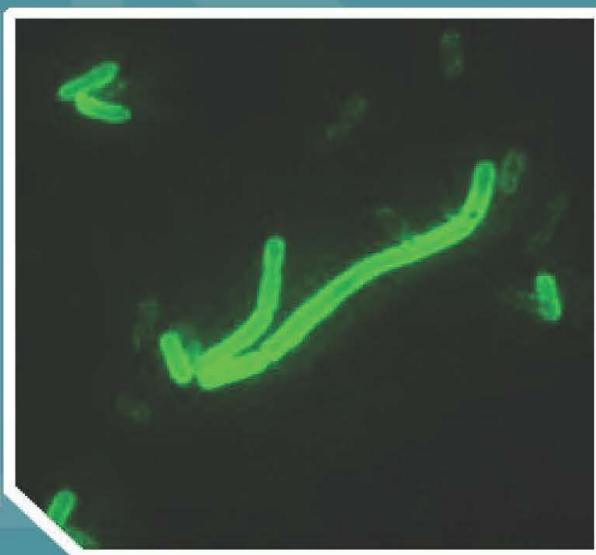
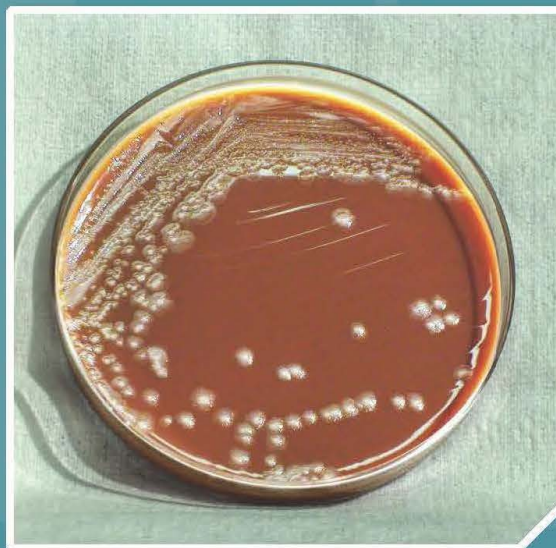


## Protocol for Detection of *Yersinia pestis* in Environmental Samples During the Remediation Phase of a Plague Incident



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**Protocol for Detection of *Yersinia pestis* in  
Environmental Samples During the  
Remediation Phase of  
a Plague Incident**

**UNITED STATES ENVIRONMENTAL PROTECTION AGENCY  
CINCINNATI, OHIO 45268**

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## **Disclaimer**

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Cover Photos: Left –Direct fluorescent antibody (DFA) of *Yersinia pestis*; Right – *Yersinia pestis* bacteria grown on chocolate agar after 72 hours (Source: Department of Health and Human Services – CDC)

Section 10 Figure 2: Left – *Yersinia pestis* colonies on SBA (Source: Public Health Image Library); Right – *Yersinia pestis* colonies on CIN agar (Source: Unite des Rickettsies - Faculte de medicine)

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## Acronyms

ABI	Applied Biosystems®
ASM	American Society for Microbiology
BD	Becton, Dickinson, and Company
BMBL	Biosafety in Microbiological and Biomedical Laboratories
BSA	Bovine serum albumin
BSC	Biological safety cabinet
BSL	Biosafety level
CBR	Chemical, biological, radiological
CDC	Centers for Disease Control and Prevention
CFR	Code of Federal Regulations
CFU	Colony forming unit
CIN	Cefsulodin-Irgasan, Novobiocin agar
CN	Cefsulodin Novobiocin
C <sub>T</sub>	Cycle threshold
DI	Deionized
DNA	Deoxyribonucleic acid
DQO	Data quality objective
EDTA	Ethylenediaminetetraacetic acid
EIC	External inhibition control
EPA	United States Environmental Protection Agency
ERLN	Environmental Response Laboratory Network
FBI	Federal Bureau of Investigation
FEM	Forum on Environmental Measurement
HCL	Hydrochloric acid
ICLN	Integrated Consortium of Laboratory Networks
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
LRN	Laboratory Response Network
NG	No growth
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
NTC	No template control
OSHA	Occupational Safety and Health Administration
PBS	Phosphate buffered saline
PBST	PBS with 0.05% Tween
PC	Positive control
PCR	Polymerase chain reaction
PMP	Paramagnetic particle
PNC	[Sample] processing negative control (blank)
PPE	Personal protective equipment
psi	Pounds per square inch
PT	Proficiency testing
QA	Quality assurance
QC	Quality control
RCF	Relative centrifugal force
RV-PCR	Rapid Viability-Polymerase Chain Reaction
rpm	Revolutions per minute

SDS	Safety data sheet
SBA	Sheep blood agar
SOP	Standard operating procedure
SWGFACT	Scientific Working Group on Forensic Analysis of Chemical Terrorism
TE	Tris(hydroxymethyl)aminomethane-hydrochloric acid-EDTA
TNTC	Too numerous to count
UF	Ultrafiltration
UNG	Uracil-DNA glycosylase
UV	Ultraviolet
WLA	Water Laboratory Alliance
YPEB	<i>Yersinia pestis</i> enrichment broth

## **Trademarked Products**

<b>Trademark</b>	<b>Holder</b>	<b>Location</b>
Acrovent™	Pall Corporation	Ann Arbor, MI
Amicon®	EMD Millipore®	Billerica, MA
Applied BioSystems®	Life Technologies™	Carlsbad, CA
BBL™	BD Diagnostics	Sparks, MD
BD Clay Adams™ Nutator Mixer	BD Diagnostics	Sparks, MD
Biopur® Safe-Lock®	Eppendorf	Hauppauge, NY
Black Hole Quencher®	Biosearch Technologies, Inc.	Novato, CA
Cole Parmer®	Cole Parmer®	Vernon Hills, IL
Costar®	Corning	Tewksbury, MA
Dispatch®	Clorox Company	Oakland, CA
Durapore®	EMD Millipore	Billerica, MA
GN-6 Metrical®	Pall Corporation	Ann Arbor, MI
Invitrogen®	Life Technologies™	Carlsbad, CA
Jiffy-Jack®	Cole Parmer®	Vernon Hills, IL
Kendall™	Covidien, Inc.	Mansfield, MA
Life Technologies™	Life Technologies™	Carlsbad, CA
MagneSil®	Promega	Madison, WI
Masterflex®	Cole Parmer®	Vernon Hills, IL
MaxQ™	Thermo Scientific Inc	Lenexa, KS
Metrical®	Pall Corporation	Ann Arbor, MI
Microcon®	EMD Millipore	Billerica, MA
MicroFunnel™	Pall Corporation	Ann Arbor, MI
Millipore®	EMD Millipore	Billerica, MA
Nalgene®	Nalge Nunc Corporation	Rochester, NY
Stomacher®	Seward	Worthington, W. Sussex, United Kingdom
TaqMan®	Life Technologies™	Carlsbad, CA
Trypticase™ soy agar	BD Diagnostics	Sparks, MD
Tween®	Sigma-Aldrich	St. Louis, MO
Ultracel®	EMD Millipore®	Billerica, MA
Ultrafree®	EMD Millipore	Billerica, MA
Vacushield™	Pall Corporation	Ann Arbor, MI
Versalon™	Covidien, Inc.	Mansfield, MA

## Introduction

The U.S. Environmental Protection Agency (EPA) is the primary federal agency responsible for the protection of indoor/outdoor structures and water infrastructure vulnerable to chemical, biological and radiological (CBR) terrorist attacks and for decontamination following a contamination incident. EPA's mission to protect human health and the environment was expanded to address such critical homeland security related needs after the 2001 terrorist attacks, including the anthrax bioterrorism incidents that resulted in human casualties and public and private facility closures.

Based on the realities of response activities after the 2001 anthrax incident and continued preparedness efforts since then, it is anticipated that in an incident involving an intentional (bioterrorist attack) or accidental release of a bioterrorism agent, several hundred to thousands of diverse environmental samples (e.g., aerosols, particulates and drinking water) will need to be rapidly processed and analyzed in order to assess the extent of contamination and support the planning of decontamination efforts. A similar number of samples may also need to be analyzed to determine the efficacy of decontamination activities during the remediation phase of the response. EPA's decision makers will need timely results from rapid sample analyses for planning the decontamination efforts and eventually, to inform recommendations for clearance decisions. The National Homeland Security Research Center (NHSRC) within the Office of Research and Development is EPA's hub for providing expertise on CBR agents and for conducting research to meet EPA's homeland security mission needs. A focus of NHSRC's research is to support the EPA's Environmental Response Laboratory Network (ERLN) and Water Laboratory Alliance (WLA), an integrated nationwide network of federal, state, local and commercial environmental testing laboratories. Along with the Centers for Disease Control and Prevention's (CDC's) Laboratory Response Network (LRN), the ERLN/WLA can be activated in response to a large-scale environmental disaster to provide analytical capability, increase capacity and produce quality data in a systematic and coordinated manner.

Since the year 541 AD, there have been three major documented pandemics due to *Yersinia pestis* (*Y. pestis*), the causative agent of plague (<http://www.cdc.gov/plague/history/>). *Y. pestis* has been used as a weapon of biological warfare for centuries; the first documented use was in 1346 AD (Reference 16.1) ([http://wwwnc.cdc.gov/eid/article/8/9/01-0536\\_article](http://wwwnc.cdc.gov/eid/article/8/9/01-0536_article)). Based on the natural occurrence of *Y. pestis* and its use as a biological weapon, it is considered a high priority agent. Therefore, combining the homeland security and natural plague outbreak related responsibilities, EPA needs to have detailed analytical methods for detection of *Y. pestis* in environmental matrices, including water. To address these critical needs, EPA NHSRC, in collaboration with CDC, has generated this protocol for detection of *Y. pestis* in environmental samples.

In the environment, *Y. pestis* is thought to survive for only short periods of time outside a host, however multiple studies evaluating seeded environmental matrices have demonstrated that *Y. pestis* is able to survive and remain infective for extended periods of time under certain environmental conditions (Reference 16.2). Strains of *Y. pestis* have been shown to survive between 74 days to 221 days in bottled water samples (Reference 16.3) and *Y. pestis* biotype Orientalis can remain viable and virulent after 40 weeks in soil (Reference 16.4). Rose et al. (Reference 16.5) reported that *Y. pestis* spotted onto coupons (e.g., glass, paper) was viable for 3 to 5 days under controlled conditions, dependent on coupon type. During an incident, analytical methods need to be available to evaluate samples for the presence and viability of *Y. pestis*. During and after cleanup, it is critical that analyses are conducted to determine if the risk to public health has been mitigated. EPA NHSRC has developed this protocol to address this need. To complement an effective sample collection strategy during a suspected *Y. pestis* release incident, a systematic approach for timely and cost-effective sample analyses is critical. Such a systematic approach also helps in effectively managing and increasing the analytical laboratory capacity. This protocol

includes three analytical approaches for the detection of *Y. pestis* in various environmental samples (aerosol, particulates [surface swabs, wipes, and Sponge-Sticks], air filters, drinking water and decontamination waste water). To detect the presence of the deoxyribonucleic acid (DNA) of *Y. pestis*, a real-time polymerase chain reaction (PCR) based sample analysis is included. To detect whether viable *Y. pestis* bacteria are present in the samples, microbiological culture and Rapid Viability-PCR (RV-PCR) analytical methods are included. This protocol has been specifically developed for use by ERLN and WLA laboratories for the analysis of environmental samples to assist in preparing for, and recovering from, disasters involving contamination from *Y. pestis*. It should be noted that the LRN laboratories providing support to EPA for environmental sample analyses may use LRN-specific protocols.

Sample processing procedures are also provided for respective analytical methods for all sample types listed earlier. Since this protocol was developed to include the analyses of diverse environmental samples, it emphasizes appropriate sample processing as well as DNA extraction and purification steps to significantly remove cells and/or PCR-inhibitory materials present in the samples. This protocol will be revised as improved sample processing procedures and real-time PCR assays become available.

For drinking water samples, large volume samples may need to be analyzed to detect low concentrations of *Y. pestis*. Therefore, the protocol also includes an ultrafiltration-based concentration procedure. For post-decontamination phase culture analyses, selected isolated colonies will be analyzed using real-time PCR to confirm the identity of *Y. pestis*, as opposed to traditional biochemical and serological testing.

Several sample processing and analysis procedures in this protocol have been derived from LRN protocols. However, these procedures have been modified, as necessary, to address EPA's homeland security mission needs during the remediation phase of a plague incident. Therefore, these modified procedures or this protocol itself must not be designated, referred to, or misconstrued as LRN procedures or as an LRN protocol.

**It should be noted that at the time of publication, this protocol has not been validated.** The real-time PCR assays included in this protocol have been only partially characterized for specificity. These assays will be updated or replaced with fully characterized and validated assays upon availability. During any *Y. pestis* related emergency situations, EPA's use of non-validated methods in the absence of validated methods must adhere to the EPA's Forum on Environmental Measurement (FEM) policy directive on method validation.

According to Agency Policy Directive FEM-2010-01, *Ensuring the Validity of Agency Methods Validation and Peer Review Guidelines: Methods of Analysis Developed for Emergency Response Situations*:

It is EPA's policy that all methods of analysis (e.g., chemical, radiochemical, microbiological) must be validated and peer reviewed prior to issuance as Agency methods. There are emergency response situations that require methods to be developed and utilized, which may or may not have previously been validated or peer reviewed prior to use. This policy directive addresses those situations in which a method must be developed, validated and/or peer reviewed expeditiously for utilization in an emergency response situation. Also, in such emergency response situations only, an analytical method may be employed that has been validated by another established laboratory network (e.g., the Center for Disease Control and Prevention's Laboratory Response Network, the U.S. Department of Agriculture/Food and Drug Administration's Food Emergency Response Network). In those instances, the responsible federal agency will indicate that the level of validation and/or peer review that their analytical method

underwent is consistent with the Integrated Consortium of Laboratory Networks' (ICLN) *Guidelines for Comparison of Validation Levels between Networks*.<sup>5</sup> The responsible federal agency may also refer to the *Validation Guidelines for Laboratories Performing Forensic Analysis of Chemical Terrorism*.<sup>6</sup> in order for the receiving federal agency to determine if the analytical method meets the intended purpose.

Any EPA regional or program office that proposes to utilize a method in an emergency response situation is responsible for establishing and documenting to what level and by what process the method has been validated and/or peer reviewed in accordance with this policy. A regional or program office may determine the level of validation and/or peer review that is necessary to provide the objective evidence that a method is suitable for its intended purpose; however, the office must document the validation and/or peer review information supporting use of the method. All documentation should be preserved in accordance with the Agency's records management policy.

<sup>5</sup> U.S. Department of Homeland Security, Integrated Consortium of Laboratory Networks (ICLN), *ICLN Guidelines for Comparison of Validation Levels between Networks*, Original Version, <http://www.icln.org/docs/sop.pdf>.

<sup>6</sup> Federal Bureau of Investigation (FBI), Scientific Working Group on Forensic Analysis of Chemical Terrorism (SWGFACT), *Validation Guidelines for Laboratories Performing Forensic Analysis of Chemical Terrorism*, Forensic Science Communications, Volume 7, Number 2, April 2005.

The above policy is available at:

[http://www.epa.gov/fem/pdfs/FEM\\_Policy2010-01\\_Emergency\\_Response\\_Methods\\_Final\\_July\\_2010.pdf](http://www.epa.gov/fem/pdfs/FEM_Policy2010-01_Emergency_Response_Methods_Final_July_2010.pdf)

Also, EPA recognizes that having analytical data of known and documented quality is critical in making proper decisions during all phases of a response to a bioterrorism incident and strives to establish data quality objectives (DQOs) for each response activity.<sup>1</sup> These DQOs are based upon needs for both quality and response time. EPA's ERLN, which is tasked with providing laboratory support following homeland security-related incidents, also has established data reporting procedures. Requirements for receiving, tracking, storing, preparing, analyzing and reporting data are specified in the *Environmental Response Laboratory Network Laboratory Requirements Document* at:

<http://epa.gov/erln/techsupport.html>; project-specific requirements also are included in individual Analytical Service Requests.

<sup>1</sup> Information regarding EPA's DQO process, considerations and planning is available at: <http://www.epa.gov/QUALITY/dqos.html>

## **Protocol for Detection of *Yersinia pestis* in Environmental Samples During the Remediation Phase of a Plague Incident**

### **1.0 Scope and Application**

The purpose of this protocol is to provide multiple procedures and methods that can be used to detect the *Yersinia pestis* (*Y. pestis*) in environmental samples. To detect the presence of the deoxyribonucleic acid (DNA) of *Y. pestis*, this protocol includes a real-time polymerase chain reaction (PCR) based method. Since traditional PCR methods cannot determine viability of *Y. pestis*, this protocol includes two additional methods: culture/plating followed by isolate confirmation by real-time PCR, and Rapid Viability-PCR (RV-PCR). **Note:** The RV-PCR procedure included in this protocol is only for water matrices. The real-time PCR assays included in this protocol have been only partially characterized for specificity. During an actual incident, validated assays from other sources (e.g., Department of Defense Critical Reagents Program or Laboratory Response Network [LRN]) may be used.

PCR assays included in this protocol will be updated or replaced with fully characterized and validated assays upon availability. This protocol will be periodically updated to include advances in sample processing and nucleic acid extraction-purification procedures. This protocol is intended for the analyses of swabs, wipes, Sponge-Sticks, air filters and water for *Y. pestis*.

### **2.0 Summary of Methods**

**2.1** Sample Analysis for Detection of *Y. pestis* DNA (Real-time PCR): Following sample processing including DNA extraction and purification, the DNA extracts are analyzed by real-time PCR using the Applied Biosystems® (ABI) 7500 Fast Real-Time PCR System. Direct DNA-based analysis of samples allows for high throughput and rapid results. Unless advised otherwise, real-time PCR based analysis should be performed using any or all three PCR assays included in Section 6.8, depending on the purpose of sample analyses.

**2.2** Sample Analyses for Detection of Viable *Y. pestis*: After samples have been appropriately processed, samples and membrane filters are cultured by either plating on Cefsulodin-Irgasan, Novobiocin agar and sheep blood agar (Reference 16.6 [culture procedure]), or inoculating into nutrient rich broth (Reference 16.7 [RV-PCR procedure]).

#### **2.2.1 Culture Procedure**

The culture option includes sample processing and plating serial dilutions of the processed sample and membrane filters on Cefsulodin-Irgasan, Novobiocin (CIN) agar and sheep blood agar (SBA) followed by rapid confirmation of typical isolated colonies using *Y. pestis* specific real-time PCR. Unless advised otherwise, real-time PCR based analysis should be performed using any or all three PCR assays included in Section 6.8, depending on the purpose of sample analyses.

#### **2.2.2 RV-PCR Procedure (Water Samples)**

The RV-PCR procedure serves as an alternative to the traditional culture-based methods for detection of viable pathogens. The RV-PCR procedure integrates high-throughput sample processing, short-incubation broth culture, and highly sensitive and specific real-time PCR assays to detect low concentrations of viable bacterial threat agents. Specifically, the procedure uses the change in real-time PCR response, referred to as the



change in cycle threshold, or  $\Delta C_T$ , between the initial cycle threshold ( $C_T$ ) at time 0 ( $T_0$ ) (just before sample incubation) and the final  $C_T$  after incubation ( $C_T T_f$ ). Example PCR response curves are shown in **Figure 3** along with the criteria for positive detection, namely  $\Delta C_T \geq 6$ . Unless advised otherwise, real-time PCR based analysis should be performed using any or all three PCR assays included in Section 6.8, depending on the purpose of sample analyses.

### **3.0 Interferences and Contamination**

- 3.1** Poor recoveries of *Y. pestis* may be caused by the presence of high numbers of competing or inhibitory organisms, background debris, or toxic substances (e.g., metals or organic compounds).
- 3.2** Metals and organic compounds may inhibit PCR reactions. Water samples suspected of containing iron or rust particles should be placed on a magnetic rack (Invitrogen® Cat. No. 123-21D or equivalent) to separate out the particulates from the samples. The supernatant should be transferred to a clean sterile bottle/tube, using care not to transfer any of the particulates.
- 3.3** Problems related to sample processing, such as clogging of filters and inefficient extraction, may also result in poor recoveries.

### **4.0 Safety**

**Note:** *This protocol should not be misconstrued as a laboratory standard operating procedure (SOP) that addresses all aspects of safety including biosafety; the laboratory should adhere to safety guidelines and requirements established by their organization or facility as well as the CDC. All wastes should be handled according to Centers for Disease Control and Prevention (CDC) & Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition, (Reference 16.8), BMBL waste management and disposal requirements.*

#### **4.1 Safety Precautions**

Direct contact of skin or mucous membranes with infectious materials, accidental parenteral inoculation, ingestion, and exposure to aerosols and infectious droplets have resulted in *Y. pestis* infection. Due to the infectious nature of this organism, all samples should be handled and analyzed using biosafety requirements as dictated by BMBL, (Reference 16.8), or the most recent version and/or organizational health and safety plans. The CDC requires biosafety level (BSL)-3 handling of this organism.

#### **4.2 Additional Safety Precautions**

- 4.2.1** Disposable materials (e.g., pipets, loops) should be used for sample manipulations.
- 4.2.2** The analyst must know and observe normal safety procedures required in a microbiology laboratory while preparing, using and disposing of media, cultures, reagents and materials. Analysts must be familiar with the operation of sterilization equipment.
- 4.2.3** Personal Protective Equipment (PPE)  
Laboratory personnel processing and conducting analyses of samples for *Y. pestis* must use appropriate PPE (e.g., gloves, lab coat). Also, laboratory personnel should familiarize themselves with the specific guidance for levels of protection and protective gear developed by the U.S. Department of Labor, Occupational Safety and Health

Administration (OSHA), as provided in Appendix B of 29 CFR 1910.120 ([http://www.osha.gov/pls/oshaweb/owadisp.show\\_document?p\\_table=STANDARDS&p\\_id=9767](http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=9767)). In addition to OSHA guidance, CDC developed recommendations for PPE based on biosafety level (BSL) (Reference 16.8, <http://www.cdc.gov/biosafety/publications/bmbl5/index.htm>).

**Note:** *Remove and don new gloves, as appropriate, to avoid contaminating hands and surfaces between processing of each sample and to prevent cross-contamination. Gloves should be disposed of (in an autoclavable biohazard bag) whenever they become visibly contaminated or the integrity of the gloves is compromised. After all work with potentially infectious materials is completed, gloves should be removed and hands should be washed with soap and water.*

- 4.2.4** This protocol does not address all safety issues associated with its use. Refer to 1) BMBL (Reference 16.8) for additional safety information; 2) Organization specific Health and Safety guidelines; 3) Select Agent Program Requirements and 4) a reference file of Safety Data Sheets (SDS).

