scan barcodes	123Scan software

	A	B	C
	Laptop or desktop computer with Google Chrome and access to the REDI-NET data portal	To connect with the handheld scanner, the thermal printer and the REDI-NET Data Portal	Locally sourced



Cryo-labels.

Desktop Thermal Transfer Ribbons - Wax/Resin 4.33 x 244 **Uline Catalog #S-18466**

SAFETY WARNINGS

RISK AND PERSONAL PROTECTION

1. Caution should be taken while processing samples as some chemicals may be harmful. Please use a fume-hood when required to avoid inhaling harmful chemicals.
2. Gloves should be worn all the time when handling samples.
3. Decontaminants such as DNA/RNaZap could irritate the skin, avoid contact with skin while preparing the workbench for nucleic acid extractions.

BEFORE START INSTRUCTIONS

BEFORE START

Note

NOTE:

- *To prevent cross contamination, nucleic acid extraction and amplification (PCR) should be performed in separate rooms.*
- *Processing can be done prior to freezing samples to save freezer space. Each location/site (edge/1m from edge) would account for 4 filter paper water samples for each sampling site.*
- *Plastic sample bags holding* 250 mL *water samples can leak after freeze/thaw. The 1190 ml/42 oz sterile bag can prevent sample loss/contamination.*

1. Make sure the feeding tube and PTFE sinker for SolVac Filter are properly clean by using 70% ethanol and allowed to air dry.
2. For the samples stored at 4 °C, pour three 250 mL samples from the same sampling location into a sterile 1190 mL sample bag.
3. If using a frozen water sample, fully thaw them at room temperature. For the frozen sample in a plastic sample bag, wipe the bag surface with 70% ethanol to remove dusts and sanitize the surface. Source three bags of 250 mL water samples from the same sampling location in a new 1190 ml sterile sample bag, then put the bagged samples in a suitable-sized container for defrosting. After samples are fully defrosted, pour the water samples into the 1190 ml outer bag and discard the original 250 ml sample bags. Hold the whole bag in a 1 L beaker.
4. Prepare 40% PEG-8000 solution for microbe aggregation. Check **Appendix 3** for the recipe.
5. Pre-cool the Bullet Blender by adding dry ice into the cooling compartment and running the cooling program.
6. Clean the work surfaces with RNaseZap, then wipe the surfaces with 70% molecular biology grade ethanol to remove additional contaminants.
7. Transfer 0.1 mm zirconium oxide beads (2 spoons, **Appendix 4**) to Clear RINO brand 1.5 ml screw-cap microcentrifuge tubes.
8. For the first time use of IndiMag pathogen kit, add 100% ethanol to Buffer AW1 and AW2, and add 100% Isopropanol to ACB as indicated on the bottles.
9. Buffer ATL may form precipitates upon storage. If necessary, warm to 56 °C until the precipitates have fully dissolved. Prepare buffer ATL-DX: add 100 µL Reagent DX to 15 mL Buffer ATL. If smaller amounts are needed, transfer 1.5 mL of Buffer ATL into a sterile 2 ml vial and add

10 µL Reagent DX. Mix well, after the addition of Reagent DX. After preparation, the mixture is stable for 6 months at Room temperature (15-25°C).

10. MagAttract Suspension G from the IndiMag pathogen kit needs to be vortexed thoroughly for 00:03:00 (before first use) or 1 minute (before subsequent uses) to ensure that the magnetic silica particles are fully resuspended.
11. Binding beads need to be vortexed thoroughly before each use.
12. Prepare a few 15 ml or 50 ml conical centrifuge tubes with nuclease-free water for preparing TNA elution in KingFisher Flex or KingFisher Duo Prime to avoid cross-contamination.

1. VACUUM PUMP SET UP

1 Wipe the surfaces with 70% ethanol to remove contaminants.

2 Use tubing to connect a 3 liter Medi-Vac Canister with vacuum pump through the vacuum outlet on the lid.

Note

If possible, the canister should be set up inside a biosafety cabinet.

3 Connect tubing with the 3 liter Medi-Vac Canister through the inlet on the lid. Close unused inlets. Turn on the pump to test the vacuum suction by feeling the airflow.