## **5.0 Supplies and Equipment**

**Note:** *Refer to Appendix A for supplies and equipment for large volume drinking water sample processing.*

### **5.1 General Laboratory Supplies**

- 5.1.1** Gloves (e.g., latex, vinyl, or nitrile)
- 5.1.2** Sterile gloves (e.g., latex, vinyl, or nitrile)
- 5.1.3** Bleach wipes (Dispatch® Cat. No. 69150 or equivalent)
- 5.1.4** Ziplock bags (large ~20" × 28", medium ~12" × 16", small ~7" × 8")
- 5.1.5** Sharps waste container
- 5.1.6** Absorbent pad, bench protector (Lab Source Cat. No. L56-149)
- 5.1.7** Medium and large autoclavable biohazard bags and wire twist ties
- 5.1.8** Sterile scalpels
- 5.1.9** Sterile stainless steel scissors
- 5.1.10** Sterile disposable forceps (Cole Palmer® Cat. No. U06443-20 or equivalent)
- 5.1.11** Squeeze bottle with 70% isopropyl alcohol
- 5.1.12** Squeeze bottle with deionized (DI) water
- 5.1.13** Autoclave tape
- 5.1.14** Autoclave bags, aluminum foil, or kraft paper
- 5.1.15** Large photo-tray or similar tray for transport of racks
- 5.1.16** Laboratory marker
- 5.1.17** Timer

- 5.1.18** Sterile disposable serological pipets: 5 mL and 50 mL
- 5.1.19** Sterile disposable aerosol barrier pipet tips: 1000 µL, 200 µL, 20 µL, 10 µL (Rainin Cat. No. SR-L1000F, SR-L200F, GP-20F, GP-10F or equivalent)
- 5.1.20** 1.5 mL Eppendorf Snap-Cap Microcentrifuge Biopur® Safe-Lock® tubes (Fisher Scientific Cat. No. 05-402-24B or equivalent)
- 5.1.21** Sterile 15 mL conical tubes (Fisher Scientific Cat. No. 339650 or equivalent)
- 5.1.22** Sterile 50 mL conical tubes (Fisher Scientific Cat. No. 06-443-18 or equivalent)
- 5.1.23** Racks for 15 mL and 50 mL conical tubes
- 5.1.24** Sterile 2 mL tubes, DNase, RNase-free, gasketed, screw caps (National Scientific Cat. No. BC20NA-PS or equivalent)
- 5.1.25** Glass beads, acid washed, 106 µm and finer (Sigma Cat. No. G4649 or equivalent)
- 5.1.26** Glass beads, acid washed, 425–600 µm and finer (Sigma Cat. No. G8772 or equivalent)
- 5.1.27** PCR 8 cap strips (VWR Cat. No. 83009-684 or equivalent)
- 5.1.28** Amicon® Ultra-0.5 Centrifugal Filter Concentrator with Ultracel® 100 Regenerated Cellulose Membrane (Millipore® Cat. No. UFC503096 or equivalent); Amicon® collection tubes (Millipore® Cat. No. UFC50VL96 or equivalent)
- 5.1.29** 0.22 µm Ultrafree®-MC GV 0.5 mL Centrifugal Filter Unit with Durapore® PVDF Membrane, Yellow Color Coded (Millipore® Cat. No. UFC30GV0S or equivalent)
- 5.1.30** 0.1 µm Ultrafree®-MC, VV Centrifugal Filter Device (Millipore® Cat. No. UFC30VV00 or equivalent)
- 5.1.31** Wide mouth screw cap containers, 120 mL (Fisher Scientific Cat. No. 14-375-459 or equivalent)
- 5.2 Supplies for Real-time PCR Method Based Sample Analysis**
  - 5.2.1** 96-well PCR plates (ABI Cat. No. 4346906 or equivalent)
  - 5.2.2** 96-well plate holders, Costar®, black (VWR Cat. No. 29442-922 or equivalent)
  - 5.2.3** Adhesive plate sealers for 96-well PCR plates (Edge Bio Cat. No. 48461 or equivalent)
  - 5.2.4** Foil seals for 96-well PCR plates (Polar Seal Foil Sealing Tape, E & K Scientific Cat. No. T592100 or equivalent), for longer storage of the plates
  - 5.2.5** Optical seals (ABI Cat. No. 4311971 or equivalent)
- 5.3 Supplies for Culture Method Based Sample Analysis**
  - 5.3.1** Sterile disposable Petri dishes, 100 mm × 15 mm
  - 5.3.2** Sterile disposable inoculating loops (10 µL) and needles
  - 5.3.3** Sterile disposable cell spreaders (such as L-shaped, Fisher Scientific Cat. No. 03-392-150 or equivalent)
  - 5.3.4** Sterile MicroFunnel™ Filter Funnels, 0.45 µm pore-size (VWR Cat. No. 55095-060 or equivalent)
  - 5.3.5** Specimen Cups, 4.5 oz. (Kendall Cat. No. 17099 or equivalent)

- 5.3.6 Racks for 15 mL and 50 mL centrifuge tubes
- 5.3.7 Sterile disposable plastic 50 mL screw cap centrifuge tubes (Becton, Dickinson and company [BD] Cat. No. 352070 or equivalent)
- 5.3.8 Sterile disposable plastic 15 mL screw cap centrifuge tubes (BD Cat. No. 352097 or equivalent)
- 5.3.9 Sterile pipet tips with aerosol filter for 1000 µL and 100 µL (Rainin Cat. No. SR-L1000F and GP-100F or equivalent)
- 5.3.10 Biotransport carrier (Nalgene®, Thermo Scientific Cat. No. 15-251-2 or equivalent)

#### **5.4 Supplies for RV-PCR Based Sample Analysis**

- 5.4.1 Disposable nylon forceps (VWR Cat. No. 12576-933 or equivalent)
- 5.4.2 50 mL conical tubes (VWR Cat. No. 21008-951 or equivalent)
- 5.4.3 Disposable serological pipets: 50 mL, 25 mL, 10 mL, 5 mL
- 5.4.4 Single 50 mL conical tube holder (Bel-Art Cat. No. 187950001 or equivalent)
- 5.4.5 Screw cap tubes, 2 mL (VWR Cat. No. 89004-298 or equivalent)
- 5.4.6 96-well tube rack(s) for 2 mL tubes (8 × 12 lay out) (Bel-Art Cat. No. 188450031 or equivalent)
- 5.4.7 2 mL Eppendorf tubes (Fisher Scientific Cat. No. 05-402-24C or equivalent)
- 5.4.8 96-well 2 mL tube rack (8 × 12 format) (Bel-Art Cat. No. 188450031)
- 5.4.9 48-well plates (E&K Scientific Cat. No. EK-2044 or equivalent)

#### **5.5 Equipment**

- 5.5.1 Biological Safety Cabinet (BSC) – Class II or Class III
  - 5.5.2 PCR preparation hood/workstation
  - 5.5.3 Balance, analytical, with Class S reference weights, capable of weighing 20 g ± 0.001 g
  - 5.5.4 ABI 7500 Fast Real-Time PCR System (Life Technologies™)
  - 5.5.5 Refrigerated centrifuge with PCR plate adapter and corresponding safety cups and rotors for 5 mL and 50 mL tubes (Eppendorf Cat. No. 5804R, 5810R or equivalent) or PCR plate spinner (placed in BSC [VWR Cat. No. 89184-608 or equivalent])
- Note:** *Swinging bucket and fixed angle rotors for the refrigerated centrifuge may also be necessary.*
- 5.5.6 Refrigerated micro-centrifuge for Eppendorf tubes with aerosol-tight rotor (Eppendorf Cat No. 5415R/5424R or equivalent)
  - 5.5.7 Vacuum pump with gauge (Cole Parmer® Model EW-07061-40 or equivalent) or vacuum source capable of < 10 pounds per square inch (psi)
  - 5.5.8 Vacuum pump filters for pump (Acrovent™ Cat. No. 4249 or equivalent)
  - 5.5.9 Vacuum trap accessories
  - 5.5.10 Single-tube vortexer (Fisher Scientific Cat. No. 50-143-447 or equivalent); optional multi-tube adapter (Scientific Industries, Inc. Cat. No. SI-V525 or equivalent)

- 5.5.11** Single-channel micropipettors (1000 µL, 200 µL, 100 µL, 20 µL, 10 µL)
- 5.5.12** Serological pipet aid
- 5.5.13** Incubator(s), microbiological type, maintained at 25.0°C–28.0°C
- 5.5.14** Autoclave or steam sterilizer, capable of achieving 121°C (15 psi) for 30 minutes
- 5.5.15** Cold block for 2 mL tubes (Eppendorf Cat. No. 3880 001.018 or equivalent)
- 5.5.16** Bead-beater (BioSpec Products, Inc. Cat. No. 607 [16 place] or equivalent)
- 5.5.17** Tube racks, 80 place (VWR Cat. No. 30128-282 or equivalent)
- 5.5.18** 40 kHz sonicator bath (Branson Ultrasonic Cleaner Model 1510, Process Equipment and Supply, Inc. Cat. No. 952-116 or equivalent)
- 5.5.19** Stomacher® 400 Circulator (Seward Cat. No. 0400/001/AJ or equivalent) with closure bags (Cat. No. BA6141/CLR or equivalent) and rack (Cat. No. BA6091 [1 place] and BA6096 [10 place] or equivalent)

## **6.0 Reagents and Standards**

- 6.1** Reagent-grade chemicals must be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (Reference 16.9). For suggestions regarding the testing of reagents not listed by the American Chemical Society, see *AnalaR Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K. (Reference 16.10); and *United States Pharmacopeia and National Formulary 24*, United States Pharmacopeial Convention, Md. (Reference 16.11).
- 6.2** Tween® 80 (Fisher Cat. No. T164 or equivalent)
- 6.3** PCR-grade water, sterile (Teknova Cat. No. W3350 or equivalent)
- 6.4** Sterile 0.01 M phosphate buffered saline (PBS) pH 7.2–7.4 (Sigma Cat. No. P3813 or equivalent)
- 6.5** 1X phosphate buffered saline with 0.05% Tween® 20 (PBST), pH 7.4, (Teknova Cat. No. P0201 or equivalent) diluted 1:1 with sterile PBS (Section 6.4)
- 6.6** TE buffer (1X Tris [10 mM] -HCl-EDTA [1 mM Ethylenediaminetetraacetic acid]) buffer, pH 8.0 (Fisher Scientific Cat. No. BP2473-500 or equivalent)
- 6.7** TaqMan® Fast Advanced PCR Master Mix (Life Technologies™ Cat. No. 4444557)
- 6.8** PCR Assays
  - YC2 (targeting a hypothetical gene on the chromosome of *Y. pestis*)
    - Forward Primer (YC2-F) – 5'- CAACGACTAGCCAGGCGAC -3'
    - Reverse Primer (YC2-R) – 5'- CATTGTTCGCACGAAACGTAA -3'
    - Probe (YC2-Pr) – 5'-6FAM- TTTTATAACGATGCCTACAACGGCTCTGCAA -BHQ1-3'
  - YpP1 (targeting the *pla* outer membrane protease gene on the pPCP1 plasmid of *Y. pestis*)
    - Forward Primer (YpP1-F) – 5'- TGGGTTCGGGCACATGATA -3'
    - Reverse Primer (YpP1-R) – 5'- CCAGCGTTAATTACGGTACCATAA -3'
    - Probe (YpP1-Pr) – 5'-6FAM- CTTACTTTCCGTGAGAAGACATCCGGCTC -BHQ1-3'

YpMT1 (targeting a putative F1 operon positive regulatory protein on the pMT1 plasmid of *Y. pestis*)

- Forward Primer (YpMT1-F) – 5'- GGTAACAGATTCGTGGTTGAAGG -3'
- Reverse Primer (YpMT1-R) – 5'- CCCCACGGCAGTATAGGATG -3'
- Probe (YpMT1-Pr) – 5'-6FAM-TCCCTTCTACCCAACAAACCTTTAAAGGACCA-BHQ1-3'

#### 6.8.1 Preparation of concentrated and primer and probe working stocks

Prior to PCR analyses, lyophilized primers and probes should be rehydrated in PCR-grade water to prepare concentrated stocks. Primary concentrated storage stocks should initially be prepared to obtain 100  $\mu\text{M}$  (0.1 nmoles/ $\mu\text{L}$ ) and 40  $\mu\text{M}$  (0.04 nmoles/ $\mu\text{L}$ ) solutions of primers and probes, respectively. These primary (concentrated) stocks will be used to prepare working stock solutions which will then be used to prepare PCR assay mixes (Section 9.7) on the day of use. Examples of rehydration of lyophilized primers/probes and dilution of rehydrated stocks to prepare working stocks are provided in **Tables 1** and **2**, respectively.

**Table 1. Example Concentrated Stock Preparation**

Lyophilized Primer/Probe (nmoles)		PCR-grade water (μL)	Concentration	
			nmoles/μL	μM
FWD Primer	29	290	0.1	100
RV Primer	35	350	0.1	100
Probe	17	425	0.04	40

**Table 2. Example Working Stock Preparation**

Concentrated Stock ( $\mu\text{L}$ )		PCR-grade water ( $\mu\text{L}$ )	Dilution	Concentration	
				nmoles/ $\mu\text{L}$	$\mu\text{M}$
FWD Primer	20	180	0.1	0.01	10
RV Primer	20	180	0.1	0.01	10
Probe	20	180	0.1	0.004	4

Working stocks will be used to prepare master mix on the day of use (Section 9.7.3).

**6.9** Positive Control (PC) – Total DNA isolated from an appropriate virulent *Y. pestis* strain containing all of the plasmids (pPCP1, pMT1). For culture analyses, *Y. pestis* A1122 strain (BSL-2 organism) or other avirulent strains may be used as a PC to meet the laboratory's BSL.

**6.10** Cefsulodin-Irgasan Novobiocin (CIN) agar, a selective *Y. pestis* medium

**6.10.1** The use of commercially prepared plates is recommended (Becton, Dickinson and Company [BD] Cat. No. 221848 or 299579 or equivalent); however, dehydrated medium (BD CN [Cefsulodin Novobiocin], Cat. No. 231961), may be used. If commercially prepared medium is not available, prepare medium using procedures in Sections 6.10.2–6.10.5.

**6.10.2** Medium Composition:

Peptone	17 g
Proteose peptone	3 g
Yeast extract	2 g
Mannitol	20 g
Sodium pyruvate	2 g
Sodium chloride	1 g
Magnesium sulfate heptahydrate	10 mg
Sodium desoxycholate	0.5 g
Sodium cholate	0.5 g
Irgasan	4 mg
Agar	13.5 g
Crystal violet	1 mg
Neutral red	30 mg
Antimicrobial supplement	10 mL
Reagent-grade water	1 L <sup>1</sup>

<sup>1</sup> The final concentration of the media components is based on the addition of 1 L of reagent-grade water. As per the manufacturer's instructions, do not adjust the volume of reagent-grade water to take into account the total weight/volume of the individual components.

**6.10.3** Antimicrobial Supplement Composition (Formula per 10 mL Vial)

Cefsulodin	4 mg
Novobiocin	2.5 mg

Rehydrate in 10 mL of sterile reagent-grade water and mix by inverting to dissolve powder.

**6.10.4** Add reagents, except antimicrobial supplement, to 950 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Bring to 1 L with reagent-grade water. Boil for 1 minute with rapid stir bar agitation to dissolve completely. Autoclave at 121°C (15 psi) for 15 minutes. Do not overheat. Cool to 45°C–50°C in a water bath.

**6.10.5** Prepare medium by aseptically adding 10 mL of rehydrated antimicrobial supplement to the cooled medium and mix well. Aseptically pour 12–15 mL into each 15 mm × 100 mm sterile Petri dish. After agar solidifies, store at 4°C for a maximum of two weeks.

**6.11** Trypticase™ Soy Agar with 5% Sheep Blood (SBA)

**6.11.1** The use of commercially prepared plates is recommended (VWR Cat. No. 90001-276 or 90001-282 or equivalent); however, dehydrated medium (BBL™ Cat. No. 227300 or equivalent), with the addition of sheep blood (Oxoid Cat. No. SR0051 or equivalent) may be used. If commercially prepared medium is not available, prepare medium using procedures in Sections 6.11.2–6.11.4.

**6.11.2** Medium Composition:



Tryptone H	15 g
Soytone	5 g
Sodium chloride	5 g
Agar	15 g
Sheep blood	50 mL
Reagent-grade water	~900 mL

**6.11.3** Add reagents except sheep blood to 850 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Boil for 1 minute with rapid stir bar agitation to dissolve completely. Adjust pH to  $7.3 \pm 0.2$  with 1.0 N HCl or 1.0 N NaOH and bring to 950 mL with reagent-grade water. Autoclave at 121°C (15 psi) for 15 minutes. Do not overheat. Cool to 45°C – 50°C in a water bath.

**6.11.4** Prepare medium by aseptically adding 50 mL of sterile sheep blood (5.0% final concentration) to the cooled medium and mix well. Aseptically pour 12 – 15 mL into each 15 × 100 mm sterile Petri dish. After agar solidifies, store at 4°C for a maximum of two weeks.

**6.12** Promega Reagents for DNA Extraction and Purification Procedure for RV-PCR:

- MagneSil® Blood Genomic, Max Yield System, Kit (Promega Cat. No. MD1360; VWR Cat. No. PAMD1360 or equivalent)
- Salt Wash (VWR Cat. No. PAMD1401 or equivalent)
- Magnesil Paramagnetic Particles (PMPs) (VWR Cat. No. PAMD1441 or equivalent)
- Lysis Buffer (VWR Cat. No. PAMD1392 or equivalent)
- Elution Buffer (VWR Cat. No. PAMD1421 or equivalent)
- Alcohol Wash, Blood (VWR Cat. No. PAMD1411 or equivalent)
- Anti-Foam Reagent (VWR Cat. No. PAMD1431 or equivalent)

**6.13** 1X *Yersinia pestis* Enrichment Broth (1X YPEB, SOP No: FERN-MIC.0004.02 <sup>1</sup>)

Prepare medium using procedures in Sections 6.13.1–6.13.2.

**6.13.1** Medium Composition:

Heart infusion broth	25 g
Yeast extract	6 g
Soytone	3 g
Ferric ammonium sulfate	0.5 g
4-Morpholinepropanesulfonic acid	8.77 g
Reagent-grade water	1000 mL

**6.13.2** Add reagents to 500 mL of reagent-grade water mix thoroughly, bring volume to 1 L. Filter-sterilize using 1-L 0.22 µm cellulose acetate filtering system with disposable bottle. Store at 4°C for a maximum of three months in screw cap containers.

<sup>1</sup> Courtesy of Doran, T., Hanes, D., Weagant, S., Torosian, S., Burr, D., Yoshitomi, K., Jinneman, K., Penev, R., Adeyemo, O., Williams-Hill, D., and Morin, P.



**6.14 2X *Yersinia pestis* Enrichment Broth (2X YPEB)**

Prepare medium using procedures in Sections 6.14.1–6.14.2.

**6.14.1 Medium Composition:**

Heart infusion broth	50 g
Yeast extract	12 g
Soytone	6 g
Ferric ammonium sulfate	1 g
4-Morpholinepropanesulfonic acid	17.54 g
Reagent-grade water	1000 mL

**6.14.2** Add reagents to 500 mL of reagent-grade water, mix thoroughly, bring volume to 1 L. Filter-sterilize using 1-L 0.22 µm cellulose acetate filtering system with disposable bottle. Store at 4°C for a maximum of three months in screw cap containers.

**6.15 10X *Yersinia pestis* Enrichment Broth (10X YPEB)**

Prepare medium using procedures in Sections 6.15.1–6.15.2.

**6.15.1 Medium Composition:**

Heart infusion broth	125 g
Yeast extract	30 g
Soytone	15 g
Ferric ammonium sulfate	2.5 g
4-Morpholinepropanesulfonic acid	43.85 g
Reagent-grade water	500 mL

**6.15.2** Add reagents to 250 mL of reagent-grade water mix thoroughly, bring volume to 500 mL. Filter-sterilize using 1-L 0.22 µm cellulose acetate filtering system with disposable bottle. Store at 4°C for a maximum of three months in screw cap containers.

**6.16 10% Bleach-pH amended (prepared daily), optional**

Add 2 parts water to 1 part bleach, then add 5% acetic acid (1 part) and remaining water (6 parts). Measure pH and add bleach (to increase pH) or acetic acid (to decrease pH) as needed to obtain a final pH between 6 and 7. A pH meter, as opposed to pH strips or kit, should be used to measure pH. When mixed, place a lid on the mixture to reduce chlorine escape and worker exposure.

**6.17 100% Ethanol (200-proof) for preparation of 70% ethanol by dilution with PCR-grade water.**

## **7.0 Calibration and Standardization**

**7.1** Check temperatures in incubators twice daily with a minimum of 4 hours between each reading to ensure operation within stated limits. Record the temperature in a log book.

- 7.2 Check temperature in refrigerators/freezers at least once daily to ensure operation is within the storage requirements for samples, reagents and media. Record daily measurements in a refrigerator/freezer log book.
- 7.3 Check thermometers, including those on instrumentation (e.g., digital display), at least annually against a National Institute of Standards and Technology (NIST) certified thermometer or one that meets the requirements of NIST Monograph SP 250-23. Check columns for breaks.
- 7.4 Calibrate pH meter prior to each use with at least two of three standards (e.g., pH 4.0, 7.0 or 10.0) closest to the range being tested.
- 7.5 Calibrate balances once per month with reference weights (e.g., ASTM Class 2).
- 7.6 Micropipettors should be calibrated at least annually and tested for accuracy on a weekly basis.
- 7.7 Follow manufacturer instructions for calibration of real-time PCR instruments.
- 7.8 Re-certify BSCs annually. Re-certification must be performed by a qualified technician.
- 7.9 Autoclave maintenance should be conducted at least annually. Autoclave temperature and total sterilization cycle time should be checked on a quarterly basis. Record the data in a log book. Spore strips or spore ampules should be used monthly as bioindicators to confirm sterilization.
- 7.10 Refrigerated centrifuges should be checked to confirm temperature and revolutions per minute (rpm) on a quarterly basis. Record the data in a log book.
- 7.11 Vacuum pressure (e.g., pumps, in house system) should be checked on a regular basis to ensure that the pressure is < 10 psi. Higher or lower vacuum pressure could negatively impact recoveries.

## **8.0 Quality Control (QC)**

- 8.1 Each laboratory that uses this protocol is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. International Organization for Standardization (ISO)/International Electrotechnical Commission (IEC) 17025 (International Standard: General requirements for the competence of testing and calibration laboratories, Section Edition 2005-05-15) provides a quality framework that could be used to develop a formal QA program.
- 8.2 Sample integrity — Samples should be checked for integrity (e.g., improperly packaged, temperature exceedance, leaking). Samples may be rejected if the integrity has been compromised. Alternately, if sample integrity has been compromised, the sample may be analyzed and the data qualified and marked accordingly (e.g., if a sample exceeded temperature during transport, the data would be flagged and marked as exceeding temperature), so that a decision can be made regarding whether the data should be considered valid/invalid.
- 8.3 Analyst qualifications — Only those analysts that have been trained and have demonstrated proficiency with these analytical techniques should perform this procedure.
- 8.4 Proficiency testing (PT) — The laboratory should have analysts analyze PT samples annually, at a minimum, to ensure they are maintaining proficiency. In addition, analysts should analyze PT samples to demonstrate proficiency prior to analyzing field samples. For laboratories not routinely using this protocol, analysts should analyze PT samples biannually. If a PT failure occurs, the

laboratory should identify and resolve any issues and then request and analyze additional PT samples. Field samples should not be analyzed until the laboratory passes the PT.

- 8.5** Media sterility check — The laboratory should test media sterility by incubating a single unit (tube or Petri dish) from each batch of medium (CIN, SBA and YPEB) at  $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for  $24 \pm 2$  hours and observe for growth. Absence of growth indicates media sterility. On an ongoing basis, the laboratory should perform media sterility checks every day that samples are analyzed.
- 8.6** PCR: Positive control (PC) — Total DNA isolated from an appropriate virulent *Y. pestis* strain containing all of the plasmids (pPCP1, pMT1) should be used as the PC. The laboratory should analyze a PC in triplicate reactions with each PCR run. Prepare the PC at a concentration of 50 pg of purified *Y. pestis* total DNA per 5  $\mu\text{L}$  of PCR-grade water. All PCs should result in a cycle threshold ( $C_T$ )  $\leq 40$  and replicates should be within  $\pm 1 C_T$  of each other.
- 8.7** Culture: Positive control (PC) — The laboratory should analyze PCs (known quantity of cells) to ensure that all media and reagents are performing properly. *Y. pestis* A1122 strain (BSL-2 organism) or other avirulent strains may be used as a PC to meet the laboratory's BSL. PCs should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should run a PC every day that samples are analyzed.
- 8.8** External inhibition control (EIC) 50 pg genomic DNA from *Y. pestis* — For determination of presence of DNA by real-time PCR, the laboratory should analyze an EIC for each environmental sample DNA extract to determine if the matrix is causing inhibition potentially resulting in false negative results. Prepare the EIC at a concentration of 50 pg of purified *Y. pestis* DNA per 1  $\mu\text{L}$  of PCR-grade water. Using a 10  $\mu\text{L}$  pipettor, carefully add 1  $\mu\text{L}$  of the DNA to the EIC wells on a PCR plate and then add 5  $\mu\text{L}$  of sample DNA extract to each well and mix thoroughly. The PCR results from the PC and EICs (both containing 50 pg of *Y. pestis* DNA) are then compared. Lower or similar  $C_T$  values for the EIC indicate there is no inhibition. A higher  $C_T$  value for the EIC ( $>3 C_T$  values) is indicative of matrix inhibition.

**Note:** *To minimize cross contamination, the EICs should not be placed next to the field samples when setting up the PCR plate.*

- 8.9** No template control (NTC) — The laboratory should analyze NTCs (5  $\mu\text{L}$  of PCR-grade water is added to the NTC wells on a PCR plate in place of the DNA or the sample DNA extract) to ensure that reagents are not contaminated. On an ongoing basis, the laboratory should analyze NTCs in triplicate PCR reactions with each PCR run. The NTC must not exhibit fluorescence above the background level (i.e., no  $C_T$  value). If  $C_T$  values are obtained as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat the analysis.
- 8.10** Field blank — The laboratory should request that the sampling team provide a field blank with each batch of samples. A field blank is defined as either a sample collection tool (e.g., wipe, swab) or sterile reagent-grade water that is taken out to the field, opened and exposed to the environment, but not used to collect a sample, and then placed in a bag and sealed and transported to the laboratory along with the field samples. The field blank is treated as a sample in all respects, including exposure to sampling location conditions, storage, preservation and all analytical procedures. Field blanks are used to assess any contamination due to sampling location conditions, transport, handling and storage. The laboratory should process and analyze this control along with each batch of environmental samples. The field blanks should not exhibit fluorescence (i.e.,  $C_T > 45$ ).

**Note:** *The field blank for large volume water samples should also be concentrated using ultrafiltration (UF) prior to analyses. A smaller volume of water (e.g., 10-20 L) may be used for the field blank to minimize the burden on the laboratory.*

- 8.11** Sample processing negative control (PNC) or method blank — The laboratory should process and analyze a PNC in the same manner as a sample to verify the sterility of equipment, materials and supplies. Absence of growth indicates lack of contamination from the target organism. Please refer to **Table 3** for appropriate PNC.
- 8.12** For RV-PCR based analysis, the T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> extracts are analyzed (in triplicate). PCR positive and negative controls must be analyzed using the same preparation of the PCR Master Mix and must be run on the same 96-well plate as the T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> extracts.

**Table 3. Sample Processing Negative Controls**

Matrix	PNC
Wipes	Clean (unused) wipe
Swabs	Clean (unused) swab
Air filters	Clean (unused) air filter
Sponge-Sticks	Clean (unused) Sponge-Stick
Drinking water and decontamination waste water	100 mL of sterile reagent-grade water
Large volume water samples	10 – 20 L of sterile reagent-grade water

## 9.0 Real-time PCR Method

Real-time PCR allows for rapid detection of *Y. pestis* in samples based simply on the presence of DNA. However, since the DNA from non-viable cells can also be detected by this method, the positive sample analysis result does not confirm the presence of viable cells. Therefore, this method is usually used for a time- and cost-effective presumptive analysis of samples. This section includes a real-time PCR method with appropriate sample processing procedures for detection of *Y. pestis*.

**Acceptable sample types:** Gauze wipes (2" × 2" 50% rayon/50% polyester [Kendall™ Versalon™ Cat. No. 8042 or equivalent]), air filters (37 mm Fluoropore™ [Millipore® Cat. No. FSLW04700 or equivalent]), swabs (macrofoam [VWR Cat. No. 89022-994 small swabs or 89022-984 extra-large swabs, or equivalent]), Sponge-Stick sampling tools (3M™ Inc. Cat. No. SSL10NB or equivalent), drinking water and decontamination waste water

### 9.1 Sample Processing for Sponge-Sticks and Wipes

**Note:** *All subsequent procedures involving manipulation of Sponge-Sticks and wipes must be carried out in a BSC using appropriate PPE. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.*

- 9.1.1** If the Sponge-Stick sponge/wipe sample is not in a Stomacher® bag, aseptically transfer it to a Stomacher® bag using sterile forceps.

**Note:** *In most cases, the Sponge-Stick handle will be removed in the field by the sampler. However, if the handle was not removed, proceed as follows. While holding the Sponge-stick handle outside the Stomacher® bag, grip the Sponge-Stick head with your other hand outside the bag and twist the head to snap off stick at the crimp line near the handle base.*

- 9.1.2** Using aseptic technique, remove the plastic stick base holding the sponge together. Place

- gloved hands on the outside of the Stomacher® bag, grip the sponge-stick head on both sides and peel the sponge away from the base and unfold the sponge. Be careful not to puncture bag with edge of stick base. Using sterile forceps, remove stick base from bag and discard in an autoclavable biohazard bag. Change forceps between samples.
- 9.1.3** Add 90 mL of PBST (0.05% Tween® 20, Section 6.5) to each bag. Set Stomacher® (Section 5.5.19) to 200 rpm.
- 9.1.4** Place the bag containing the sample into the Stomacher® (Section 5.5.19) so the Sponge-Stick sponge/wipe rests evenly between the paddles and homogenize each sample for 1 minute at 200 rpm.
- 9.1.5** Open the door of the Stomacher® (Section 5.5.19) and remove the bag. Grab the sponge/wipe from the outside of the bag with your hands. With the bag closed, move the sponge/wipe to the top of the bag while using your hands to squeeze excess liquid from the sponge/wipe.
- 9.1.6** Open the bag, remove the sponge/wipe using sterile forceps and discard in an autoclavable biohazard bag.
- 9.1.7** Follow the steps described above for each sample, changing forceps between samples.
- 9.1.8** Allow bags to sit for 10 minutes to allow elution suspension foam to settle.
- 9.1.9** Gently mix the elution suspension in the Stomacher® bag up and down 3 times with a sterile 50 mL pipet. Remove half of the suspension volume (~47 mL) and place it in a labeled 50 mL screw cap centrifuge tube. Place the remaining suspension (~47 mL) into a second 50 mL tube. Adjust the suspension volumes in both the tubes to ensure they are similar.
- 9.1.10** Process the elution suspensions for each sample as described above.
- 9.1.11** Place 50 mL tubes into sealing centrifuge buckets and decontaminate the outside of the centrifuge buckets before removing them from the BSC.
- 9.1.12** Centrifuge tubes at a maximum speed ( $\sim 3200 \times g$ ) with the brake off, for 15 minutes in a swinging bucket rotor at 4°C.
- 9.1.13** Using a sterile 50 mL pipet for each sample, remove 44 mL of the supernatant from each sample tube and discard it in an autoclavable biohazard container. The pellet may be easily disturbed and not visible, so keep the pipet tip away from the bottom of the tube.
- 9.1.14** Set the vortexer (Section 5.5.10) to the high setting. Set the sonicator water bath to high.
- 9.1.15** Vortex the tubes for 30 seconds and transfer the tubes to the sonicator water bath and sonicate for 30 seconds. Repeat the vortex and sonication cycles two more times.
- Note:** *As an alternative to sonication, tubes may be vortexed for two minutes in 10 second bursts using a vortexer. If possible use a vortexer with a multi-tube adapter to reduce processing time for multiple samples. Repeat the vortex cycle two more times.*
- 9.1.16** Remove the suspension from both tubes with a sterile 5 mL pipet and combine into a 15 mL conical tube. Measure final volume of suspension with 5 mL pipet and record the result on the tube and data sheet.
- 9.1.17** Centrifuge tubes at  $3200 \times g$  with the brake off, for 15 minutes in a swinging bucket rotor at 4°C.

- 9.1.18** Using a sterile 5 mL pipet remove 3 mL of supernatant (from ~ 6 mL), and discard in an autoclavable biohazard bag.
- 9.1.19** Vortex tube for 1 minute in 10 second bursts.
- 9.1.20** Repeat processing steps for each sample.
- 9.1.21** Use a 1.5 mL aliquot for DNA extraction using bead-beating, as described in Section 9.6.

## **9.2 Sample Processing for Swabs**

**Note:** *All subsequent procedures involving manipulation of swabs and membranes must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.*

- 9.2.1** If the swabs are not in 15 mL centrifuge tubes, transfer each swab to a sterile, labeled 15 mL centrifuge tube using sterile forceps.
- 9.2.2** If necessary, cut the handle of the swab to fit into the tube using sterile forceps and scissors for each sample.
- 9.2.3** Add 2 mL of sterile PBS for smaller swabs and for larger swabs add 3 mL of sterile PBS to each tube and vortex at the highest setting for two minutes. Additional PBS may be added in 0.5 mL increments to ensure that a minimal volume of 2 mL is available for PCR analysis.
- 9.2.4** Using sterile forceps, remove the swab from the 15 mL centrifuge tube. Use the forceps to press the tip of the swab against the inside of the tube to remove extra liquid from the foam tip before discarding the swab in an autoclavable biohazard bag.
- 9.2.5** Repeat steps described above for each swab.
- 9.2.6** Use 1.5 mL aliquot for DNA extraction using bead-beating, as described in Section 9.6.

## **9.3 Sample Processing for Air Filters**

**Note:** *All subsequent procedures involving manipulation of air filters must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.*

- 9.3.1** If the air filters are not in 50 mL tubes, aseptically transfer each sample to a sterile 50 mL tube using sterile forceps. Change forceps between samples.
- 9.3.2** Add 3 mL of PBS to each tube and vortex at the highest setting for two minutes.
- 9.3.3** Open the tube and using a sterile transfer pipet, depress the air filter against the side of the tube to expel as much liquid as possible before discarding filter in an autoclavable biohazard bag.
- 9.3.4** Repeat steps described above for each sample.
- 9.3.5** Use 1.5 mL aliquot for DNA extraction using bead-beating, as described in Section 9.6.

## **9.4 Sample Processing for Water Samples (Large Volume [10 L – 100 L], Drinking Water)**

Please see Appendix A for primary (Section 2.0) and secondary (Section 3.0) concentration of large volume (10 L-100 L) water samples. For water samples < 10 L and ≥ 50 mL, please refer to Appendix A, Section 3.0, secondary concentration.



**Note:** *All subsequent procedures involving manipulation of water samples and membranes must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.*

- 9.4.1** If the membranes are not in 50 mL tubes, aseptically transfer each membrane to a sterile 50 mL tube using sterile forceps. Change forceps between samples.
  - 9.4.2** Add 5 mL of sterile PBS to 50 mL screw cap tube containing a membrane and vortex at the highest setting for two minutes in 10 seconds bursts.
  - 9.4.3** Using sterile forceps remove membrane from the tube and discard in an autoclavable biohazard bag.
  - 9.4.4** Repeat steps described above for each sample.
  - 9.4.5** Use 1.5 mL aliquot for DNA extraction using bead-beating, as described in Section 9.6.
- 9.5 Sample Processing for Water Samples (Small Volume [ $< 50$  mL], Surface or Drinking Water)**
- 9.5.1** Transfer no more than 30 mL of the water sample to a 50 mL screw cap tube.
  - 9.5.2** Add 10 mL of sterile PBS and mix by vortexing for 30 seconds.
  - 9.5.3** Centrifuge at  $3200 \times g$ , with the brake off, for 15 minutes at  $4^{\circ}\text{C}$ .
  - 9.5.4** Remove 37 mL of the supernatant without disturbing/dislodging the pellet. The volume of supernatant remaining should not be below the conical portion of the tube. Resuspend the pellet by vortexing for 30 seconds in the remaining volume.
  - 9.5.5** Use 1.5 mL aliquot for DNA extraction using bead-beating, as described in Section 9.6.

## **9.6 Sample Processing: DNA Extraction and Purification**

**Note:** *Alternate DNA extraction-purification procedures may be used (e.g., MagNA-Pure LC instrument).*

- 9.6.1** In a clean room, using the 8 cap strips, transfer two level capfuls ( $\sim 100$  mg) of the  $106\ \mu\text{m}$  glass beads and two level capfuls ( $\sim 100$  mg) of the  $425 - 600\ \mu\text{m}$  glass beads (using a clean strip of caps between bead sizes) into each gasketed, capped 2 mL bead-beating tube.
- 9.6.2** In the BSC, pipet 1.5 mL of the suspension (sample eluent) into a pre-labeled, gasketed, capped bead-beating 2 mL tube containing glass beads. Replace cap on tube securely. Wipe outside of tube with a 10% pH amended bleach solution (Section 6.16) or bleach wipes (Section 5.1.3). Store the remaining suspension at  $4^{\circ}\text{C}$ . Repeat for each sample.
- 9.6.3** Insert tubes in tube holders of the bead-beater (Section 5.5.16) and set the timer for three minutes (180 seconds). Bead-beat at 3450 oscillations/minute to disrupt cells to release the DNA.
- 9.6.4** Remove tubes from bead-beater (tubes will be warm), and place in a cold block for two minutes (or until cool to touch). If any tubes leak during bead-beating, wipe tubes and bead-beater thoroughly with a 10% pH amended bleach solution (Section 6.16) or bleach wipes (Section 5.1.3).

### **9.6.5 Supernatant Separation and Transfer**

- For each sample, label the following: one 1.5 mL microcentrifuge tube, two yellow-top Ultrafree<sup>®</sup>-MC filter units (Section 5.1.29; Millipore<sup>®</sup> Cat. No. UFC30GV0S), two Amicon<sup>®</sup> Ultra filter inserts, and six Amicon<sup>®</sup> Ultra collection tubes (Section 5.1.28; Millipore<sup>®</sup> Cat. No. UFC503096) with sample ID for each bead-beating tube (Section 9.6.4); and one 0.1 µm Ultrafree<sup>®</sup>-MC centrifugal filter device (Section 5.1.30; Millipore<sup>®</sup> Cat. No. UFC30VV00).

**Note:** *It may not be necessary to label all the collection tubes as long as the Amicon<sup>®</sup> Ultra filter insert is clearly labeled.*

- In a BSC, centrifuge the bead-beating tubes (Section 9.6.4) at 7000 rpm for two minutes in a microcentrifuge using a fixed angle rotor to pellet beads and particulate matter.
- Using a micropipettor, carefully transfer 0.5 mL of the supernatant to each of the two yellow-top filter units (Section 5.1.29; Millipore<sup>®</sup> Cat. No. UFC30GV0S). Avoid beads and particulate matter at bottom of bead-beating tube). Cap the filter units.
- Centrifuge (Section 5.5.6; Eppendorf 5415R/5424R) at 7000 rpm for three minutes at 4°C.

**Note:** *Ensure that the supernatant has been filtered. Centrifuge for an additional two minutes if there is any supernatant in the filter.*

- Open the filter units; remove the yellow-top filter inserts with sterile disposable forceps (gripping only on the sides) and discard in an autoclavable biohazard bag. Transfer 0.5 mL of the filtrate from the collection tube to Amicon<sup>®</sup> Ultra filter inserts (Section 5.1.28; Millipore<sup>®</sup> Cat. No. UFC503096). Do not transfer any particulate matter that may be evident at bottom of the tubes. Place filter inserts into new collection tubes (Section 5.1.28; Millipore<sup>®</sup> Cat. No. UFC50VL96). Cap the filter units.
- Centrifuge (Section 5.5.6; Eppendorf 5415R/5424R) at 7000 rpm for two minutes at 4°C.
- Open the filter units. Remove the Amicon<sup>®</sup> Ultra filter inserts (Section 5.1.28; Millipore<sup>®</sup> Cat. No. UFC503096) with disposable forceps (gripping only the sides) and transfer to new collection tubes (Section 5.1.28; Millipore<sup>®</sup> Cat. No. UFC50VL96). Dispose of old collection tubes with filtrate as per CDC BSL-3 requirements (in an autoclavable biohazard bag).
- Transfer the remaining (0.5 mL) filtrate from all of the second yellow-top filter units to the corresponding Amicon<sup>®</sup> Ultra filter inserts (Section 5.1.28; Millipore<sup>®</sup> Cat. No. UFC503096). Do not transfer any particulate matter that may be evident at bottom of tubes. Cap the filter units.
- Centrifuge at 7000 rpm for three minutes at 4°C.
- Open the filter units. Remove the Amicon<sup>®</sup> Ultra filter inserts using disposable forceps (gripping only the sides) and transfer to new collection tubes (Section 5.1.28; Millipore<sup>®</sup> Cat. No. UFC50VL96). Dispose of old collection tubes with filtrate as per CDC BSL-3 requirements (in an autoclavable biohazard bag).



#### **9.6.6 First Wash**

- Add 400 µL of 1X TE buffer (Section 6.6) to the Amicon® Ultra filters.
- Centrifuge at 7000 rpm for two minutes at 4°C.
- Open the filter units. Carefully remove the retentate from the top of the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC503096), avoiding any particulate matter visible on filter surface (tilt the tube for better viewing) and transfer liquid into new Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC503096) inserted in the collection tubes (Section 5.1.28; Millipore; Cat. No. UFC50VL96). Discard the used Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC503096) and collection tubes (Section 5.1.28; Millipore® Cat. No. UFC50VL96) in an autoclavable biohazard bag.

#### **9.6.7 Second Wash**

- Add 400 µL 1X TE buffer (Section 6.6) to the Amicon® Ultra filters. Cap the filter units.
- Centrifuge at 7000 rpm for three minutes at 4°C.
- Open the filter units. Transfer the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC503096) with disposable forceps (gripping only the sides) to new collection tubes (Section 5.1.28; Millipore® Cat. No. UFC50VL96).

#### **9.6.8 Third Wash**

- Add 400 µL 1X TE buffer (Section 6.6) to the Amicon® Ultra filters. Cap the filter units.
- Centrifuge at 7000 rpm for three minutes at 4°C.
- Open the filter units. Transfer the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC503096) with disposable forceps (gripping only the sides) to new collection tubes (Section 5.1.28; Millipore® Cat. No. UFC50VL96).

#### **9.6.9 Fourth Wash**

- Add 400 µL of PCR-grade water (Section 6.3) to the Amicon® Ultra filters. Cap the filter units.
- Centrifuge at 7000 rpm for 1 minute at 4°C.
- Check fluid level in the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC503096). If fluid level is above 200 µL, pulse spin for about 10 seconds (or less) until about 100 µL of fluid is retained on top of white base.
- If there is less than 100 µL of extract, transfer DNA extract back to the same Amicon® Ultra filter insert (Section 5.1.28; Millipore® Cat. No. UFC503096) and add 100 µL PCR-grade water and pulse spin to obtain about 100 µL on filter.

**Note:** *Very dirty samples may require additional washes to remove any potential inhibitors.*

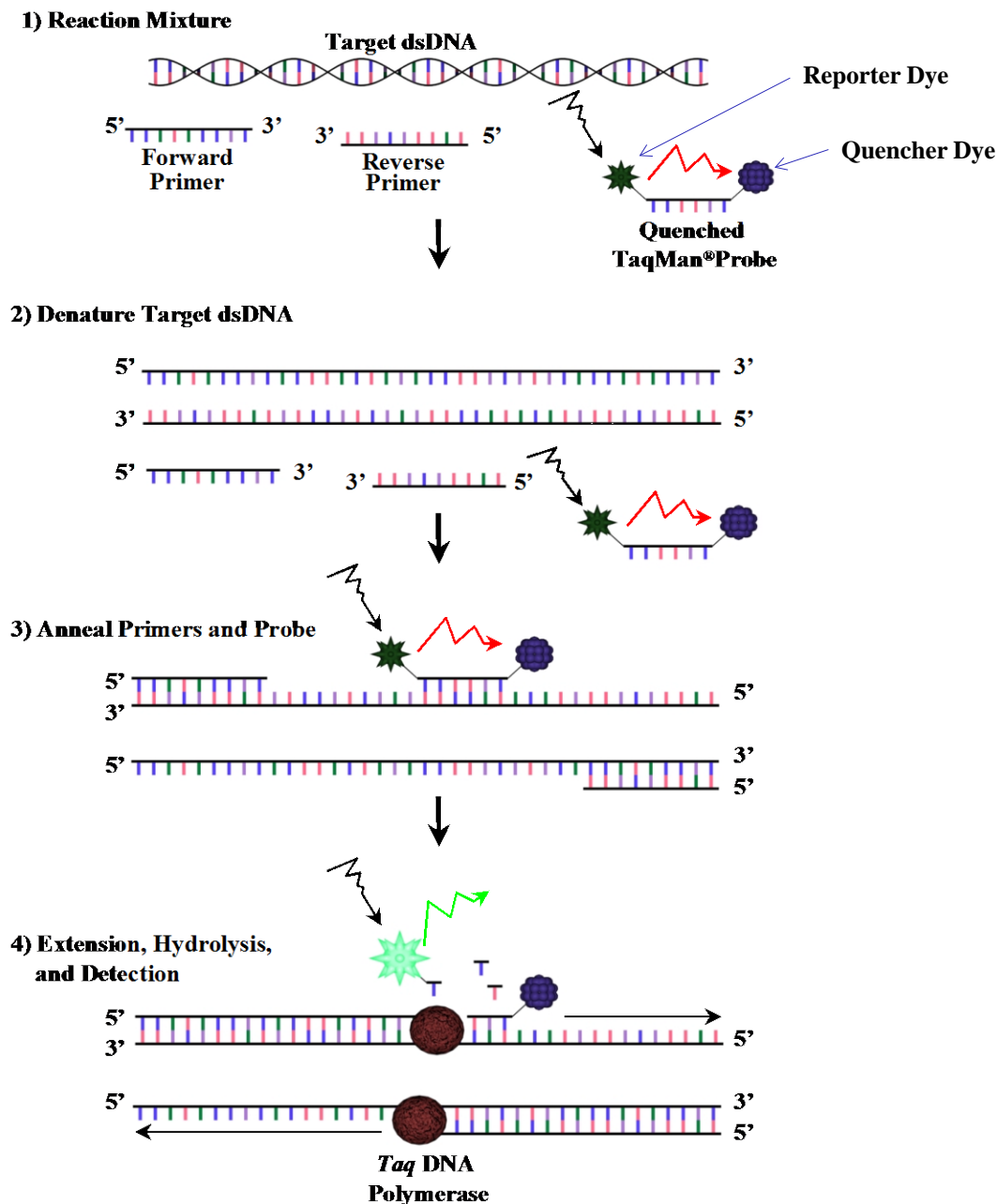
#### **9.6.10 Filtration of DNA Extract using 0.1 µm Centrifugal Filter Device (Section 5.1.30)**

Centrifugal filtration with 0.1-µm Ultrafree®-MC filter device following extraction of DNA allows for the removal of any *Y. pestis* cells which may have contaminated DNA preparations, making the samples safe without compromising the sensitivity of the real-time PCR assay (Reference 16.12).