2. WATER SAMPLE PRE-FILTRATION

4

Note

NOTE: *If the water sample contains floating plants and debris, filter the sample with coffee filter paper with a funnel or filter mesh by gravity to remove the big pieces that can clot the tubing. If the water is very dirty, before coffee filter paper filtration, filter the water with a sterile 8-ply gauze on a funnel using gravity to remove mud or micro algae, it could be done multiple times.*

Assemble seal gasket, housing base, and vacuum port adaptor of SolVac filter holder on the top of a new 1L sterile glass or plastic bottle. Connect the tubing from the Medi-Vac Canister to the vacuum port adaptor on the housing base.

Note

NOTE: *See* Appendix 1 *for the SolVac Filter compartments.*

5 Wipe the housing base with 70% ethanol and let ethanol air dry (Vacuum pump can be turned on to help drying).

6 Clip a thumb clamp on the feeding tubing.

7 Connect upper housing, feeding tubing, and PTFE sinker together.

8 Place a 5 µm Versapor membrane disc filter on the housing base.

9 Seal the membrane with a membrane seal gasket and the upper housing.

10 Place the feeding tubing with PTFE sinker in the 750 mL sample (see steps 1 and 2 from Before start section).

11 Turn on the pump (<15 psi). Release the thumb clamp of the feeding tube to allow the water sample to pass through. The water sample will be filtered and collected in the 1L sterile bottle. Turn off the vacuum pump after the water sample runs out.

Note

If clogging happens, replace the membrane disc filter with a new one, reassemble the filter system and collect all the filtrates in the same bottle

12 Include a positive control for each batch of samples: transfer 37.5 µL ZymoBIOMICS Microbial Community Standard and 100 µL EBV, and 100 µL HIV standard into 180 mL sterile 1x PBS.

13 Include a negative control for each batch of samples: 180 mL sterile 1x PBS.

14 Add 250 mL of PEG-8000 solution to the pre-filtered 750 mL water sample and 60 mL of PEG-8000 solution in each control (the final solution of PEG-8000 is 10%). Mix well by shaking.

15 Store the sample at 4 °C Overnight .

3. MICROORGANISM COLLECTION

16

Note

NOTE: *See* Appendix 2 *for the 300 ml MicroFunnel ST filter funnel compartments.*

Disconnect the SolVac filter holder from the Medi-Vac Canister tubing.

17 Place a 300 ml MicroFunnel ST Filter funnel on a wire rack.

Note

Do not open the lid and touch the inner side of the funnel.

18 Connect the tubing from the Medi-Vac Canister to the base of a 300 ml MicroFunnel ST Filter Funnel.

Note

Three adaptors are provided in the box of the MicroFunnel ST Filter Funnel, use adapter for connection if necessary.

19 Turn on the pump (< 15 psi). Pour the PEG-8000 treated water sample into the filter funnel (Stir the water sample while pouring).

20 If possible, filter the entire 750 mL water sample using one filter funnel (the filtrate will be collected in the Medi-Vac Canister as waste). When clogging happens, use a new funnel to filter the rest.

21 Disassemble the filter funnel. Carefully use an autoclaved forceps (or wipe with 70% ethanol and let dry) to move the GN-6 membrane on the supporting pad to a sterile 60 mm Petri dish.

22 If not processing the membrane immediately, evenly distribute 250 µL DNA/RNA Shield Reagent on the membrane in the Petri dish, seal the Petri dish with parafilm and store at -20 °C for short-term and -80 °C for long-term until DNA/RNA extraction.

4. SAMPLE LYSIS

23 Pre-cool the Bullet Blender by adding dry ice into the cooling compartment and running the cooling program.

24 Clean the work surfaces with RNaseZap, then wipe the surfaces with 70% molecular biology grade ethanol to remove additional contaminants.

Transfer 0.1 mm zirconium oxide beads (2 spoons, Appendix 4, see Guidelines & Warnings tab) and four

25 3.5 mm UFO beads to Clear RINO brand 1.5 ml screw-cap microcentrifuge tubes.

26 Add 500 µL of ATL-DX buffer and 135 µL VXL buffer to the Clear RINO brand 1.5 ml screw-cap micro-centrifuge tubes containing 0.1 mm and 3.5 mm UFO beating beads.