- Using a micropipettor, carefully remove all of the retentate (~ 100 µL) from the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC503096) and transfer to corresponding 0.1 µm Ultrafree®-MC filter devices (Section 5.1.30; Millipore® Cat. No. UFC30VV00). Do not allow the micropipettor tip to touch the filter membrane. Avoid transferring any particulate matter that may be evident at bottom of the tubes. Close the caps. Discard the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC503096) with collection tubes (Section 5.1.28; Millipore® Cat. No. UFC50VL96) in an autoclavable biohazard bag.
- Repeat the above step for all the samples/retentates.
- Place the Ultrafree®-MC filter devices (Section 5.1.30; Millipore® Cat. No. UFC30VV00) into a centrifuge (Section 5.5.6; Eppendorf 5415R/5424R) and balance the rotor head.
- Centrifuge at  $8000 \times g$  (approximately 9200 rpm) for two minutes at 4°C (Reference 16.12).
- Carefully open the caps and remove the Ultrafree®-MC filter inserts (Section 5.1.30; Millipore® Cat. No. UFC30VV00) using disposable forceps (gripping only the sides), cap the collection tubes and dispose of the Ultrafree®-MC inserts (Section 5.1.30; Millipore® Cat. No. UFC30VV00) in an autoclavable biohazard bag. Place the collection tubes in a cold block.
- Carefully wipe the outside of the collection tubes containing sample DNA extract with a 10% pH amended bleach solution (Section 6.16) or bleach wipes (Section 5.1.3).
- Using clean gloves, place the cold block with the tubes containing filter extracts in DNA loading station/hood in preparation for PCR analyses (Section 9.7).

## **9.7 Real-time PCR Analyses**

As compared to traditional PCR, real-time PCR uses a sequence-specific hybridization probe sequence internal to the amplification primers, in addition to two target gene-specific amplification primers. The probe is fluorescently labeled at the 5' end with a reporter dye/fluorophore and at the 3' end with a quencher dye (usually, Black Hole Quenchers). The emission of light/fluorescence by the reporter dye is normally quenched by virtue of its proximity to the quencher dye. At the annealing step in a PCR, along with the amplification primers, depending upon its orientation, the probe sequence also hybridizes to its target site on the DNA strand downstream from the binding site of one of the primers. During the enzymatic extension step, when the probe comes in contact with the Taq DNA polymerase enzyme, the 5' exonuclease activity of the enzyme hydrolyzes the probe sequence by cleaving individual nucleotides from the 5' end. Cleavage of the probe releases the reporter dye from the proximal quencher, allowing emission of measurable fluorescence. Therefore, this assay is also known as the 5' exonuclease assay, as it relies on the 5' to 3' exonuclease activity of the Taq DNA polymerase enzyme to hydrolyze the probe. Thus, the PCR amplification of a specific gene sequence can be detected by monitoring the increase in fluorescence (**Figure 1**).



**Figure 1. Real-Time PCR Amplification.**

As the amplification reaction proceeds, more amplicons become available for probe binding and hydrolysis, and consequently, the fluorescence signal intensity per cycle increases. The increase in fluorescence can be detected in real time on PCR thermocyclers. When the fluorescence level crosses a set threshold value at a certain cycle number during the PCR, the result indicates the presence of the target gene sequence in the DNA in the sample, which in turn indicates the presence of a target pathogen in the sample. The PCR can specifically amplify a single copy of target gene sequence and generate millions of copies in a matter of minutes.

The TaqMan® fluorogenic probe hydrolysis-based real-time PCR assays are commonly used in

biodetection. Using established computer software (e.g., Primer Express) and genome sequence databases, bioagent-specific primers and probe nucleotide sequences for these assays are selected in such a way that they are present only in a specific location on the unique gene and/or virulence factor gene of interest for the detection and identification of a specific pathogen. These primers and probe sequences are absent in any other gene of that pathogen or in the genes of any near neighbor organisms. The primers generate a PCR product (amplicon) of a definite length/size. For a high-confidence identification of pathogens, PCR assays for multiple pathogen-specific genes are usually used. Therefore, in this protocol, three single-plex PCR assays are included. The YC2 PCR assay targets a hypothetical gene on the *Y. pestis* chromosome, while the YpMT1 and YpP1 PCR assays target a putative F1 operon positive regulatory protein gene on the pMT1 plasmid and the *pla* outer membrane protease gene on the pPCP1 plasmid, respectively.

It should be noted that the real-time PCR assays included in this protocol have been only partially characterized for specificity. These assays will be updated or replaced with fully characterized and validated assays upon availability.

**Note:** *This procedure is to be carried out in an area designated for PCR only. A PCR-workstation that is equipped with an ultraviolet (UV) light for sterilization must be used for PCR Master Mix preparation. Micropipets and corresponding sterile, aerosol-resistant pipet tips are used throughout this procedure for the addition of reagents. Aseptic technique must be used throughout, and all reagents must be kept at or near 4°C.*

**9.7.1** Decontaminate the PCR workstation by treating all work surfaces with a 10% pH amended bleach solution (Section 6.16) or bleach wipes (Section 5.1.3), allowing the bleach to contact the work surface for a minimum of 15 minutes prior to rinsing with sterile water. Turn on UV light for 15 minutes. After decontamination, discard gloves in an autoclavable biohazard bag and replace with a new, clean pair.

**Note:** *If gloves become contaminated, they should be disposed of in an autoclavable biohazard bag and fresh gloves donned. Only open one tube at a time throughout the process. At no point should more than one tube be open. Do not allow hands (gloved or otherwise) to pass over an open tube, PCR plate, or any reagent container. All used pipet tips, gloves and tubes must be discarded in an autoclavable biohazard bag.*

**9.7.2** Determine the number of reactions that are to be run. Include four replicate reactions each (for each assay) for an NTC (Section 8.9), PC (Section 8.6) and three replicates of the PNC (Section 8.11) per run. In addition, include three reactions for each sample including field blanks (Section 8.10) and two reactions for the EIC (Section 8.8) for each sample. Prepare a sufficient volume of Master Mix to allow for a minimum of one extra reaction for every 10 reactions, so that there is enough Master Mix regardless of pipetting variations. *For example, if 10 samples are to be analyzed for each PCR assay, a total of 61 reactions would be required [e.g., 4-NTC, 4-PC, 3-PNC, 30-samples and 20-EICs]. Therefore, the volume of PCR Master Mix prepared should be sufficient for 70 reactions per PCR assay.*

**9.7.3** Based on the example provided above (i.e., 10 samples), the amount of Master Mix required for each assay would be as indicated in **Table 4**.

**Table 4. Example Yp Single-plex PCR Master Mix Preparation for 70 Reactions**

Reagent	Volume per reaction (μL)	Total Volume (μL)	Final Concentration (μM)
TaqMan® 2X Universal Master Mix	12.5	875	1X
Forward primer, 10 μM	0.5	35	0.20
Reverse primer, 10 μM	0.5	35	0.20
Probe, 4 μM	0.4	28	0.064
PCR-grade water	6.1	427	N/A
<b>Total Volume</b>	20	1400	

**Note:** *The PC and NTC controls must be analyzed prior to sample analyses to verify that the Master Mix works properly and is free of contamination.*

**9.7.4** In a clean PCR-preparation hood, pipet 20 μL of Master Mix to four wells of the PCR plate. Label two wells as NTC and two as PC.

**9.7.5** Add 5 μL of PCR-grade water into the NTC wells.

**9.7.6** Cover the plate with adhesive plate sealer and transfer the PCR plate to the BSC. Remove the seal and add 5 μL of the PC (*Y. pestis* DNA [10 pg/μL]) to the PC wells.

**Note:** *This step must be performed in the BSC outside the PCR clean room set-up area. Change gloves.*

**9.7.7** Seal PCR plate with optical seal, using plate sealer for good contact. Change gloves.

**9.7.8** Centrifuge sealed PCR plate for 1 minute at 2000 rpm and 4°C, using the PCR plate safety cups or mini-plate centrifuge in the BSC.

**9.7.9** Open the centrifuge safety cup and transfer PCR plate to the ABI® 7500 Fast thermocycler.

**9.7.10** The PCR cycling conditions on the ABI® 7500 Fast are provided in **Table 5**. Fluorescence is automatically measured at the end of the 60°C annealing-extension combined step.

**Table 5. PCR Cycling Conditions <sup>a, b</sup>**

Steps	UNG <sup>c</sup> Incubation	AmpliTaq Gold Activation	PCR, 45 cycles <sup>d</sup>	
	Hold	Hold	Denaturation	Annealing/Extension
Temperature	50°C	95°C	95°C	60°C
Time	2 minutes	10 minutes	5 seconds	20 seconds <sup>e</sup>

<sup>a</sup> Run Mode: Fast 7500

<sup>b</sup> Reaction volume 25 μL

<sup>c</sup> Uracil-DNA glycosylase

<sup>d</sup> Fast Ramp: 3.5°C/seconds up and 3.5°C/seconds down

<sup>e</sup> 30 seconds for ABI® 7500 Fast Dx instrument

**9.7.11** If the Master Mix test results show “True Positive” assay detection for the PC and “True Negative” assay detection for the NTC, then proceed with analyses of samples. If the results are not “True” then repeat the PCR Master Mix preparation and testing protocol and reanalyze.

**9.7.12** In a clean PCR-preparation hood, pipet 20  $\mu$ L of Master Mix into the required number of wells of a new PCR plate (as per the number of samples to be analyzed). An eight-channel micropipettor can be used to add the Master Mix to the plate. Label two wells as NTC and two as PC. Label the rest of the wells such that there are five wells for each sample (three wells for actual sample analyses and two wells for EICs for each sample).

**9.7.13** Add 5  $\mu$ L of PCR-grade water into the NTC wells.

**9.7.14** Cover the plate with adhesive plate sealer and transfer the PCR plate to the BSC. Remove the seal and add 5  $\mu$ L of the PC (*Y. pestis* DNA [10 pg/ $\mu$ L]) to the PC wells.

**Note:** *These steps must be performed in the BSC outside the PCR clean room set-up area. Change gloves.*

**9.7.15** Add 5  $\mu$ L of the PNC extract to the three PNC wells.

**9.7.16** Add 5  $\mu$ L of each sample DNA extract to the respective sample wells and EIC wells.

**9.7.17** Add 1  $\mu$ L of the PC (*Y. pestis* DNA [50 pg/ $\mu$ L]) to all the EIC wells.

**Note:** *To minimize cross contamination, the EICs should not be placed next to the field samples when setting up the PCR tray.*

**9.7.18** Seal PCR plate with optical seal, using a plate sealer for good contact. Change gloves.

**9.7.19** Centrifuge sealed PCR plate for 1 minute at 2000 rpm and 4°C, using the PCR plate safety cups or mini-plate centrifuge in the BSC.

**9.7.20** Transfer PCR plate to the ABI® 7500 Fast thermocycler.

**9.7.21** Run PCR using the thermocycling conditions as described in Section 9.7.10.

**9.7.22** After completion of thermocycling, discard the sealed PCR plate in an autoclavable biohazard bag.

**Note:** *PCR plates with amplified product should not be opened in the laboratory.*

**9.7.23** Laboratory cleanup procedures

- Dispose of all biological materials in autoclavable biohazard bags (double bagged).
- Autoclave all waste materials at the end of the work day.
- Decontaminate counters and equipment with a 10% pH amended bleach solution (Section 6.16) or bleach wipes (Section 5.1.3), followed by 70% isopropyl and a DI water final rinse.

**9.7.24** Refer to Section 12.1 for data analyses and calculations.

## 10.0 Culture Method

**Acceptable sample types:** Gauze wipes (2" × 2" 50% rayon/50% polyester [Kendall™ Versalon™ Cat. No. 8042 or equivalent]), air filters (37 mm Fluoropore™ [Millipore Cat. No. FSLW04700 or equivalent]), swabs (macrofoam [VWR Cat. No. 89022-994 small swabs or 89022-984 large swabs, or equivalent]), Sponge-Stick sampling tools (3M™ Inc. Cat. No. SSL10NB or equivalent) and drinking water and decontamination waste water.

**Note:** *Neutralization of decontamination agent(s) may be required prior to sample processing and analyses. The EPA technical lead for this protocol may be contacted for guidance.*

Media sterility checks (Section 8.5) and positive controls (Section 8.7) should be analyzed every day that samples are analyzed, to ensure that all media and reagents are performing properly.

### 10.1 Sample Processing and Plating for Sponge-Sticks and Wipes

**Note:** *All subsequent procedures involving manipulation of Sponge-Sticks and wipes must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.*

#### 10.1.1 Recover Bacteria from Sponge-Sticks and Wipes

- If the Sponge-Stick sponge/wipe sample is not in a Stomacher® bag, aseptically transfer it to a Stomacher® bag using sterile forceps.

**Note:** *In most cases, the Sponge-Stick handle will be removed in the field by the sampler. However, if the handle was not removed, proceed as follows. While holding the sponge-stick handle outside the Stomacher® bag, grip the Sponge-Stick head with your other hand outside the bag and twist the head to snap off stick at the crimp line near the handle base.*

- Using aseptic technique, remove the plastic stick base holding the sponge together. Place gloved hands on the outside of the Stomacher® bag, grip the sponge-stick head on both sides and peel the sponge away from the base and unfold the sponge. Be careful not to puncture bag with edge of stick base. Using sterile forceps, remove stick base from bag and discard in an autoclavable biohazard bag. Change forceps between samples.
- Add 90 mL of PBST (0.05% Tween® 20, Section 6.5) to each bag. Set Stomacher® (Section 5.5.19) to 200 rpm.
- Place the bag containing the sample into the Stomacher® (Section 5.5.19) so the Sponge-Stick sponge/wipe rests evenly between the paddles and homogenize each sample for 1 minute at 200 rpm.
- Open the door of the Stomacher® (Section 5.5.19) and remove the bag. Grab the wipe from the outside of the bag with your hands. With the bag closed, move the sponge/wipe to the top of the bag while using your hands to squeeze excess liquid from the sponge/wipe.
- Open the bag and remove sponge/wipe using sterile forceps. Retain the sponge/wipe in a labeled specimen cup (Section 5.3.5).
- Follow the steps described above for each sample, changing forceps between samples.



- Allow bags to sit for 10 minutes to allow elution suspension foam to settle.
- Gently mix the elution suspension in the Stomacher® bag up and down 3 times with a sterile 50 mL pipet. Remove half of the suspension volume (~47 mL) and place it in a labeled 50 mL screw cap centrifuge tube. Place the remaining suspension (~47 mL) into a second 50 mL tube. Please adjust the suspension volumes in both the tubes to ensure they are similar.
- Process elution suspension for each sample, as described above.
- Place 50 mL tubes into sealing centrifuge buckets and decontaminate centrifuge buckets before removing them from the BSC.
- Centrifuge tubes at a maximum speed ( $\sim 3200 \times g$ ) with the brake off, for 15 minutes in a swinging bucket rotor at 4°C.
- Using a sterile 50 mL pipet for each sample, remove 44 mL of the supernatant from each sample tube and discard it in an autoclavable biohazard container. The pellet may be easily disturbed and not visible, so keep the pipet tip away from the bottom of the tube.
- Set the vortexer (Section 5.5.10) to the high setting. Set the sonicator water bath to high.
- Vortex the tubes for 30 seconds and transfer the tubes to the sonicator bath and sonicate for 30 seconds. Repeat the vortex and sonication cycles two more times.

**Note:** *As an alternative to sonication, tubes may be vortexed for two minutes in 10 second bursts using a vortexer with a multi-tube adapter.*

- Remove suspension from one tube with a sterile 5 mL pipet and combine it with the suspension in the other tube from the same sample. Measure final volume of suspension with 5 mL pipet and record the result on the tube and data sheet.
- Follow vortexing and sonication steps for each sample.

#### **10.1.2 Serially Dilute the Suspension in PBS**

- Vortex the suspension on high for 30 seconds.
  - a. Transfer 1 mL of the suspension from the 50 mL tube to a 15 mL tube containing 9 mL of PBS. Recap the tube and vortex it on high for 30 seconds. This is the  $10^{-1}$  suspension.
  - b. Open the cap of the  $10^{-1}$  suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBS. Recap the tube and vortex on high for 30 seconds. This is the  $10^{-2}$  suspension.
  - c. The above results in 3 cell suspensions: the initial wipe elution suspension (undiluted) and 2 serial dilutions of the suspension in PBS ( $10^{-1}$  and  $10^{-2}$ ).
- Repeat steps (a) and (b) for each sample.



### 10.1.3 Culture Cell Suspensions on CIN and SBA

To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature, or dried using a laminar-flow hood.

**Note:** *Plating of 0.1 mL results in an additional 1:10 dilution of each of the suspensions.*

Each of the following will be conducted in triplicate, resulting in the evaluation of 18 spread plates per sample:

- a. After vortexing tubes, pipet 0.1 mL of undiluted suspension onto the surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-1}$ ).
  - b. After vortexing tubes, pipet 0.1 mL of  $10^{-1}$  suspension onto surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-2}$ ).
  - c. After vortexing tubes, pipet 0.1 mL of  $10^{-2}$  suspension onto surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-3}$ ).
- After pipetting the 6 spread plates for each dilution, beginning with the  $10^{-3}$  dilution, use a sterile L-Shaped spreader to distribute the inoculum over the surface of the medium by rotating the dish by hand or on a turntable. Please ensure that inoculum is evenly distributed over the entire surface of the plate. Use a different sterile spreader for each plate. Repeat for the next two dilutions  $10^{-2}$  and  $10^{-1}$ , in that order.
  - Allow inoculum to absorb into the medium completely.

### 10.1.4 Incubate and Enumerate Plates

Invert the SBA and CIN plates and incubate them at 25°C–28°C for a maximum of 5 days. Observe plates daily for growth.

- SBA plates
  - a. *Y. pestis* produces gray-white, translucent colonies, usually too small to be seen as individual colonies at 24 hours. After incubation for 48 hours, colonies are approximately 1–2 mm in diameter, gray-white to slightly yellow and opaque (**Figure 2**). After 48–72 hours of incubation, colonies have a raised, irregular “fried egg” appearance, which becomes more prominent as the culture ages. Colonies also can be described as having a “hammered copper,” shiny surface. There is little or no hemolysis of the sheep red blood cells.
  - b. Count the number of *Y. pestis* colonies on each plate and record results.
- CIN plates
  - a. *Y. pestis* produces “bull’s-eye” colonies, colorless with deep red center on CIN agar. Colonies are usually 1–2 mm after incubation for 48 hours (**Figure 2**).
  - b. Count the number of *Y. pestis* colonies on each plate and record results.



**Figure 2. *Y. pestis* Colonies on SBA and CIN Agar after 48 Hours.**

- Plate counts
  - a. If the number of colonies is  $\leq 250$ /plate, record the actual number.
  - b. If the number of colonies is  $> 250$ /plate, record as “too numerous to count” (TNTC).
  - c. If no target colonies are observed, record as “None detected” and proceed to evaluation of growth on MicroFunnel™ plates (Section 10.1.5).

A minimum of 3 typical colonies should be confirmed using real-time PCR (Section 10.5).

#### **10.1.5 Capture Cells on Microfunnel™ Filter Membranes and Culture**

- Place two 0.45  $\mu\text{m}$  (pore-size) MicroFunnel™ filter funnels (Section 5.3.4) on the vacuum manifold and moisten membrane with 5 mL PBS. All filtering should be done with a vacuum pressure  $< 10$  psi.
- With the vacuum valve closed (and vacuum pressure released), place 10 mL of PBS into each filter cup. Add 1.0 mL of the undiluted elution suspension (Section 10.1.1) to each of two MicroFunnel™ cups.
- Open the vacuum valve and filter the suspension. Close the valve and release the vacuum pressure. Rinse the walls of each MicroFunnel™ cup with 10 mL of PBS and filter. Open the valve and complete filtration by pouring remaining rinsate through the filter.
- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard the cup in an autoclavable biohazard bag. Remove the membranes with sterile forceps and place them grid-side up on labeled SBA and CIN plates. Make sure that the filters are in contact with the surface of the agar. If an air pocket occurs under the filter, use the sterile forceps to lift the edge of the filter to release the air pocket.
- Record the exact volume of the suspension filtered (e.g., 1.0 mL) on each plate.
- Repeat steps (Section 10.1.5) described above for each sample.

- 10.1.6** Invert and incubate SBA and CIN plates at 25°C–28°C for a maximum of 5 days. Plates should be examined daily for growth. Count the number of colonies and record results. Confirm 1–3 colonies using real-time PCR (Section 10.5).

**Note:** *For faster sample analysis results during the initial stages of an incident (e.g., incident characterization) and during post-decontamination/clearance phase, it is recommended that the remainder of all suspensions (e.g., undiluted,  $10^{-1}$  and  $10^{-2}$  dilutions) be filtered using an additional MicroFunnel™ and plated as described above, instead of proceeding with enrichment in 2X YPEB.*

#### 10.1.7 Enrich in 2X YPEB

- Add the remainder of the undiluted,  $10^{-1}$  and  $10^{-2}$  suspensions to the specimen cup containing the corresponding sponge/wipe. Add 25 mL of 2X YPEB to the specimen cup. Repeat for each sample. Incubate tubes at 25°C–28°C for 18–24 hours.
- Evaluate the 2X YPEB Enrichment
  - a. If broth is not cloudy, record as no growth (NG); incubate for an additional 24 hours and recheck for growth.
  - b. If broth is cloudy, record as positive growth and proceed to next step.
  - c. Cap the cup tightly and mix 2X YPEB with growth for 30 seconds. Remove a loopful of broth with a sterile 10 µL loop and streak on a SBA plate for isolation. Repeat two times for a total of three SBA isolation plates.
  - d. Incubate the isolation plates and 2X YPEB with growth for 18–24 hours at 25°C– 28°C.
  - e. Examine plates for *Y. pestis*-suspect colonies. If any colonies are isolated, proceed to PCR confirmation (Section 10.5).
  - f. If no suspect colonies are observed, perform PCR on 2X YPEB with growth according to Section 10.5.