Note

NOTE: For the preparation of the ATL-DX buffer, see step 9 from Before start section under the Guidelines & Warnings tab.

27 Cut the membrane with a new razor blade into 2 halves.

28 Place a half of the filter membrane in a new Petri dish and store the unused half membrane in the Petri dish at -20 °C for future use.

29 Use 70% ethanol to wipe forceps and surgical scissors. Use the forceps to fold the half membrane into a smaller size sector and directly cut the sector (into tiny pieces, smaller than 1 mm x 3 mm) into the 1.5 mL RINO tube with lysis buffer and beads.

30 Add 20 µL Proteinase K from IndiMag kit and incubate the tube at 56 °C in the heat block shaker set up at 1400 rpm, 00:20:00 .

Note

If heat block shaker is not available, vortex the tube every 5 min.

31 Load the sample/bead tubes in the Bullet Blender.

32 Set the speed at 12 and time at 3. Press Start.

33 Let the samples settle for 00:01:00 and then repeat step 32. 1m

Note

STOPPING POINT: Lysed samples can be stored at 4 °C Overnight.

5. INSTRUMENT SET UP

34

Note

NOTE: KingFisher Flex only, if using KingFisher Duo Prime, go to section 6

Confirm 96 deep-well magnetic head and 96 well deep-well heat block are being used.

35 Ensure the program **IndiMag_Pathogen_KF_Flex_4wash** has been downloaded and loaded onto the KingFisher Flex instrument.

5.1 SET UP THE PROCESSING PLATES

36 Set up the Wash, Elution, and Tip Comb Plates outside the instrument according to the following table.

Note

NOTE: DO NOT *use the elution buffer provided by the kit for TNA elution. The ingredients in the elution buffer inhibit the downstream DNA sequencing efficiency.*

	A	B	C	D	E
	Plate ID	Plate position	Plate type	Reagent	Volume per well
	Tip comb	7	Place a 96 Deep-well Tip comb in a deep-well plate		

	A	B	C	D	E
	Elution	6	Deep-Well	Nuclease-free water	75 µL
	Wash 4	5	Deep-Well	100 % ethanol	750 µL
	Wash 3	4	Deep-Well	80% ethanol	750 µL
	Wash 2	3	Deep-Well	Buffer AW2	700 µL
	Wash 1	2	Deep-Well	Buffer AW1	700 µL
	Sample	1	Sample Lysate	Lysate and lysis buffer	985 µL

5.2 EXTRACTION

37 Centrifuge the bead tubes with lysate from step 33 for 12000 x g, 00:05:00. 5m

38 Transfer 425 µL supernatant without any particle carryover to the wells of the Deep-well plate. This plate becomes the Sample Plate.

39 Add 540 µL Buffer ACB, and 20 µL MagAttract Suspension G to each sample in the sample plate. For multiple samples, make a master mix with 10% overage. Invert slowly to mix the master mix, avoid foaming (can be mixed on Hula mixer for 2 min). Add 560 µL mixture to each sample.

40 Select the program **IndiMag_Pathogen_KF_Flex_4wash** on the instrument.

41 Start the run, then load the prepared plates into the positions when prompted by the instrument.

5.3 QUANTIFICATION AND STORAGE

42 After the running protocol is completed (~35 minutes), immediately remove the elution plate from the instrument and cover the plate or transfer the eluate to the final tube or plate of choice for final storage.

43 In a 0.6 mL microcentrifuge tube, use 3 µL total nucleic acid for DNA and RNA concentration measurement using Qubit 4 Fluorometer following manufacturer instructions.

Note

Kits needed: Qubit 1X dsDNA HS Assay Kit and Qubit RNA HS Assay Kit. (see Appendix 5 and Appendix 6).

44 Proceed with sample testing following the REDI-NET SOP W-4 Water Testing or store at -20 °C for less than 2 weeks.

Note

For long-term storage the sample needs to be stored at -80 °C following the REDI-NET SOP W-3 Water Storage.

6. INSTRUMENT SET UP

45

Note

NOTE: KingFisher Duo Prime only, if using KingFisher Flex, go to section 5

Confirm 12-tip magnetic head and 12 deep-well heat blocks are being used.

46 Ensure the program **IndiMag_Pathogen_KF_Duo_4wash** has been downloaded and loaded onto the KingFisher Duo Prime instrument.