### 10.2 Sample Processing and Plating for Swabs

**Note:** *All subsequent procedures involving manipulation of swabs must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.*

#### 10.2.1 Recover Bacteria from Swabs

- If the swabs are not in screw cap centrifuge tubes, transfer each swab to sterile, plastic 15 mL screw cap centrifuge tube using sterile forceps.
- If necessary, cut the handle of the swab to fit into the tube using sterile scissors. Use sterile forceps and scissors for each sample.
- Add 5 mL of PBS to each tube and vortex on high for two minutes.
- Using sterile forceps, remove the swab from the 15 mL centrifuge tube. Use the forceps to press the tip of the swab against the inside of the tube to remove extra liquid from the foam tip.
- Place the swab into a labeled 50 mL tube with 5 mL 2X YPEB and set aside.
- Repeat steps described above for each swab sample.

#### 10.2.2 Serially Dilute Elution Suspension in PBS

- Vortex the elution suspension on high for 30 seconds.

- a. Transfer 1 mL of the suspension from the 50 mL tube to a 15 mL tube containing 9 mL of PBS. Recap the tube and vortex it on high for 30 seconds. This is the  $10^{-1}$  suspension.
  - b. Open the cap of the  $10^{-1}$  suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBS. Recap the tube and vortex on high for 30 seconds. This is the  $10^{-2}$  suspension.
  - c. The above results in 3 cell suspensions: the initial wipe elution suspension (undiluted) and 2 serial dilutions of the suspension in PBS ( $10^{-1}$  and  $10^{-2}$ ).
- Repeat steps (a) and (b) for each sample.

### **10.2.3 Culture Cell Suspensions on CIN and SBA**

To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

**Note:** *Plating of 0.1 mL results in an additional 1:10 dilution of each of the suspensions.*

Each of the following will be conducted in triplicate, resulting in the evaluation of 18 spread plates per sample:

- a. After vortexing tubes, pipet 0.1 mL of undiluted suspension onto the surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-1}$ ).
  - b. After vortexing tubes, pipet 0.1 mL of  $10^{-1}$  suspension onto surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-2}$ ).
  - c. After vortexing tubes, pipet 0.1 mL of  $10^{-2}$  suspension onto surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-3}$ ).
- After pipetting the 6 spread plates for each dilution, beginning with the  $10^{-3}$  dilution, use a sterile L-Shaped spreader to distribute the inoculum over the surface of the medium by rotating the dish by hand or on a turntable. Please ensure that inoculum is evenly distributed over the entire surface of the plate. Use a different sterile spreader for each plate. Repeat for the next two dilutions  $10^{-2}$  and  $10^{-1}$ , in that order.
  - Allow inoculum to absorb into the medium completely.

### **10.2.4 Incubate and Enumerate Plates**

Invert the SBA and CIN plates and incubate them at 25°C–28°C for a maximum of 5 days. Observe plates daily for growth.

- SBA plates
  - a. *Y. pestis* produces gray-white, translucent colonies, usually too small to be seen as individual colonies at 24 hours. After incubation for 48 hours, colonies are approximately 1–2 mm in diameter, gray-white to slightly yellow and opaque (Figure 2). After 48–72 hours of incubation, colonies have a raised, irregular “fried egg” appearance, which becomes more prominent as the culture ages. Colonies also can be described as having a “hammered copper,” shiny surface. There is little or no hemolysis of the sheep red blood cells.
  - b. Count the number of *Y. pestis* colonies on each plate and record results.
- CIN plates

a. *Y. pestis* produces “bull’s-eye” colonies, colorless with deep red center on CIN agar. Colonies are usually 1–2 mm after incubation for 48 hours (Figure 2).

b. Count the number of *Y. pestis* colonies on each plate and record results.

- Plate counts

a. If the number of colonies is  $\leq 250$ /plate, record the actual number.

b. If the number of colonies is  $> 250$ /plate, record as “too numerous to count” (TNTC).

c. If no target colonies are observed, record as “None detected” and proceed to evaluation of growth on MicroFunnel™ plates (10.2.5).

A minimum of 3 typical colonies should be confirmed using real-time PCR (Section 10.5).

#### **10.2.5 Capture Cells on MicroFunnel™ Filter Membranes and Culture**

- Place two, 0.45  $\mu\text{m}$  (pore-size) MicroFunnel™ filter funnels (Section 5.3.4) on the vacuum manifold and moisten membrane with 5 mL PBS. All filtering should be done with a vacuum pressure  $< 10$  psi.
- With the vacuum valve closed (and vacuum pressure released), place 10 mL of PBS into each filter cup. Add 1.0 mL of the undiluted elution suspension (Section 10.2.1) to each of two MicroFunnel™ cups.
- Open the vacuum valve and filter the suspension. Close the valve and release the vacuum pressure. Rinse the walls of each MicroFunnel™ cup with 10 mL of PBS and filter. Open the valve and complete filtration by pouring remaining rinsate through the filter.
- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard the cup in an autoclavable biohazard bag. Remove the membranes with sterile forceps and place them grid-side up on labeled SBA and CIN plates. Make sure that the filters are in contact with the surface of the agar. If an air pocket occurs under the filter, use the sterile forceps to lift the edge of the filter to release the air pocket.
- Record the exact volume of the suspension filtered (1.0 mL) on each plate.
- Repeat steps (Section 10.2.5) described above for each sample.

**10.2.6** Invert and incubate SBA and CIN plates at 25°C–28°C for a maximum of 5 days. Plates should be examined daily for growth. Count the number of colonies and record results. Confirm 1–3 colonies using real-time PCR (Section 10.5).

**Note:** *For faster sample analysis results during the initial stages of an incident (e.g., incident characterization) and during post-decontamination/clearance phase, it is recommended that the remainder of all suspensions (e.g., undiluted,  $10^{-1}$  and  $10^{-2}$  dilutions) be filtered using an additional MicroFunnel™ and plated as described above, instead of proceeding with enrichment in 2X YPEB.*

#### **10.2.7 Enrich in 2X YPEB**

- Add the remainder of the undiluted,  $10^{-1}$  and  $10^{-2}$  suspensions to the specimen cup containing the corresponding swab. Add 25 mL 2X YPEB to the specimen cup. Repeat for each sample. Incubate tubes at 25°C–28°C for 18–24 hrs.
- Evaluate the 2X YPEB

- a. If broth is not cloudy, record as no growth (NG); incubate for an additional 24 hours and recheck for growth.
- b. If broth is cloudy, record as positive growth and proceed to next step.
- c. Cap the cup tightly and mix 2X YPEB with positive growth for 30 seconds. Remove a loopful of broth with a sterile 10  $\mu$ L loop and streak on a SBA plate for isolation. Repeat 2 times for a total of three SBA isolation plates.
- d. Incubate the isolation plates and 2X YPEB with growth for 18–24 hours at 25°C–28°C.
- e. Examine plates for *Y. pestis*-suspect colonies. If any colonies are isolated, proceed to PCR confirmation (Section 10.5).
- f. If no suspect colonies are observed, perform PCR on 2X YPEB with growth according to Section 10.5.

### **10.3 Sample Processing and Plating for Air Filters**

**Note:** *All subsequent procedures involving manipulation of air filters must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.*

#### **10.3.1 Recover Bacteria from Air Filters**

- If the air filters are not in 50 mL tubes, aseptically transfer each sample to a sterile 50 mL tube using sterile forceps. Change forceps between samples.
- Add 5 mL of sterile PBS to each tube and vortex on high for two minutes.
- Transfer liquid to a sterile, labeled 50 mL tube.
- Place air filter in a specimen cup and set aside.
- Repeat the steps described above for each air filter.

#### **10.3.2 Serially Dilute the Suspension in PBS**

- Vortex the elution suspension on high for 30 seconds.
  - a. Transfer 1 mL of the suspension from the 50 mL tube to a 15 mL tube containing 9 mL of PBS. Recap the tube and vortex it on high for 30 seconds. This is the  $10^{-1}$  suspension.
  - b. Open the cap of the  $10^{-1}$  suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBS. Recap the tube and vortex on high for 30 seconds. This is the  $10^{-2}$  suspension.
  - c. Open cap of the  $10^{-2}$  suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBS. Recap the PBS tube and vortex on high for 30 seconds. This is the  $10^{-3}$  suspension.
  - d. The above results in 4 cell suspensions: the initial sock elution suspension (undiluted) and three serial dilutions of the suspension in PBS ( $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$ ).
- Repeat steps (a) through (c) for each sample.

#### **10.3.3 Culture Cell Suspensions on CIN and SBA**



To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature, or dried using a laminar-flow hood.

**Note:** *Plating of 0.1 mL results in an additional 1:10 dilution of each of the suspensions.*

Each of the following will be conducted in triplicate, resulting in the evaluation of 24 spread plates per sample:

- a. After vortexing tubes, pipet 0.1 mL of undiluted suspension onto the surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-1}$ ).
  - b. After vortexing tubes, pipet 0.1 mL of  $10^{-1}$  suspension onto surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-2}$ ).
  - c. After vortexing tubes, pipet 0.1 mL of  $10^{-2}$  suspension onto surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-3}$ ).
  - d. After vortexing tubes, pipet 0.1 mL of  $10^{-3}$  suspension onto surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-4}$ ).
- After pipetting the 6 spread plates for each dilution, beginning with the  $10^{-4}$  dilution, use a sterile L-Shaped spreader to distribute the inoculum over the surface of the medium by rotating the dish by hand or on a turntable. Please ensure that inoculum is evenly distributed over the entire surface of the plate. Use a different sterile spreader for each plate. Repeat for the next three dilutions  $10^{-3}$ ,  $10^{-2}$  and  $10^{-1}$ , in that order.
  - Allow inoculum to absorb into the medium completely.

#### **10.3.4 Incubate and Enumerate Plates**

Invert the SBA and CIN plates and incubate them at 25°C–28°C for a maximum of 5 days. Observe plates daily for growth.

- SBA plates
  - a. *Y. pestis* produces gray-white, translucent colonies, usually too small to be seen as individual colonies at 24 hours. After incubation for 48 hours, colonies are approximately 1–2 mm in diameter, gray-white to slightly yellow and opaque (Figure 2). After 48–72 hours of incubation, colonies have a raised, irregular “fried egg” appearance, which becomes more prominent as the culture ages. Colonies also can be described as having a “hammered copper,” shiny surface. There is little or no hemolysis of the sheep red blood cells.
  - b. Count the number of *Y. pestis* colonies on each plate and record results.
- CIN plates
  - a. *Y. pestis* produces “bull’s-eye” colonies, colorless with deep red center on CIN agar. Colonies are usually 1–2 mm after incubation for 48 hours (Figure 2).
  - b. Count the number of *Y. pestis* colonies on each plate and record results.
- Plate counts
  - a. If the number of colonies is  $\leq 250$ /plate, record the actual number.
  - b. If the number of colonies is  $> 250$ /plate, record as “too numerous to count” (TNTC).
  - c. If no target colonies are observed, record as “None detected” and proceed to evaluation of growth on MicroFunnel™ plates (Section 10.3.5).

A minimum of 3 typical colonies should be confirmed using real-time PCR (Section 10.5).

### 10.3.5 Capture Cells on MicroFunnel™ Filter Membranes and Culture

- Place two, 0.45 µm (pore-size) MicroFunnel™ filter funnels (Section 5.3.4) on the vacuum manifold and moisten membrane with 5 mL PBS. All filtering should be done with a vacuum pressure < 10 psi.
- With the vacuum valve closed (and vacuum pressure released), place 10 mL of PBS into each filter cup. Add 1.0 mL of the undiluted elution suspension (Section 10.3.1) to each of two MicroFunnel™ cups.
- Open the vacuum valve and filter the suspension. Close the valve and release the vacuum pressure. Rinse the walls of each MicroFunnel™ cup with 10 mL of PBS and filter. Open the valve and complete filtration by pouring remaining rinsate through the filter.
- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard the cup in an autoclavable biohazard bag. Remove the membranes with sterile forceps and place them grid-side up on labeled SBA and CIN plates. Make sure that the filter is in contact with the surface of the agar. If an air pocket occurs under the filter, use the sterile forceps to lift the edge of the filter to release the air pocket.
- Record the exact volume of the suspension filtered (1.0 mL) on each plate.
- Repeat steps (Section 10.3.5) described above for each sample.

**10.3.6** Invert and incubate SBA and CIN plates at 25°C–28°C for a maximum of 5 days. Plates should be examined daily for growth. Count the number of colonies and record results. Confirm 1–3 colonies using real-time PCR (Section 10.5).

**Note:** *For faster sample analysis results during the initial stages of an incident (e.g., incident characterization) and during post-decontamination/clearance phase, it is recommended that the remainder of all suspensions (e.g., undiluted, 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup> dilutions) be filtered using an additional MicroFunnel™ and plated as described above, instead of proceeding with enrichment in 2X YPEB.*

### 10.3.7 Enrich in 2X YPEB

- Add the remainder of the undiluted, 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup> suspensions to the specimen cup containing the corresponding air filter. Add 30 mL 2X YPEB to the specimen cup. Repeat for each sample. Incubate specimen cups at 25°C–28°C for 18–24 hours.
- Evaluate the 2X YPEB
  - a. If broth is not cloudy, record as no growth (NG); incubate for an additional 24 hours and recheck for growth.
  - b. If broth is cloudy, record as positive growth and proceed to next step.
  - c. Cap the cup tightly and mix 2X YPEB with positive growth for 30 seconds. Remove a loopful of broth with a sterile 10 µL loop and streak on a SBA plate for isolation. Repeat 2 times for a total of three SBA isolation plates.
  - d. Incubate the isolation plates and 2X YPEB with growth for 18–24 hours at 25°C–28°C.



- e. Examine plates for *Y. pestis*-suspect colonies. If any colonies are isolated, proceed to PCR confirmation (Section 10.5).
- f. If no suspect colonies are observed, perform PCR on 2X YPEB with growth according to Section 10.5.

#### **10.4 Sample Processing and Plating for Water Samples**

Please see Appendix A for primary (Section 2.0) and secondary (Section 3.0) concentration of large volume (10 L-100 L) water samples. For water sample volumes ranging from 10 mL to < 10 L, please refer to Appendix A, Section 3.0, secondary concentration.

**Note:** *All subsequent procedures involving manipulation of water samples and membranes must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.*

##### **10.4.1 Recover Bacteria from the MicroFunnel™ Membrane** (from secondary water concentration, Appendix A, Section 3.0).

- If the membranes are not in 50 mL tubes, aseptically transfer each membrane to a sterile 50 mL tube using sterile forceps. Change forceps between samples.
- Add 5 mL of sterile PBS to each tube and vortex on high for two minutes.
- Transfer liquid to a sterile, labeled 50 mL tube.
- Place the membrane in a specimen cup and set aside.
- Repeat the steps described above for each membrane.

##### **10.4.2 Serially Dilute the Suspension in PBS**

- Vortex the elution suspension on high for 30 seconds.
  - a. Transfer 1 mL of the suspension from the 50 mL tube to a 15 mL tube containing 9 mL of PBS. Recap the tube and vortex it on high for 30 seconds. This is the  $10^{-1}$  suspension.
  - b. Open the cap of the  $10^{-1}$  suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBS. Recap the tube and vortex on high for 30 seconds. This is the  $10^{-2}$  suspension.
  - c. Open cap of the  $10^{-2}$  suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBS. Recap the PBS tube and vortex on high for 30 seconds. This is the  $10^{-3}$  suspension.
  - d. The above results in 4 cell suspensions: the initial sock elution suspension (undiluted) and three serial dilutions of the suspension in PBS ( $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$ ).
- Repeat steps (a) through (c) for each sample.

##### **10.4.3 Culture Cell Suspensions on CIN and SBA**

To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature, or dried using a laminar-flow hood.

**Note:** *Plating of 0.1 mL results in an additional 1:10 dilution of each of the suspensions.*

Each of the following will be conducted in triplicate, resulting in the evaluation of 24 spread plates per sample:

- a. After vortexing tubes, pipet 0.1 mL of undiluted suspension onto the surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-1}$ ).
  - b. After vortexing tubes, pipet 0.1 mL of  $10^{-1}$  suspension onto surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-2}$ ).
  - c. After vortexing tubes, pipet 0.1 mL of  $10^{-2}$  suspension onto surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-3}$ ).
  - d. After vortexing tubes, pipet 0.1 mL of  $10^{-3}$  suspension onto surface of pre-dried SBA plate and a pre-dried CIN plate (labeled  $10^{-4}$ ).
- After pipetting the 6 spread plates for each dilution, beginning with the  $10^{-4}$  dilution, use a sterile L-Shaped spreader to distribute the inoculum over the surface of the medium by rotating the dish by hand or on a turntable. Please ensure that inoculum is evenly distributed over the entire surface of the plate. Use a different sterile spreader for each plate. Repeat for the next three dilutions  $10^{-3}$ ,  $10^{-2}$  and  $10^{-1}$ , in that order.
  - Allow inoculum to absorb into the medium completely.

#### **10.4.4 Incubate and Enumerate Plates**

Invert the SBA and CIN plates and incubate them at 25°C–28°C for a maximum of 5 days. Observe plates daily for growth.

- SBA plates
  - a. *Y. pestis* produces gray-white, translucent colonies, usually too small to be seen as individual colonies at 24 hours. After incubation for 48 hours, colonies are approximately 1–2 mm in diameter, gray-white to slightly yellow and opaque (Figure 2). After 48–72 hours of incubation, colonies have a raised, irregular “fried egg” appearance, which becomes more prominent as the culture ages. Colonies also can be described as having a “hammered copper,” shiny surface. There is little or no hemolysis of the sheep red blood cells.
  - b. Count the number of *Y. pestis* colonies on each plate and record results.
- CIN plates
  - a. *Y. pestis* produces “bull’s-eye” colonies, colorless with deep red center on CIN agar. Colonies are usually 1–2 mm after incubation for 48 hours (Figure 2).
  - b. Count the number of *Y. pestis* colonies on each plate and record results.
- Plate counts
  - a. If the number of colonies is  $\leq 250$ /plate, record the actual number.
  - b. If the number of colonies is  $> 250$ /plate, record as “too numerous to count” (TNTC).
  - c. If no target colonies are observed, record as “None detected” and proceed to evaluation of growth on MicroFunnel™ plates (10.4.5).

A minimum of 3 typical colonies should be confirmed using real-time PCR (Section 10.5).

#### 10.4.5 Capture Cells on MicroFunnel™ Filter Membranes and Culture

- Place two, 0.45 µm (pore-size) MicroFunnel™ filter funnels (Section 5.3.4) on the vacuum manifold and moisten membrane with 5 mL PBS. All filtering should be done with a vacuum pressure < 10 psi.
- With the vacuum valve closed (and vacuum pressure released), place 10 mL of PBS into each filter cup. Add 1.0 mL of the undiluted elution suspension (Section 10.4.1) to each of two MicroFunnel™ cups.
- Open the vacuum valve and filter the suspension. Close the valve and release the vacuum pressure. Rinse the walls of each MicroFunnel™ cup with 10 mL of PBS and filter. Open the valve and complete filtration by pouring remaining rinsate through the filter.
- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard the cup in an autoclavable biohazard bag. Remove the membranes with sterile forceps and place them grid-side up on labeled SBA and CIN plates. Make sure that the filter is in contact with the surface of the agar. If an air pocket occurs under the filter, use the sterile forceps to lift the edge of the filter to release the air pocket.
- Record the exact volume of the suspension filtered (1.0 mL) on each plate.
- Repeat steps (Section 10.4.5) described above for each sample.

- 10.4.6** Invert and incubate SBA and CIN plates at 25°C–28°C for a maximum of 5 days. Plates should be examined daily for growth. Count the number of colonies and record results. Confirm 1–3 colonies using real-time PCR (Section 10.5).

**Note:** *For faster sample analysis results during the initial stages of an incident (e.g., incident characterization) and during post-decontamination/clearance phase, it is recommended that the remainder of all suspensions (e.g., undiluted, 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup> dilutions) be filtered using an additional MicroFunnel™ and plated as described above, instead of proceeding with enrichment in 2X YPEB.*

#### 10.4.7 Enrich in 2X YPEB

- Add the remainder of the undiluted, 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup> suspensions to the specimen cup containing the corresponding membrane filter. Add 30 mL 2X YPEB to the specimen cup. Repeat for each sample. Incubate specimen cups at 25°C–28°C for 18–24 hours.
- Evaluate the 2X YPEB Enrichment
  - a. If broth is not cloudy, record as no growth (NG); incubate for an additional 24 hours and recheck for growth.
  - b. If broth is cloudy, record as positive growth and proceed to next step.
  - c. Cap the cup tightly and mix 2X YPEB with positive growth for 30 seconds. Remove a loopful of broth with a sterile 10 µL loop and streak on a SBA plate for isolation. Repeat 2 times for a total of three SBA isolation plates.
  - d. Incubate the isolation plates and 2X YPEB with growth for 18–24 hours at 25°C–28°C.
  - e. Examine plates for *Y. pestis*-suspect colonies. If any colonies are isolated, proceed to PCR confirmation (Section 10.5).

- f. If no suspect colonies are observed, perform PCR on 2X YPEB with growth according to Section 10.5.

## **10.5 Confirm *Y. pestis* Colonies by Real-time PCR Analysis**

### **10.5.1 DNA Preparation from Cultured Cells**

- Cells grown on solid culture medium
  - a. Pipet 100  $\mu$ L of PCR-grade water into a 1.5 mL Eppendorf microcentrifuge tube (Section 5.1.20).
  - b. Use a disposable 1  $\mu$ L inoculating loop or pre-wetted swab to remove bacterial growth from a typical *Y. pestis* colony grown on SBA or CIN. Growth from the SBA plate would be preferred; however, it may be necessary to use isolates from the CIN if there are no isolated colonies on SBA due to overgrowth by background organisms.

**Note:** *In some cases, it may be difficult to remove the bacterial growth with a loop. If this happens, use a sterile applicator swab. Be sure to pre-wet the swab with PCR-grade water before removing the bacterial growth.*

- c. Insert the loop or swab into the tube containing the PCR-grade water and immerse the bacterial growth in the liquid.
  - d. Gently spin the loop or swab in the liquid to remove and resuspend the bacterial growth in the water. Press the tip of the swab against the tube to remove the liquid from the tip prior to discarding in an autoclavable biohazard bag. Proceed to Section 10.5.2.
- Cells grown in liquid culture medium:
  - a. Transfer 50  $\mu$ L of broth with growth to a microcentrifuge tube.
  - b. Place tube into a refrigerated microcentrifuge and spin at  $12,000 \times g$  for two minutes.
  - c. Remove and discard the supernatant in an autoclavable biohazard container. Add 100  $\mu$ L of PCR-grade water to the tube containing the bacterial pellet.
  - d. Resuspend the pellet by flicking the tube. Proceed to Section 10.5.2.

### **10.5.2 Preparation of Lysate**

- Cap the microcentrifuge tubes containing the bacterial suspension with cap-holding tabs to prevent the tubes from popping open during heating, and briefly vortex.
- Place the capped tubes in a floating rack if using the water bath. Otherwise, place the capped tube in the heat block at  $95^{\circ}\text{C} - 98^{\circ}\text{C}$ .
- Heat the sample for 20 minutes. Heating for 20 minutes will ensure all organisms are killed; this allows the sample to be handled outside of the BSL-3 laboratory.
- Remove the tubes from the water bath or heat block and place them directly in a cold block. Chill the tubes for a minimum of two minutes.
- Remove the cap-holding tabs and place the microcentrifuge tubes in the refrigerated microcentrifuge. Centrifuge at 12,000 rpm for two minutes.