6.1 SET UP SAMPLE PLATE AND ELUTION STRIP

47 Set up the Sample Plate according to the table below:

	A	B	C	D
	Row ID	**Plate Row**	**Reagent**	**Volume per well**

	A	B	C	D
	Sample row	A	Lysate and lysis buffer	985 µL
	Wash 1	B	Buffer AW1	700 µL
	Wash 2	C	Buffer AW2	700 µL
	Wash 3	D	80 % ethanol	750 µL
	Wash 4	E	100 % ethanol	750 µL
	Tip Comb Wash 2	F	12-Tip comb	
		G	Empty	
		H		

48 Set up the Elution Strip according to the table below:

Note

NOTE: DO NOT *use the elution buffer provided by the kit for TNA elution. The ingredients in the elution buffer inhibit the downstream DNA sequencing efficiency.*

	A	B	C	D
	Row ID	**Plate Row**	**Reagent**	**Volume per well**
	Elution	A	Nuclease-free water	75 µL

6.2 EXTRACTION

49 Centrifuge the bead tubes with lysate from step 33 for 12000 x g, 00:05:00. 5m

50 Transfer 425 µL supernatant without any particle carryover to the wells of the Deep-well plate. This plate becomes the Sample Plate.

51 Add 540 µL Buffer ACB, and 25 µL MagAttract Suspension G to each sample in the sample plate. For multiple samples, make a master mix with 10% overage. Invert slowly to mix the master mix, avoid foaming. Add 565 µL mixture to each sample.

52 Select the program IndiMag_Pathogen_KF_Duo_4wash on the instrument.

53 Start the run, then load the prepared plates into position when prompted by the instrument.

54 Keep the door open while extraction is in process. The chamber of the KingFisher Duo Prime is small. Closing the door makes the ethanol vapor restrained inside the chamber and increases the ethanol contamination.

6.3 QUANTIFICATION AND STORAGE

55 After the running protocol is completed (~35 minutes), immediately remove the elution plate from the instrument and cover the plate or transfer the eluate to the final tube or plate of choice for final storage.

56 In a 0.6 mL microcentrifuge tube, use 3 µL total nucleic acid for DNA and RNA concentration measurement using Qubit 4 Fluorometer following manufacturer instructions. (see Appendix 5 and Appendix 6).

Note

Kits needed: Qubit 1X dsDNA HS Assay Kit and Qubit RNA HS Assay Kit.

57 Proceed with sample testing following the REDI-NET SOP W-4 Water Testing or store at -20 °C for less than 2 weeks).

Note

For long-term storage the sample needs to be stored at -80 °C following the REDI-NET SOP W-3 Water Storage.

APPENDIX 3. PEG-8000 Preparation

58 Preparation of 40% PEG-8000 Solution

	A	B
	PEG-8000	400 g
	NaCl	70 g
	Add 1x PBS to final volume	1 L

The final concentrations: 40% PEG-8000 and 1.2M NaCl.

59 Add stir bar, PEG-8000 and NaCl into an empty 1L sterile bottle (plastic or glass, autoclavable).

60 Add sterile 1xPBS to final volume 1L.

61 Autoclave the whole bottle with lid at 121℃ for 30 min. After autoclave, place the hot solution on a stirring hot plate and stir the solution until it cools down to room temperature.

62 If autoclave is not available, stir the solution on a stirring hot plate until the crystals are fully dissolved and filter the solution through 0.45 µm Corning Disposable Vacuum Filter/Storage Systems.

63 Store the 40% PEG-8000 solution at 4℃.

APPENDIX 5. DNA and RNA Measurement using Qubit Fluorom...

64 DNA quantification:

According to the volume of sample used, add the 1xHS dsDNA Qubit Assay for a final volume of 200 µL (i.e., if using 3 µL of sample, add 197 µL of 1x HS dsDNA Qubit Assay.

65 RNA Quantification:

In a new microcentrifuge tube/falcon tube (depending on the number of samples processed), prepare a working solution of the Qubit HS RNA Assay:

66 In a new 0.6 ml tube, mix 197 µL of Qubit HS RNA Assay working solution and 3 µL of the sample. Incubate for 1 minute at room temperature before reading.