### 10.5.3 Filtration of Lysate using a 0.1 µm Centrifugal Filter Device (Section 5.1.30)

Centrifugal filtration with 0.1-µm Ultrafree®-MC filter devices following extraction of DNA allows for the removal of *Y. pestis* cells which may contaminate DNA preparations, making the samples safe without compromising the sensitivity of the real-time PCR assay (Reference 16.12).

- Remove top cap from the 0.1 µm Ultrafree®-MC filter device (Section 5.1.30; Millipore® Cat. No. UFC30VV00).
- Hold each filter device vertical with the filter cup opening facing up. Using a micropipettor tip, transfer the supernatant from each microcentrifuge tube into the corresponding 0.1 µm Ultrafree®-MC filter device (Section 5.1.30; Millipore® Cat. No. UFC30VV00). Do not allow the micropipettor tip to touch the filter membrane. Avoid transferring any particulate matter that may be evident at the bottom of the tube. Close the cap. Discard the microcentrifuge tube in an autoclavable biohazard bag.
- Place the Ultrafree®-MC filter devices into a centrifuge (Section 5.5.6; Eppendorf 5415R/5424R) and balance the rotor head.
- Centrifuge at  $8000 \times g$  (approximately 9200 rpm) for two minutes at 4°C.

**Note:** *If the supernatant has not passed completely through the filter, centrifuge for an additional two minutes. Repeat as necessary until all the supernatant has passed through the filter.*

- Carefully open the caps and remove the Ultrafree®-MC filter inserts (Section 5.1.30; Millipore® Cat. No. UFC30VV00) using disposable forceps (gripping only the sides), close the caps of the collection tubes and dispose of the Ultrafree®-MC inserts in an autoclavable biohazard bag.
- If there is concern regarding the biosafety of the filtered material, it is recommended that the laboratory perform a sterility check on the filtered material according to internal laboratory procedures.
- Wipe the outside of the tubes containing lysates with 10% pH amended bleach (Section 6.16) or bleach wipes (Section 5.1.3). Samples lysates are safe to remove from the BSL-3 after filtration and disinfection of the tube.
- Using clean gloves, place the cold block with the tubes containing the lysates in DNA loading station/hood in preparation for PCR analyses (Section 9.7)
- If PCR analysis will not be completed the same day the lysates are prepared, aliquot lysates and freeze them at -20°C.

**Note:** *DNA extracted by this procedure should not be stored for more than one week.*

**10.5.4** Use 5 µL of the lysate as the DNA template to run the PCR analysis in triplicate using the YC2 assay. Optionally, for virulent strains, any combination or all three PCR assays can be used.

**Note:** *DNA obtained from cell lysates should be diluted (e.g., 1:10 or 1:100) prior to testing to avoid excess DNA template, which can cause false negative results.*

**10.5.5** For real-time PCR, follow instructions provided in Sections 9.7.1–9.7.23 with the following exceptions and changes:

- No PNC and EIC controls are required for the samples.
- For each batch of sample colonies, PCR Master Mix should be made for 4 PCs, 4 NTCs and 3 replicates for DNA extracts per colony.

**10.5.6** Refer to Sections 12.1 and 12.2.3 for data analyses and calculations.

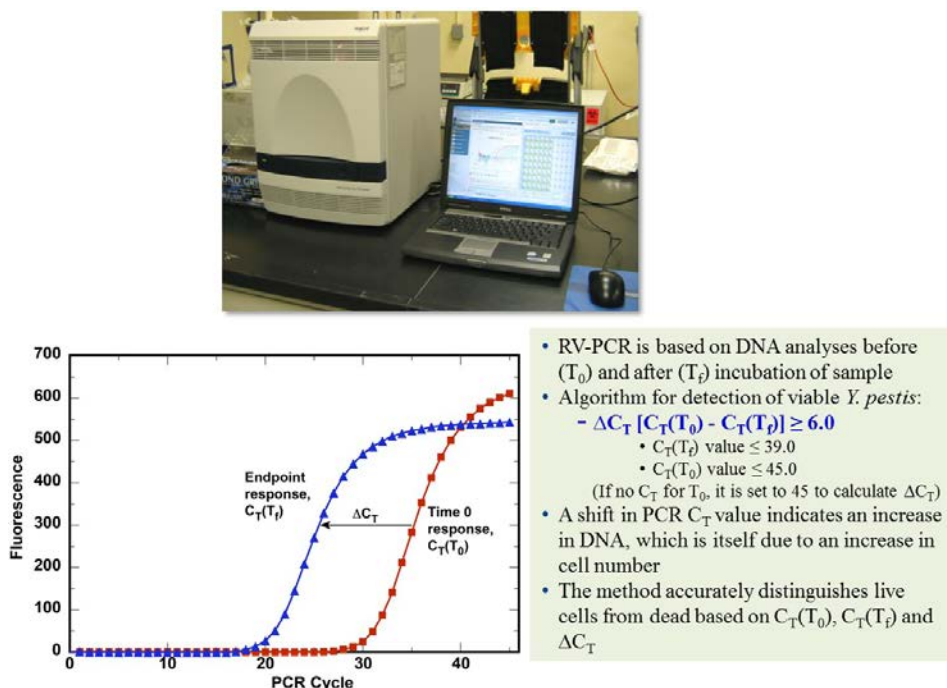
## 11.0 Rapid Viability-Polymerase Chain Reaction (RV-PCR) Method

**Acceptable sample types:** Drinking water and decontamination waste water

### 11.1 RV-PCR

The RV-PCR method (**Figures 3 and 4**) serves as an alternative to the traditional culture-based methods for detection of viable pathogens. The RV-PCR method integrates high-throughput sample processing, short-incubation broth culture, and highly sensitive and specific real-time PCR assays to detect low concentrations of viable *Y. pestis* (Reference 16.7). This section includes a RV-PCR method with appropriate sample processing procedures for detection of *Y. pestis* in water samples.

The RV-PCR method not only generates rapid results, but also may provide a higher throughput capability compared to the traditional culture-based methods, and hence, increases the laboratory capacity for sample analysis. In place of multiple dilutions, plates, and enrichment culture per sample used by the culture method, the RV-PCR method uses a single well on a 48-well plate per sample for *Y. pestis* (Figure 3).



**Figure 3. Example Real-time PCR Amplification Curves for the Initial  $T_0$  Aliquot and the  $T_f$  (Final) Endpoint Aliquot.**



The RV-PCR protocol steps and some of the equipment for *Y. pestis* are shown in Figure 3. After mixing the water sample with growth medium, an aliquot is withdrawn for baseline (time 0) analysis before incubating the broth culture in the filter vial at 30°C for 24 hours. This is the T<sub>0</sub> aliquot and is stored at 4°C for immediate processing or at -20°C for an extended period until analysis. After the broth culture is incubated for 24 hours or more, another aliquot is withdrawn. This is the T<sub>24</sub> or T<sub>f</sub> aliquot. Both the T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> aliquots are then processed to extract and purify *Y. pestis* total DNA. The T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> DNA extracts are then analyzed, in triplicate, using real-time PCR to detect the presence of *Y. pestis* DNA. The C<sub>T</sub> values for both the T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> DNA extracts are recorded and compared. A change in C<sub>T</sub> for the T<sub>24</sub> or T<sub>f</sub> aliquot relative to the C<sub>T</sub> for the T<sub>0</sub> aliquot is calculated as follows:  $\Delta C_T = (C_T [T_0] - C_T [T_{24} \text{ or } T_f])$ . A  $\Delta C_T \geq 6$  (i.e., the endpoint PCR C<sub>T</sub> of  $\leq 39$  for the T<sub>24</sub> or T<sub>f</sub> DNA extract in a 45-cycle PCR) is set as a cut-off value for a positive detection of viable *Y. pestis* in the sample. The  $\Delta C_T \geq 6$  criterion represents an approximate two log increase in DNA concentration at T<sub>24</sub> or T<sub>f</sub> relative to T<sub>0</sub>. The increase in DNA concentration at T<sub>24</sub> or T<sub>f</sub> is as a result of the presence of viable *Y. pestis* bacteria in the sample that grew during the 24 or more hours of incubation in growth medium. Incubation time could be extended to 36–48 hours to greatly eliminate the possibility of a false negative result, especially for very dirty and/or post-decontamination clearance samples. The current protocol provides qualitative (presence or absence) results.

As stated in Section 9, for a high-confidence identification of pathogens, PCR assays for multiple pathogen-specific genes are usually used. Therefore, in this protocol, three single-plex PCR assays are included. The YC2 PCR assay targets a hypothetical gene on the *Y. pestis* chromosome while the YpMT1 and YpP1 PCR assays target a putative F1 operon positive regulatory protein gene on the pMT1 plasmid and the *pla* outer membrane protease gene on the pPCP1 plasmid, respectively. However, for sample analysis during a confirmed plague incident for which the *Y. pestis* strain has already been identified and characterized, only the most sensitive real-time PCR assay targeting a strain-specific gene on a plasmid may be performed. In the absence of such a gene, the assay targeting the chromosomal marker should be used. Although, if needed, all three assays or any combination thereof can be performed using the same sample DNA extract.

**Note:** *The real-time PCR assays included in this protocol have been only partially characterized for specificity. These assays will be updated or replaced with fully characterized and validated assays upon availability.*

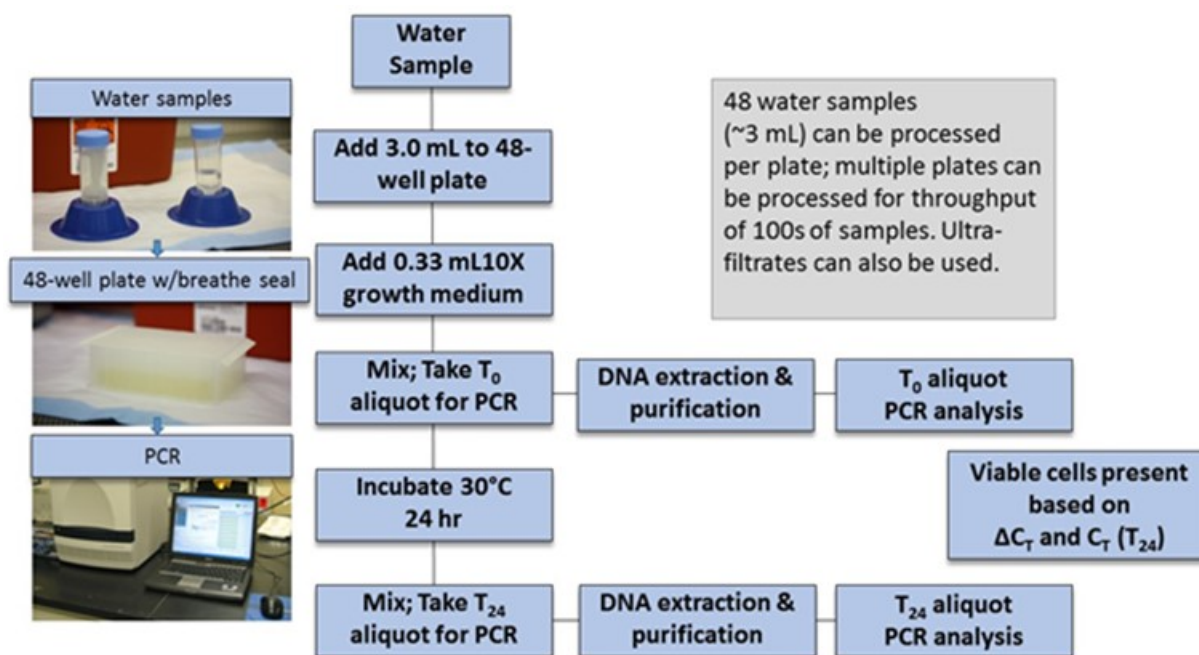


Figure 4. Flow Chart for RV-PCR Analysis of *Y. pestis* Cells from Water Samples.

## 11.2 RV-PCR Sample Processing and Plating for Water Samples

**Note:** All subsequent procedures involving manipulation of water samples and membranes must be carried out in a BSC using appropriate PPE). Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.

### 11.2.1 Concentrate Water Sample by Centrifugation

**Note:** If the water sample has not been previously stabilized by buffer addition to maintain cell viability, add 4.5 mL of 10X PBS to 40 mL water sample (final ~1X PBS concentration). If the sample volume is greater than 40 mL, adjust the PBS volume to achieve a final concentration of ~1X PBS.

- Using a 50-mL serological pipet, transfer 40 mL of sample to a 50 mL screw cap centrifuge tube. If the sample volume is greater than 40 mL, divide the final volume into equal volumes and dispense into multiple tubes.
- Repeat the step above for each sample.
- Make sure tubes are balanced and place tubes into sealing centrifuge buckets. Decontaminate centrifuge buckets with a 10% pH amended bleach solution (Section 6.16) or bleach wipes (Section 5.1.3) before removing them from the BSC.
- Centrifuge tubes at  $3500 \times g$ , with the brake off, for 15 minutes in a swinging bucket rotor.
- Remove the supernatant from each tube with a sterile 50 mL serological pipet and discard, leaving approximately 3 mL in each tube (or 3 mL total if combining pellets).



from multiple tubes per sample). The pellet may be easily disturbed and not visible, so keep the pipet tip away from the tube bottom.

- Remove suspension (or combined suspension) from one tube with a sterile 5 mL pipet (recording the volume) and transfer to one well of the 48-well plate.
- Repeat for each sample.

#### **11.2.2 Add Concentrated Growth Medium and Process for RV-PCR analysis**

- Add 333  $\mu$ L of 10X YPEB to each well of the 48-well plate containing sample using a 1000  $\mu$ L pipettor (final YPEB concentration  $\sim$  1X). Mix well.
- Transfer 500  $\mu$ L from each well of the 48-well plate to a screw cap tube. This is a  $T_0$  aliquot. Repeat for each sample.
- Store aliquots on ice or in cold block (4°C).

#### **11.2.3 Seal and Incubate 48-well Plate**

- Seal the 48-well plate with a sterile, breathable seal.
- Place in ziplock bag and seal.
- Incubate the 48-well plate at 28°C for 24 hours in a shaker incubator at 180 rpm.

**Note:** *The incubation time could be extended to 36-48 hours to minimize the possibility of false negative results due to dirty and/or post-decontamination clearance samples.*

#### **11.2.4 Process $T_0$ Aliquots for DNA Extraction**

- Centrifuge tubes at 14,000 rpm (20,800 relative centrifugal force [RCF]) for 10 minutes at 4°C.
- Remove 300 mL of supernatant and dispose to waste. Store pellets on ice or in cold block (4°C). Alternatively, the pellet may be stored at -20°C until it is ready to be processed for DNA extraction.

#### **11.2.5 Process $T_{24}$ or $T_f$ Aliquots for DNA Extraction**

**Note:** *For dirty and post-decontamination samples the incubation time could be extended to 36-48 hours.*

- After 24 hour or longer incubation, transfer 500  $\mu$ L from each well to a 2-mL screw cap tube. This is a  $T_{24}$  or  $T_f$  aliquot.
- Centrifuge tubes at 14,000 rpm (20,800 RCF) for 10 minutes at 4°C.
- Remove 300 mL of supernatant and dispose to waste. Store pellets on ice or in cold block (4°C). Alternatively, the pellet may be stored at -20°C until it is ready to be processed for DNA extraction.

### **11.3 RV-PCR: DNA Extraction/Purification Procedure**

**11.3.1** Thaw  $T_0$  and  $T_{24}$  or  $T_f$  aliquots if they were stored at -20°C.

**11.3.2** Add 800  $\mu$ L of Lysis Buffer (VWR, Cat. No. PAMD1392 or equivalent) using a 1000  $\mu$ L pipettor with a new tip for each sample. Cap the tubes and mix by vortexing on high ( $\sim$ 1800 rpm) for 30 seconds and place in 96-well tube rack at room temperature. Change gloves as necessary between samples.

- 11.3.3** Vortex each screw cap tube briefly (low speed, 5–10 seconds) and transfer the sample volume to a 2 mL Eppendorf tube (ensure the tubes are labeled correctly during transfer). Change gloves in between each sample. Incubate the T<sub>0</sub> and T<sub>24</sub> lysate tubes hereafter referred to as “T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes” at room temperature for five minutes.
- 11.3.4** Vortex the paramagnetic particles (PMPs) on high (~1800 rpm) for 30–60 seconds, or until they are uniformly resuspended. Keep PMPs in suspension by briefly vortexing (3–5 seconds) before adding to each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> lysate tube.
- 11.3.5** Uncap one tube at a time and add 600 µL of PMPs using a new tip for each sample, to each T<sub>0</sub> and T<sub>24</sub> tubes (containing 1 mL sample). Discard used tips in a sharps container. Mix by vortexing for 3–5 seconds.
- 11.3.6** Repeat Section 11.3.5 for all T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes, vortexing the PMPs suspension between each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tube.
- 11.3.7** Vortex each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tube for 5–10 seconds (high), incubate at room temperature for five minutes, briefly vortex, and then place on the magnetic stand with hinged-side of the tube facing toward the magnet. After all the tubes are in the stand, invert tubes 180 degrees (upside-down) turning away from you, then right side-up, then upside down toward you, then right side-up (caps up) position. This step allows all PMPs to contact the magnet. Check to see if any beads are in the caps and if so, repeat the tube inversion cycle again. Let the tubes sit for 5–10 seconds before opening. Maintain the tube layout when transferring tubes between the magnetic stand and the 96-well tube rack.
- 11.3.8** Uncap each tube one at a time and withdraw all liquid using a 1000 µL pipettor with the pipet tip placed in the bottom of 2 mL tube, taking care not to disturb the PMPs. Ensure that all the liquid is removed. Use a new pipet tip to remove any residual liquid, if necessary. If liquid remains in the tube cap, remove by pipetting. Dispose tip and liquid in a sharps container. Recap tube. Change gloves.
- Note:** *Section 11.3.8 can be combined with Section 11.3.9. After withdrawing the liquid in Section 11.3.8, add 360 µL of Lysis Buffer using a separate pipettor and a new tip.*
- 11.3.9** Uncap each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tube one at a time, and add 360 µL of Lysis Buffer using a 1000 µL pipettor. Use a new tip for each sample and discard used tips in a sharps container. Cap and vortex on low setting for 5–10 seconds, then transfer to 96-well tube rack.
- 11.3.10** After adding Lysis Buffer to all of the T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes, vortex each tube for 5–10 seconds (low) and place back on the magnetic stand. After all tubes are in the stand, follow tube inversion cycle, as described in Section 11.3.7.
- 11.3.11** Remove all the liquid as described in Section 11.3.8, except that a glove change between samples is not required. Use a new tip for each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tube (discard used tips in a sharps container). Recap the tube.
- 11.3.12** Repeat Sections 11.3.9–11.3.11 for all tubes.
- Note:** *Section 11.3.11 can be combined with Section 11.3.13. After withdrawing the liquid in Section 11.3.11, add 360 µL of Salt Wash solution using a separate pipettor and new tip. If the steps are combined, cap the tube after the Salt Wash addition.*
- 11.3.13** 1st Salt Wash: Uncap each T<sub>0</sub> and T<sub>24</sub> tube one at a time, and add 360 µL of Salt Wash solution (Section 6.12). Use a new tip for each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tube and discard used tips in a sharps container. Cap and transfer to 96-well tube rack.

**11.3.14** After adding the Salt Wash solution to all of the T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes, vortex each tube for 5-10 seconds (low) and place on the magnetic stand. After all tubes are in the stand, follow tube inversion cycle, as described in Section 11.3.7.

**11.3.15** Remove liquid as described in Section 11.3.8, except that a glove change between T<sub>0</sub> and T<sub>24</sub> tubes is not required. Use a new tip for each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tube and discard used tips in a sharps container. Recap the tube. Repeat for all T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes.

**Note:** *Section 11.3.15 can be combined with Section 11.3.16. After withdrawing the liquid in Section 11.3.15, add 360 µL of Salt Wash solution using a separate pipettor and new tip. If the steps are combined, cap the tube after the Salt Wash addition.*

**11.3.16** 2nd Salt Wash: Repeat Sections 11.3.13–11.3.15 for all T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes.

**Note:** *Section 11.3.16 can be combined with Section 11.3.17. After withdrawing the liquid in Section 11.3.16, add 500 µL of Alcohol Wash buffer using a separate pipettor and new tip. If the steps are combined, cap the tube after the Alcohol Wash addition.*

**11.3.17** 1st Alcohol Wash: Uncap each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tube, one at a time, and add 500 µL of Alcohol Wash (Section 6.12). Use a new tip for each sample and discard used tips in a sharps container. Cap and transfer to 96-well tube rack.

**11.3.18** After adding the Alcohol Wash (Section 6.12) to all of the T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes, vortex each tube for 5–10 seconds (low speed) and place on the magnetic stand. After all T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes are in the stand, follow the tube inversion cycle, as described in Section 11.3.7.

**11.3.19** Remove liquid as described in Section 11.3.8, except that a glove change between T<sub>0</sub> and T<sub>24</sub> tubes is not required. Use a new tip for each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tube and discard used tips in a sharps container. Recap the tube.

**Note:** *Section 11.3.19 can be combined with Section 11.3.20. After withdrawing the liquid in Section 11.3.19, add 500 µL of Alcohol Wash using a separate pipettor and new tip. If the steps are combined, cap the tube after the Alcohol Wash addition.*

**11.3.20** 2nd Alcohol Wash: Repeat Sections 11.3.17–11.3.19, except use 70% ethanol wash solution for all tubes. After the liquid is removed, recap the tube and transfer to the 96-well tube rack.

**Note:** *Section 11.3.20 can be combined with Section 11.3.21. After withdrawing the liquid in Section 11.3.20, add 500 µL of Alcohol Wash using a separate pipettor and new tip. If the steps are combined, cap the tube after the ethanol wash addition.*

**11.3.21** 3rd Alcohol Wash: Repeat Sections 11.3.17–11.3.19 for all T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes except use 70% ethanol wash solution. After the liquid is removed, recap the tube and transfer to the 96-well tube rack.

**11.3.22** Open all T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes and air dry for two minutes.

**11.3.23** Heat the open T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tubes in the heat block (placed in the BSC) at 80°C until the PMPs are dry (~20 minutes). Allow all the alcohol solution to evaporate, since alcohol may interfere with analysis.

**11.3.24** DNA elution: While they are in the heating block add 200 µL of Elution Buffer to each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tube, and close tube.

**11.3.25** Vortex for 10 seconds and let the tubes sit in the heating block for 80 seconds.

**11.3.26** Briefly vortex the tubes (5–10 seconds), taking care to prevent the liquid from entering the tube cap. Let the tube sit in the heating block for 1 minute.

**11.3.27** Repeat Section 11.3.26 four more times.

**11.3.28** Remove the tubes from the heating block, place them in a 96-tube rack in the BSC, and let them sit at room temperature for at least five minutes.

**11.3.29** Briefly vortex each tube (5–10 seconds) on low speed. Optional: Centrifuge at 2000 rpm at 4°C for 1 minute. Place tube in 96-well tube rack.

**11.3.30** Briefly vortex each tube and place on the magnetic stand for at least 30 seconds. Bring the cold block to the BSC.

**11.3.31** Collect elution liquid from each T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> tube with a micropipettor and transfer to a clean, labeled, 1.5 mL tube (~80–90 µL) on a cold block (check tube labels to ensure the correct order). Use a new tip for each tube and discard tips in a sharps container. Visually verify absence of PMP carryover during final transfer. If magnetic bead carryover occurred, place the 1.5 mL tube on magnet, collect liquid, and transfer to a clean, labeled, 1.5 mL tube (ensure the tubes are labeled correctly during transfer).

**11.3.32** Centrifuge tubes at 14,000 rpm at 4°C for five minutes to pellet any particles remaining with the eluted DNA; carefully remove supernatant and transfer to a new 1.5 mL tube using a new tip for each tube (ensure the tubes are labeled correctly during transfer).

**Note:** *If analyses need to be conducted outside of a BSL-3, the DNA extract may be filtered using a 0.1 µm Ultrafree®-MC filter insert, as described in Section 9.6.10.*

**11.3.33** Store T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> DNA extract tubes at 4°C until PCR analysis (use photo-tray to transport 1.5 mL tubes in a rack).

**Note:** *If PCR cannot be performed within 24 hours, freeze DNA extracts at -20°C.*

**11.3.34** Laboratory cleanup procedures

- Dispose of all biological materials in autoclavable biohazard bags (double bagged).
- Autoclave all waste materials at the end of the work day.
- Decontaminate counters and equipment with fresh 10% pH amended bleach (Section 6.16), followed by 70% isopropyl, and a DI water final rinse.

#### **11.4 Real-time PCR Analysis of T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> DNA Extracts**

**Note:** *PCR Master Mix for 6 reactions per sample is required to accommodate the T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> DNA extracts. For each batch of samples, PCR Master Mix should be made for 4 PCs, 4 NTCs, 3 PNCs and 6 DNA extracts per sample (3 for T<sub>0</sub> and 3 for T<sub>24</sub> or T<sub>f</sub> DNA extracts). No EIC control is required for the samples.*

**11.4.1** Prepare PCR Mix according to the **Table 6**.

**11.4.2** Set up 96-well PCR plate with PCR mix according to plate layout in PCR-preparation hood, seal, and transfer to BSC.

**11.4.3** Analyze T<sub>0</sub> and T<sub>24</sub> or T<sub>f</sub> DNA extracts on same PCR plate.

**11.4.4** If DNA extracts were frozen, transfer them to BSC and let them thaw to room temperature.

- 11.4.5** Perform 1:10 dilution of DNA extracts. Alternatively, only run samples undiluted (5 µL plus 20 µL PCR Master Mix).
- 11.4.6** Add 90 µL of PCR-grade water to wells of a sterile 96-well plate. Note: 10-fold dilutions may also be made in screw cap tubes or 1.5 mL Eppendorf tubes.
- 11.4.7** Mix DNA extracts by vortexing (5 seconds at low speed) and transfer 10 µL to the plate wells, following the plate layout.
- 11.4.8** Mix diluted DNA extracts by vortexing (5 seconds at low speed) and transfer 5 µL from each plate well or tube to the PCR plate (with PCR Mix). Seal PCR plate with adhesive plate sealer.
- 11.4.9** Centrifuge sealed PCR plate for 1 minute at 2000 rpm.
- 11.4.10** Open the centrifuge safety cup in BSC, place plate on photo-tray, change gloves, transfer PCR plate to ABI® thermocycler.
- 11.4.11** The PCR cycling conditions on the ABI® 7500 Fast are provided in **Table 7**. Fluorescence is automatically measured at the end of the 60°C annealing-extension combined step.
- 11.4.12** After cycle completion, discard sealed PCR plate to waste and autoclave. PCR plates with amplified product are never to be opened in the laboratory.
- 11.4.13** Follow laboratory cleanup procedure (11.3.34).

**Table 6. PCR Mix for All Selected *Y. pestis* Assays**

Reagent	Volume (µL)	Final Concentration (µM)
TaqMan® 2X Universal Master Mix	12.5	1X
Forward primer, 10 µM	0.5	0.20
Reverse primer, 10 µM	0.5	0.20
Probe, 4 µM	0.4	0.064
Molecular Biology grade water	6.1	N/A
Template DNA	5	Variable
TOTAL	25	

**Table 7. PCR Thermal Cycling Conditions <sup>a, b</sup>**

Steps	UNG <sup>c</sup> Incubation	AmpliTaq Gold Activation	PCR , 45 cycles <sup>d</sup>	
	Hold	Hold	Denaturation	Annealing/Extension
Temperature	50°C	95°C	95°C	60°C
Time	2 minutes	10 minutes	5 seconds	20 seconds <sup>e</sup>

<sup>a</sup> Run Mode: Fast 7500

<sup>b</sup> Reaction volume 25 µL

<sup>c</sup> Uracil-DNA glycosylase

<sup>d</sup> Fast Ramp: 3.5°C/s up and 3.5°C/s down

<sup>e</sup> 30 seconds for ABI® 7500 Fast Dx instrument

## 12.0 Data Analysis and Calculations

### 12.1 Real-time PCR Analysis

Calculate the average  $C_T$  from the replicate reactions for each sample DNA extract, PC and the EIC, where applicable.

$$\frac{\sum_{j=1}^N C_T(j)}{N} = \text{Average } C_T,$$

where  $N$  is the number of replicate reactions

Example:

Where 3 replicate reactions produce  $C_T$  values of 20, 25, and 24,

$$\frac{\sum_{j=1}^3 C_T(1) + C_T(2) + C_T(3)}{3} = \frac{\sum 20 + 25 + 24}{3} = \frac{69}{3} = 23 = \text{Average } C_T$$

The average  $C_T \leq 40$  and the presence of a logarithmic curve in the real-time graph for the sample DNA extract indicates a positive result, suggesting the presence of *Y. pestis* in the sample. A minimum of two out of three replicates must show  $C_T \leq 40$  for a sample result to be considered positive. If only one out of three PCR replicates for any sample DNA extract gives  $C_T \leq 40$ , the PCR analysis of the DNA extract for that sample must be repeated.

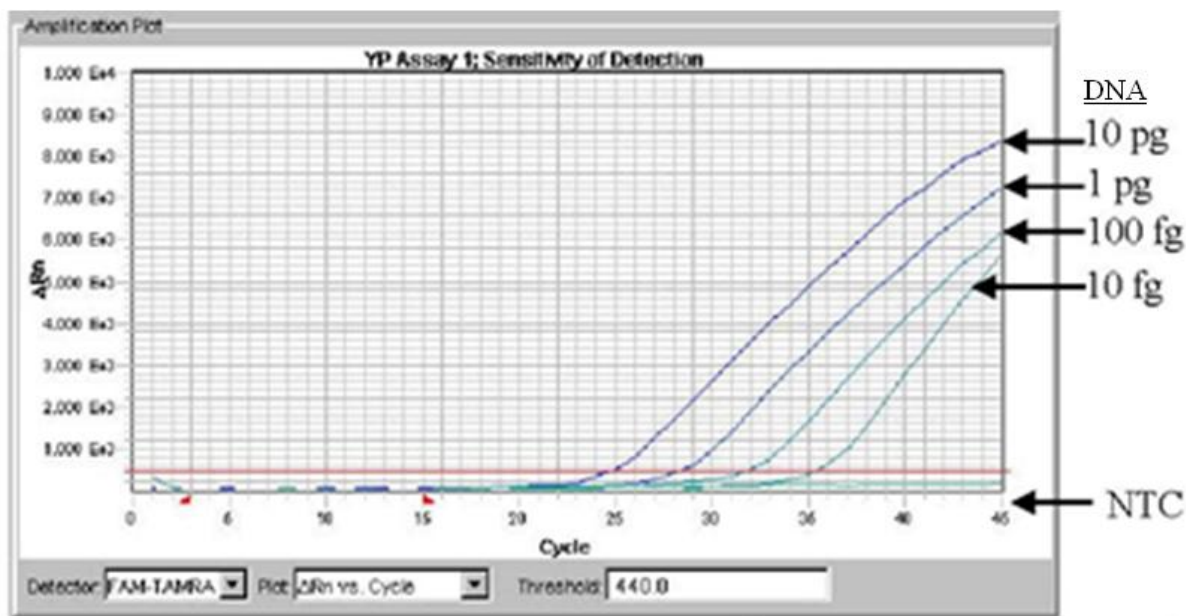


Figure 5. Example Logarithmic Curve for Fluorogenic PCR for *Y. pestis*.

If the EIC (with 50 pg of the *Y. pestis* DNA) for a sample results in a  $C_T$  value ( $\geq 3$ ) compared to the  $C_T$  value for the positive control, there may be matrix inhibition. If the corresponding sample DNA extract is negative ( $C_T > 40$ ) for *Y. pestis*, the sample DNA extract should be diluted 1:4 and 1:10 and the PCR assay should be repeated for that sample, along with the EIC with diluted sample DNA extracts. Negative controls should not yield any measurable  $C_T$  values. If  $C_T$  values



are obtained, check for potential cross-contamination and repeat analysis. In addition, field blank samples should not yield any measurable  $C_T$  values. If  $C_T$  values are obtained as a result of a possible contamination or cross-contamination, depending upon the  $C_T$  value, a careful interpretation of the  $C_T$  values for the sample DNA extracts must be done for the final result or the PCR analyses must be repeated.

## 12.2 Culture Analysis

### 12.2.1 Serial Dilution Plating

Count the number of typical colonies (Figure 2) on replicate culture plates and calculate the average number of colonies per plate. Apply the following guidelines (a – e) when counting the colonies and report results based on the number of characteristic *Y. pestis* colonies.

Media sterility checks should not exhibit growth. Growth should also not be present on CIN or SBA plates from field blank samples. If growth is observed on plates, colony morphology should be evaluated to determine if contamination is due to the target organism and potential source of contamination. Depending on the situation, results should be qualified if QC plates are contaminated with *Y. pestis*.

- a. If the number of colonies is  $\leq 250$ /plate, record the actual number.
- b. If the number of colonies is  $> 250$ /plate, record as “TNTC.”
- c. Ideally, plates with 25–250 colonies should be used to calculate the number of colony forming units (CFU) per sample, as described below.
- d. If there are no plates with 25–250 colonies, chose the plates with the counts closest to the acceptable range of 25–250 colonies. For example, if all plate counts are greater than 250, chose the plates that have counts closest to 250. Likewise, if all of the plate counts are less than 25, the plates with counts closet to 25 would be used to calculate the number of CFUs per sample.
- e. If no target colonies are observed, record as “None detected.”

To determine the number of CFUs per sample, average the CFU counts of colonies in the dilution series which produced  $\leq 250$  colonies/plate. In this case assume the colony counts were 210, 193, and 200 for the  $10^{-1}$  dilution series and 25, 19, and 26 for the  $10^{-2}$ . Divide the total number of colonies on plates with 25–250 colonies by the total volume plated to obtain the number of colonies in 1 mL, and then multiply by the total suspension volume, as in the following equation:

$$\frac{\sum_{j=1}^N CFU(j)}{\sum_{j=1}^N Volume(j)} \times Total\ Suspension\ Volume = CFUs\ per\ sample,$$

where  $N$  is the number of plates for which the CFU Count is between 25 and 250

Example:

$$\left[ \left( \frac{210\ CFU + 193\ CFU + 200\ CFU + 25\ CFU + 26\ CFU}{0.1\ mL + 0.1\ mL + 0.1\ mL + 0.01\ mL + 0.01\ mL} \right) \times 5\ mL \right] \\ = 10,220\ CFUs\ per\ sample$$

### 12.2.2 MicroFunnel™ Filter Plating

Count the number of typical colonies (Figure 2) on each filter and record. Apply the following (a – e) when counting the colonies and report results based on the number of characteristic *Y. pestis* colonies.

Media sterility checks should not exhibit growth. Growth should also not be present on CIN or SBA plates from field blank samples. If growth is observed on filters, colony morphology should be evaluated to determine if contamination is due to the target organism and potential source of contamination. Depending on the situation, results should be qualified if QC plates are contaminated with *Y. pestis*.

- a. If the number of colonies is  $\leq 80$ /plate, record the actual number.
- b. If the number of colonies is  $> 80$ /plate, record as “TNTC.”
- c. Ideally, plates with 20–80 colonies should be used to calculate the number of CFUs per sample, as described below.
- d. If there are no plates with 20–80 colonies, chose the plates with the counts closest to the acceptable range of 20–80 colonies. For example, if all plate counts are greater than 80, chose the plates that have counts closest to 80. Likewise, if all of the plate counts are less than 20, the plates with counts closet to 20 would be used to calculate the number of CFUs per sample.
- e. If no target colonies are observed, record as “None detected”.

To determine the number of CFUs per sample, average the number of colonies on the duplicate filters which produced 20–80 colonies/plate. In this case assume the colony counts were 57 colonies/filter and 63 colonies/filter on the 2 respective filters. Since each filter received 1.0 mL of the suspension, then the average colony count for the filters would then be 60 colonies/mL. Multiply the average colony count per mL by the total suspension volume per sample, as in the following equation:

$$60 \frac{CFU}{mL} \times 5 \frac{mL}{sample} = 300 CFUs \text{ per sample}$$

### 12.2.3 Enrichment in 2X YPEB

Evaluate post-enrichment streaked SBA plates for the presence of *Y. pestis* colonies (Figure 2). If no suspect colonies are observed, broth should be evaluated for the presence of *Y. pestis*. Typical isolates or 2X YPEB with growth must be confirmed using real-time PCR prior to reporting results. Since the sample was enriched, only qualitative (presence/absence) results can be reported.

Media sterility checks should not exhibit growth. Growth should also not be present in 2X YPEB from field blank samples. If growth is observed on plates, colony morphology should be evaluated to determine if contamination is due to the target organism and the potential source of contamination. Depending on the situation, results should be qualified if QC samples are contaminated with *Y. pestis*.

### 12.2.4 Confirmation of Colonies by Real-time PCR

Presence of *Y. pestis* typical colonies on the culture plate indicates the presence of viable *Y. pestis* cells in the sample. A minimum of three typical colonies should be confirmed using real-time PCR and the YC2 real-time PCR assay. Optionally for virulent strains any combination or all three PCR assays can be used. The  $C_T \leq 40$  and the presence of a



logarithmic curve in the real time graph (Figure 3) for the sample indicates a positive result suggesting the presence of *Y. pestis* in the respective sample. Report the results based on the number of confirmed colonies. Negative controls should not yield any measurable  $C_T$  values above the background level. If  $C_T$  values are obtained as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat the analysis.

### **12.3 RV-PCR Analysis**

Calculate an average  $C_T$  from the replicate reactions for  $T_0$  and  $T_{24}$  or  $T_f$  DNA extracts of each sample. Subtract the average  $C_T$  of the  $T_{24}$  or  $T_f$  DNA extract from the average  $C_T$  of the  $T_0$  DNA extract. If there is no  $C_T$  for the  $T_0$  DNA extract (i.e., the  $T_0$  is non-detect), use 45 (total number of PCR cycles used) as the  $C_T$  to calculate the  $\Delta C_T$  for the sample. The change (decrease) in the average  $C_T$  value from  $T_0$  to  $T_{24}$  or  $T_f$  ( $\Delta C_T$ )  $\geq 9$  indicates a positive result suggesting the presence of viable *Y. pestis* cell in the sample. A  $\Delta C_T$  criterion of  $\geq 6$  (an approximate two log difference in DNA concentration) and a corresponding  $T_{24}$  or  $T_f$   $C_T$  of  $\leq 39$ , was set. If an incubation time longer than 24 hours was used for the RV-PCR, instead of  $T_{24}$ , appropriate  $T_f$  (incubation time) should be used (i.e., 36 hour for post-decontamination, field samples with high concentrations of dead *Y. pestis* cells). However, ( $\Delta C_T$ )  $\geq 6$  algorithm should still be used for a positive result. A minimum of two out of three  $T_0$  PCR replicates must result in  $C_T$  values  $\leq 44$  (in a 45-cycle PCR) to calculate the average  $T_0$   $C_T$ . A minimum of two out of three  $T_{24}$  or  $T_f$  PCR replicates must result in  $C_T$  values  $\leq 39$  to calculate the average  $C_T$  for a sample result to be considered positive. Negative controls (NTCs) should not yield any measurable  $C_T$  values above the background level. If  $C_T$  values are obtained as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat analysis. In addition, field blank samples should not yield any measurable  $C_T$  values. If  $C_T$  values are observed as a result of a possible contamination or cross-contamination, a careful interpretation of the  $C_T$  values for the sample DNA extracts and field blanks must be done to determine if the data is considered valid or if the PCR analyses must be repeated.

## **13.0 Method Performance**

To be completed upon protocol verification and/or validation.

## **14.0 Pollution Prevention**

The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be discarded. If there is any possibility of the materials having been contaminated, they must be disposed of according to CDC BSL-3 procedure (in an autoclavable biohazard container).

## 15.0 Waste Management

- 15.1** It is the laboratory's responsibility to comply with all federal, state and local regulations governing waste management, especially the biohazard and hazardous waste rules and land disposal restrictions. Following these regulations protects the air, water and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.
- 15.2** Samples, reference materials and equipment known or suspected to be contaminated with or to contain viable *Y. pestis* must be decontaminated prior to disposal.
- 15.3** Large volume water filtrates should be decontaminated using bleach (10% final concentration) for a minimum of 30 minutes prior to disposing to the sanitary sewer (e.g., pouring down the drain).
- 15.4** For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* (Reference 16.13) and *Less Is Better: Laboratory Chemical Management for Waste Reduction* (Reference 16.14), both authored by the American Chemical Society.

## 16.0 References

- 16.1** Wheelis M. Biological warfare at the 1346 siege of Caffa. *Emerg Infect Dis* [serial online] 2002 Sep. Available from <http://wwwnc.cdc.gov/eid/article/8/9/01-0536>
- 16.2** Pawlowski, D.R., Metzger, D.J., Raslawsky, A., Howlett, A., Siebert, G., Karalus, R.J., Garrett, S., and Whitehouse, C.A. 2011. Entry of *Yersinia pestis* into the Viable but Nonculturable State in a Low-Temperature Tap Water Microcosm. *PLoS One* 6(3) e17585: 1-9.
- 16.3** Torosian, S.D., Regan, P.M., Taylor, M.A., and Margolin, A. 2009. Detection of *Yersinia pestis* over time in seeded bottled water samples by cultivation on heart infusion agar. *Canadian Journal of Microbiology* 55: 1125-1129.
- 16.4** Ayyadurai, S., Houhamdi, L., Lepidi, H., Nappez, C., Raoult, D., and Drancourt, M. 2008. Long-term persistence of virulent *Yersinia pestis* in soil. *Microbiology* 154: 2865-2871.
- 16.5** Rose, L.J., Donlan, R., Banerjee, S.N., and Arduino, M.J. 2003. Survival of *Yersinia pestis* on Environmental Surfaces. *Applied and Environmental Microbiology* 69(4): 2166-2171.
- 16.6** American Society for Microbiology. 2014. *Sentinel Level Clinical Laboratory Guidelines for Suspected Agents of Bioterrorism and Emerging Infectious Diseases – Yersinia pestis*. Washington, DC. <http://www.asm.org/images/PSAB/LRN/Ypestis316.pdf> [Accessed April 19, 2016]
- 16.7** S. Létant, G. Murphy, T. Alfaro, J. Avila, S. Kane, E. Raber, T. Bunt, and S. Shah. 2011. Rapid Viability Polymerase Chain Reaction for detection of Virulent *Bacillus anthracis* from Environmental Samples. *Appl. Env. Microbiol* 77: 6570-6578, 2011.
- 16.8** U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Institutes of Health. 2009. *Biosafety in Microbiological and Biomedical Laboratories* (BMBL), 5th Edition. HHS Publication No. (CDC) 21-1112 <http://www.cdc.gov/biosafety/publications/bmbl5/index.htm> [Accessed April 19, 2016]
- 16.9** American Chemical Society (ACS). 2005. *Reagent Chemicals: Specifications and Procedures*, 10<sup>th</sup> Edition Oxford University Press (USA), New York.

- 16.10** British Drug Houses, Ltd. 1984. *AnalaR Standards for Laboratory Chemicals* 8<sup>th</sup> Edition. BDH Ltd., Poole, Dorset, U.K.
- 16.11** United States Pharmacopeia. 2005. *United States Pharmacopeia and National Formulary 24*. United States Pharmacopeial Convention, Rockville, MD.
- 16.12** Dauphin, L. A., and Bowen, M. D. 2009. A simple method for the rapid removal of *Bacillus anthracis* spores from DNA preparations. *Journal of Microbiological Methods* 76: 212–214.
- 16.13** American Chemical Society (ACS) 1990. *The Waste Management Manual for Laboratory Personnel*. American Chemical Society, Department of Government Relations and Science Policy, Washington, DC.
- 16.14** American Chemical Society (ACS). 2002. *Less Is Better: Laboratory Chemical Management for Waste Reduction*. American Chemical Society Taskforce on RCRA (Resource Conservation and Recovery Act of 1976), Washington, DC. <http://goo.gl/2yIHPr> [Accessed April 19, 2016]

## Appendix A

### Concentration of Water Samples using Ultrafiltration (UF) for the Detection of Bioterrorism Threat (BT) Agents in Potable Water Samples

Large volume water samples (10 L–100 L) may need to be concentrated using ultrafiltration (UF) to detect low levels of biothreat agents prior to sample analysis. UF is an integral component of the sample processing procedures provided in Sections 9 and 10 of the protocol and should not be misconstrued as sample collection.

**Note:** *This protocol should not be misconstrued as a laboratory standard operating procedure (SOP) that addresses all aspects of safety; the laboratory should adhere to their established safety guidelines.*

*At a MINIMUM, these procedures should be performed in a Biological Safety Level (BSL)-3 facility using BSL-3 practices. All sample manipulations should be performed within a Class II (or higher) biological safety cabinet (BSC).*

#### 1.0 Sample Preparation

**Note:** *Water samples should be concentrated as soon as possible after collection and analyses should be initiated immediately, if possible. However, if analyses cannot be accomplished immediately, the concentrated sample may be stored at 2°C–8°C for up to 24 hours.*

##### 1.1 Laboratory Supplies

**1.1.1** Asahi Kasai Rexeed 25S Dialyzers (Dial Medical Supply Cat. No. 25S or equivalent)

**1.1.2** Masterflex® L/S #36 silicon tubing (Cole Parmer® Cat. No. EW-96410-36 or equivalent)

**Note:** *An alternative to the Masterflex® tubing is #36 BioPharm Silicone tubing (Cole Parmer® Cat. No. EW-96420-36).*

**1.1.3** Masterflex® L/S #24 silicon tubing (Cole Parmer® Cat. No. EW-96410-24 or equivalent)

**1.1.4** Masterflex® tubing reducing connectors (Cole Parmer® Cat. No. EW-40610-08 or equivalent)

**1.1.5** 3-prong extension clamp (Cole Parmer® Cat. No. EW-08021-36 or equivalent)

**1.1.6** Ring stand (Fisher Cat. No. 14-670C or equivalent)

**1.1.7** Clamp connector/holder (Cole Parmer® Cat. No. EW-08041-20 or equivalent)

**1.1.8** Nalgene® Analytical Filter Unit, 0.45 µm (Fisher Scientific Cat. No. 09-740-21B or equivalent)

**1.1.9** Heavy duty pinchcock, metal clamp (Cole Parmer® Cat. No. EW-08126-0302 or equivalent)

**1.1.10** GN-6 Metrical® Membrane Filters, 0.45 µm, 47 mm mixed cellulose ester (VWR Cat. No. 28148-926 or equivalent)

**1.1.11** Forceps, sterile, disposable (Cole Palmer® Cat. No. U06443-20 or equivalent)

- 1.1.12** Hose clamps (Cole Parmer®)
  - (a) Large: #10 and #12 white, plastic (Cat. No. EW-06832-10, EW-06832-12 or equivalent)
  - (b) Medium size: #6 and #8 white, plastic (Cat. No. EW-06832-06, EW-06832-08 or equivalent) or 7/32" to 5/8" stainless steel (Cat. No. EW-06403-11 or equivalent)
  - (c) Small: #4 white, plastic (Cat. No. EW-06832-04 or equivalent) or 7/32" to 5/8" stainless steel (Cat. No. EW-06403-11 or equivalent)
- 1.1.13** 1 L heavy duty polypropylene vacuum bottle (Fisher Scientific Cat. No. 06-443A or equivalent)
- 1.1.14** 3 port filling/venting closure cap with tubing (Fisher Scientific Cat. No. 02-923-13Y or equivalent)
- 1.1.15** Barbed reducing Y connector 1/4 × 3/8 (Cole Parmer® Cat. No. EW-30726-33 or equivalent)
- 1.1.16** DIN adapters (filter connectors)
  - (a) Small connectors for 24 tubing (Molded Products Cat. No. MPC-855NS.250PP or equivalent)
  - (b) Large connectors for 36 tubing (Molded Products Cat. No. MPC-855NS.375PP or equivalent)
- 1.1.17** Blood Port Storage Cap (screw cap), polypropylene (Molded Products Cat. No. MPC-40PP or equivalent)
- 1.1.18** Ice bucket and ice
- 1.1.19** Flow regulator (Keck) tubing clamps (Cole Parmer® Cat. No. A-06835-07 or equivalent)
- 1.1.20** Ziplock bags
- 1.1.21** Parafilm
- 1.1.22** Bleach Wipes (Dispatch® Cat. No. 69150 or equivalent)
- 1.1.23** Bottle, sterile, 100 mL
- 1.1.24** Tubes, sterile, 15 mL (Fisher Scientific Cat. No. 339650 or equivalent)
- 1.1.25** Bottle, 1 L sterile polypropylene (Thermo Scientific-Nalgene® Cat. No. 2105-0032 or equivalent)
- 1.1.26** Filter flask, sterile, 500 mL, glass or polypropylene (Fisher Scientific Cat. No. FB-300-500; 10-182-50A; or equivalent)
- 1.1.27** 10 mL plastic pipets, sterile, T.D. bacteriological (Fisher Scientific Cat. No. 13-678-12E or equivalent)
- 1.1.28** 50 mL plastic pipets, sterile, T.D. bacteriological (Fisher Scientific Cat. No. 13-678-14C or equivalent)
- 1.1.29** Pliers
- 1.1.30** Graduated cylinder (1 L) or graduated beaker (1 L)
- 1.1.31** Syringe, 60 mL

- 1.1.32** #7 rubber stopper, 500 mL flask, with hole (Fisher Scientific Cat. No. 14-135L or equivalent)
- 1.1.33** Collapsible containers, 10 L or 20 L (Cole Parmer® Cat. No. EW-06100-30 or EW-06100-40 or equivalent)
- 1.1.34** Vacushield™ Vent Device (HEPA filter) (VWR Cat. No. 55095-006 or equivalent)

## **1.2 Equipment**

- 1.2.1** Masterflex® Console Drive (Cole Parmer® Cat. No. EW-07554-90 or equivalent)
- 1.2.2** Masterflex® EasyLoad II Pump Head (Cole Parmer® Cat. No. SI-77200-52 or equivalent)
- 1.2.3** Jiffy-Jack® apparatus positioner (Cole Parmer® Cat. No. A-08057-40 or equivalent)
- 1.2.4** BD Clay Adams™ Nutator Mixer (VWR Cat. No. 15172-203 or equivalent)
- 1.2.5** Biological safety cabinet (BSC) – Class II or Class III
- 1.2.6** Vortex mixer (Fisher Scientific Cat. No. 02-215-365 or equivalent)

## **1.3 Reagents**

- 1.3.1** 1000X (10%) Sodium Poly-Phosphate (NaPP): Add 10 g of NaPP per 100 mL of sterile reagent-grade water in a sterile 100-mL bottle. Cap the bottle and shake vigorously by hand for 1 minute to mix the solution. If the water is cold (e.g., from refrigerator), let the solution dissolve for 2 hours at room temperature, mixing for 1 minute approximately every 15 minutes. If water is initially at room temperature, the NaPP should dissolve within 30 minutes (mixing vigorously by hand every 15 minutes). If the NaPP is not completely dissolved in the water, place the NaPP solution in a water bath at 50°C and incubate for 2 hours. Continue incubating until the NaPP is completely in solution. Store the 10% NaPP in refrigerator for up to 2 months.
- 1.3.2** 10% Tween 80-1% Antifoam Y-30 solution: Pipet 0.1 mL Antifoam Y-30 Emulsion (Sigma Cat. No. A5758 or equivalent) and 1 mL Tween 80 (Fisher Cat. No. T164 or equivalent) into 15 mL conical tube containing 8.9 mL reagent-grade water. Vortex for 30 seconds to mix. Solution can be stored at room temperature for 1 month.
- 1.3.3** Elution solution (0.01% Tween® 80, 0.01% NaPP and 0.001% Antifoam Y-30): Add 0.5 mL of the 10% Tween 80-1% Antifoam Y-30 solution and 0.5 mL of 10% NaPP to 500 mL of sterile reagent-grade water. Swirl to mix. Solution can be made up to 24 hours in advance and stored in a refrigerator. Bring to room temperature prior to use.

**Note:** *The elution solution should be made in or transferred to a sterile 1 L heavy duty polypropylene bottle with closed cover.*

- 1.3.4** 0.01% NaPP solution (filter wash) – 1 L: Add 1 mL of 10% NaPP solution to 999 mL of reagent-grade water and swirl to mix. Solution can be made up to 24 hours in advance and stored in a refrigerator. Bring to room temperature prior to use.
- 1.3.5** 1% Bleach solution (for tubing decontamination) – 500 mL: Add 5 mL bleach to 495 mL reagent grade or deionized (DI) water and swirl to mix. Solution can be stored at room temperature for 1 week.
- 1.3.6** 70% Ethanol solution (Fisher Scientific Cat. No. 04-355-56 or equivalent)

**1.3.7** Sodium thiosulfate solution: Prepare 10% w/v sodium thiosulfate by adding 100 g sodium thiosulfate (Fisher Scientific Cat. No. S446 or equivalent) to 1 L of sterile reagent-grade water and mix well. Store at 4°C for up to 1 month.

**1.3.8** Dialyzed Fetal Bovine Serum (FBS) (Fisher Scientific Cat. No. SH3007303 or equivalent)

**1.3.9** Phosphate buffered saline (PBS) (Fisher Scientific Cat. No. BP3991 or equivalent)

**1.4 Pre-treat the Rexeed Ultrafilter with Dialyzed 5% FBS to Block Non-specific Protein Binding**

The ultrafilter comes filled with sterile water that must be removed prior to use. Prepare the ultrafilter by removing all caps from top, bottom and side ports. Hold the ultrafilter upright to allow all sterile water to settle on one end. The sterile water can be poured out the side ports into a sink until all visible water is removed.

**1.4.1** To make 150 mL of 5% FBS, add 7.5 mL of FBS to 142.5 mL of reagent-grade water.

**1.4.2** Before adding the FBS to the filter, secure the ultrafilter to ring stand with a 3 prong clamp. Ensure the bottom port is securely closed with a blood port storage screw cap. Place the white port caps that come with the ultrafilter onto both side ports, leaving them loosened to allow air to escape.

**1.4.3** Load the ultrafilter with approximately 120 mL (no more than 150 mL) of 5% FBS by injecting it into the top port of the ultrafilter (a 60 mL syringe with no needle works well for this application). Attach 3" of #24 tubing to a small filter connector, secure with a #4 clamp, screw into the filter port, and insert the tip of the syringe into the #24 tubing. Pour 60 mL of FBS solution into the syringe and use the plunger to push the solution into the ultrafilter. Repeat for remaining volume.

**1.4.4** When the ultrafilter is loaded, close the open end of the ultrafilter with a second blood port storage screw cap and ensure side ports are closed with white port caps (they will "click" into place).

**1.4.5** Place the ultrafilter on a rocker panel at room temperature; rock for 30 minutes.

**Note:** *Do not store the filter after pretreating, as this will encourage growth of contaminating bacteria which may clog the filter.*

**1.5 Pre-treat the Water Sample**

**Note:** *Any procedure in which sample containers are opened should be performed inside a BSC.*

**1.5.1** If sodium thiosulfate was not added to the sample at the time of collection, add 0.5 mL/L of a 10% w/v solution of sodium thiosulfate immediately upon receipt of the sample.

**1.5.2** Pretreat the water sample with NaPP to reach a final concentration of 0.01%. To achieve a 0.01% concentration of NaPP, add 1 mL of 10% NaPP per 1 L of water sample.

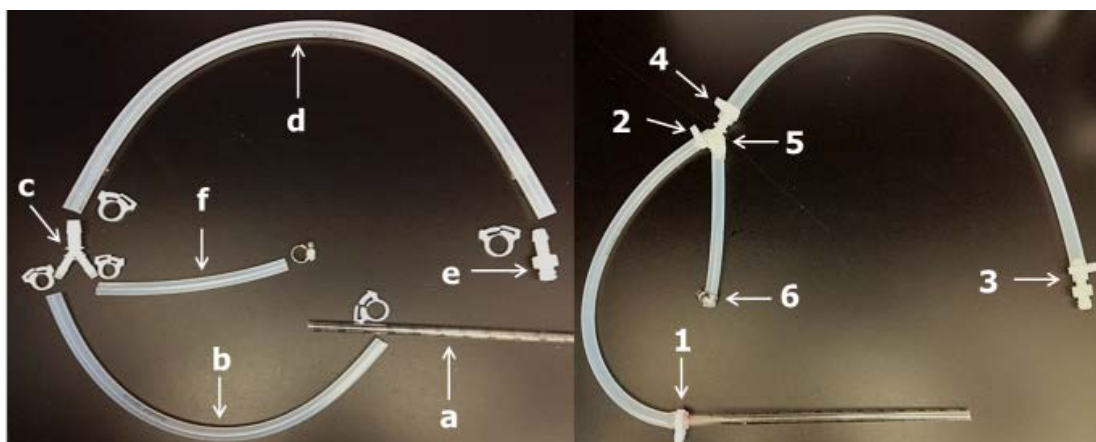
**1.6** Prepare the empty filtrate container(s) by adding a sufficient volume of bleach such that the final concentration is at least 1% bleach (e.g., 200 mL bleach in a 20 L container).

**1.7 Assembly of the Sample Tubing Set**

**1.7.1** Remove both ends (tip and end containing cotton material) of a 10 mL pipet by carefully breaking off the ends while the pipet is within its plastic wrapping (Figure 1, a). Keep the plastic sleeve to place the sample pipet in during changing of sample containers.



- 1.7.2** Connect the 10 mL pipet to 14" of #24 Masterflex® (MF) tubing (Figure 1, b) and secure with a medium hose clamp (#8 plastic) (Figure 1, #1).
- 1.7.3** Connect the #24 tubing to one of the small 1/4" barbs on the Y connector (Figure 1, c) and secure with a small hose clamp (#4 plastic or 7/32" to 5/8" stainless steel) (Figure 1, #2).
- 1.7.4** Connect 21" of #36 tubing (Figure 1, d) to the end of the large filter connector (Figure 1, e) and secure connection with large hose clamp (#10 plastic) (Figure 1, #3).
- 1.7.5** Connect the other end of the #36 tubing to the large 3/8" barb on the Y connector (Figure 1, c) and secure with a large hose clamp (#8 plastic) (Figure 1, #4).
- 1.7.6** Connect 6" of #24 tubing (Figure 1, f) to the open small 1/4" barb on the Y connector (Figure 1, c) and secure with a small hose clamp (#4 plastic or 7/32" to 5/8" stainless steel) (Figure 1, #5). Connect the opposite end to the empty port on the 1 L vented cap bottle and secure with a stainless steel clamp (vented cap bottle not shown in Figure 1, #6; see Figure 7a, #10).



**Figure 1. Sample tubing set assembly.**

## **1.8 Assembly of the Retentate Return Tubing Set**

- 1.8.1** Connect 15" of #24 tubing (Figure 2, a) to the small filter connector (Figure 2, b); secure connection with a small hose clamp (#4 plastic or 7/32" to 5/8" stainless steel) (Figure 2, c).
- 1.8.2** Attach a flow regulator tubing clamp (Figure 2, d) to the #24 tubing so that the wide end is facing the 1 L vented cap bottle (Figure 2, e).
- 1.8.3** Connect the opposite end of the #24 tubing to the port on the 1 L vented cap bottle that is attached to the shorter internal tubing (Figure 2, f; Figure 7a, #9); secure with a stainless steel clamp (Figure 2, g).



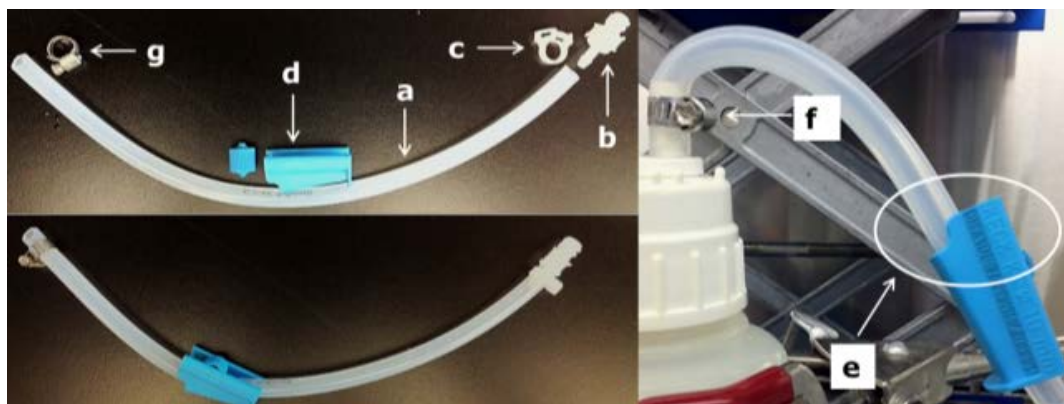


Figure 2. Retentate return tubing set assembly.

### 1.9 Assembly of the Filtrate Tubing Set

- 1.9.1 Remove both ends (tip and end containing cotton material) of a 10 mL pipet by carefully breaking off the ends while the pipet is within its plastic wrapping (Figure 3, a).
- 1.9.2 Connect the 10 mL pipet to 16" of #36 MF tubing and secure with a medium hose clamp (#8 plastic) (Figure 3, b).

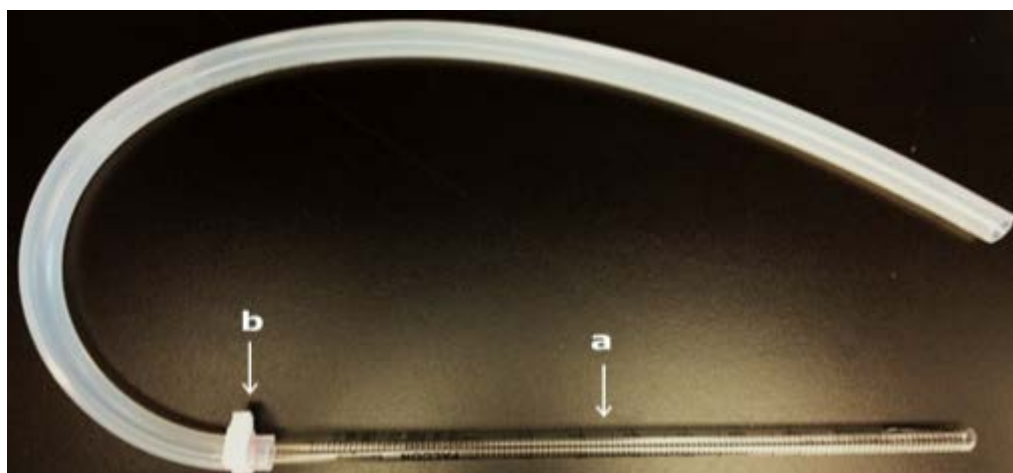


Figure 3. Filtrate tubing set assembly.

### 1.10 Connection of the Pumping Station

**Note:** *The pumping station and ultrafilter setup may be assembled inside of a BSC or on the bench top (bench top allows ease of assembly, but it is recommended that this protocol not be performed on the bench). Additionally, absorbent underpads/diapers may be placed under the UF setup and containers to capture any potential leaks or spills that may occur during sample processing or disassembly.*

- 1.10.1 Raise the pump to a height equal to or above the top of the reservoirs by placing the pump on a shelf or raising it on a variable height platform such as the Jiffy-Jack® apparatus positioner (Section 1.2.3). Also, secure the filter and the 1 L vented cap bottle in a vertical position by using adjustable metal clamps and a ring stand.

- 1.10.2** Connect the sample reservoir to the ultrafilter using the sample tubing set. Attach the large filter connector on the end of the sample tubing set to the top port of the ultrafilter. Feed the #36 tubing through the pump head, and ensure the tubing remains securely clamped to the large filter connector.
- 1.10.3** Attach the small filter connector on the end of the retentate return tubing set to the bottom port of the ultrafilter.
- 1.10.4** Connect the ultrafilter to the filtrate reservoir by connecting the filtrate return tubing set to the top side port of the ultrafilter (may secure with #12 plastic clamp, but not required) and placing the 10 mL pipet into the filtrate reservoir. Ensure the other side port is closed with the plastic cap provided with the filter.
- 1.10.5** The assembled UF setup should appear as shown in Figures 7a and 7b.
- 1.10.6** Place a 1 L beaker next to the UF setup and another 1 L beaker next to the filtrate setup. Use beakers to hold the sample and filtrate tubing pipets when changing sample and filtrate containers to prevent potential water droplets from dripping onto the BSC.
- 1.10.7** Verify that the MF tubing is threaded through the pump head correctly (Figure 4).
- 1.10.8** Set the pump to ~50% power. If using a digital pump, the flow rate should be set to 1450 mL/min.
- 1.10.9** If the pump has a flow direction toggle switch, confirm the flow direction is set to the right (Figure 4).



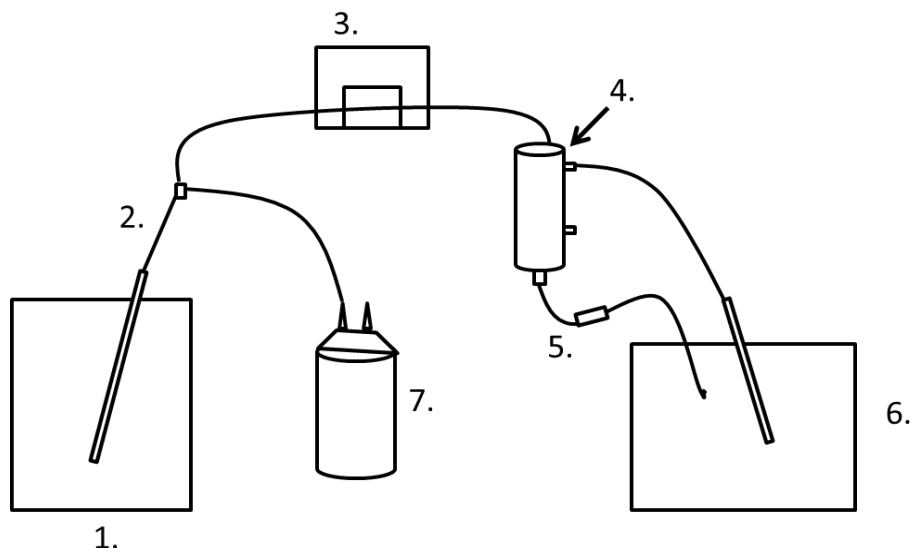
**Figure 4. Photo of the pump head.**

## **1.11 Washing the Ultrafilter**

- 1.11.1** Wash the 5% FBS from the ultrafilter by placing the sample tubing end (Figure 5, #2) into the 1 L bottle containing 0.01% NaPP filter wash prepared in Section 1.3.4 (Figure 5, #1).
- 1.11.2** Detach the retentate return tubing set (Figure 5, #5) from the 1 L vented cap bottle (Figure 5, #7) and place the end in the filtrate reservoir (Figure 5, #6) so that the retentate return tubing set and the filtrate tubing are both in the filtrate container.
- 1.11.3** Apply the pinchcock to the tubing from port C (clamp the tubing from vented cap bottle to the Y connector, getting as close to the Y connector as possible) (Figure 7a, #1).
- 1.11.4** Start the pump (Figure 5, #3) and flush the 0.01% NaPP filter wash through the lines and the ultrafilter (Figure 5, #4).

**Note:** While flushing with the NaPP filter wash, be sure to check the tubing and connections for any leaks or drips throughout the system.

**1.11.5** When the filter wash is completed, reset the tubing as shown in Figure 7a.



**Figure 5. Schematic of Filter Wash**

(1) Sterile water wash, (2) Sample tubing, (3) Pump, (4) Ultrafilter,  
(5) Retentate tubing set, (6) Filtrate reservoir, (7) 1 L vented cap bottle

## 2.0 Primary Water Sample Concentration

**2.1** With the sample container in place and the tubing set per Figure 7a, check to make sure the pinchcock is applied to the tubing from port C (Figures 7a and 7c, #1), the vented cap is securely tightened to the bottle, and the rubber cap is removed from the vented cap.

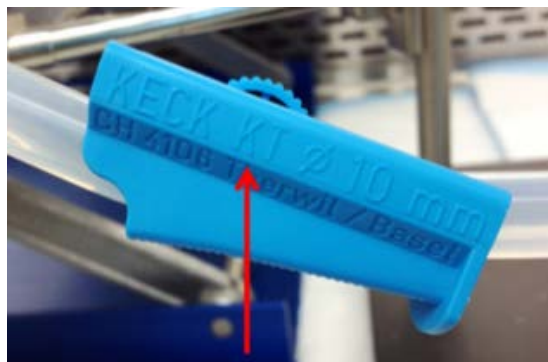
**Note:** When applying the pinchcock clamp to the tubing, make sure it is clamped as close to the Y connector as possible to completely block and/or stop the flow of water through tubing (Figure 7c, #1).

**2.2** Start the pump with the flow switch turned to the right and the pump speed set to the maximum (~ 2900 mL/min flow rate).

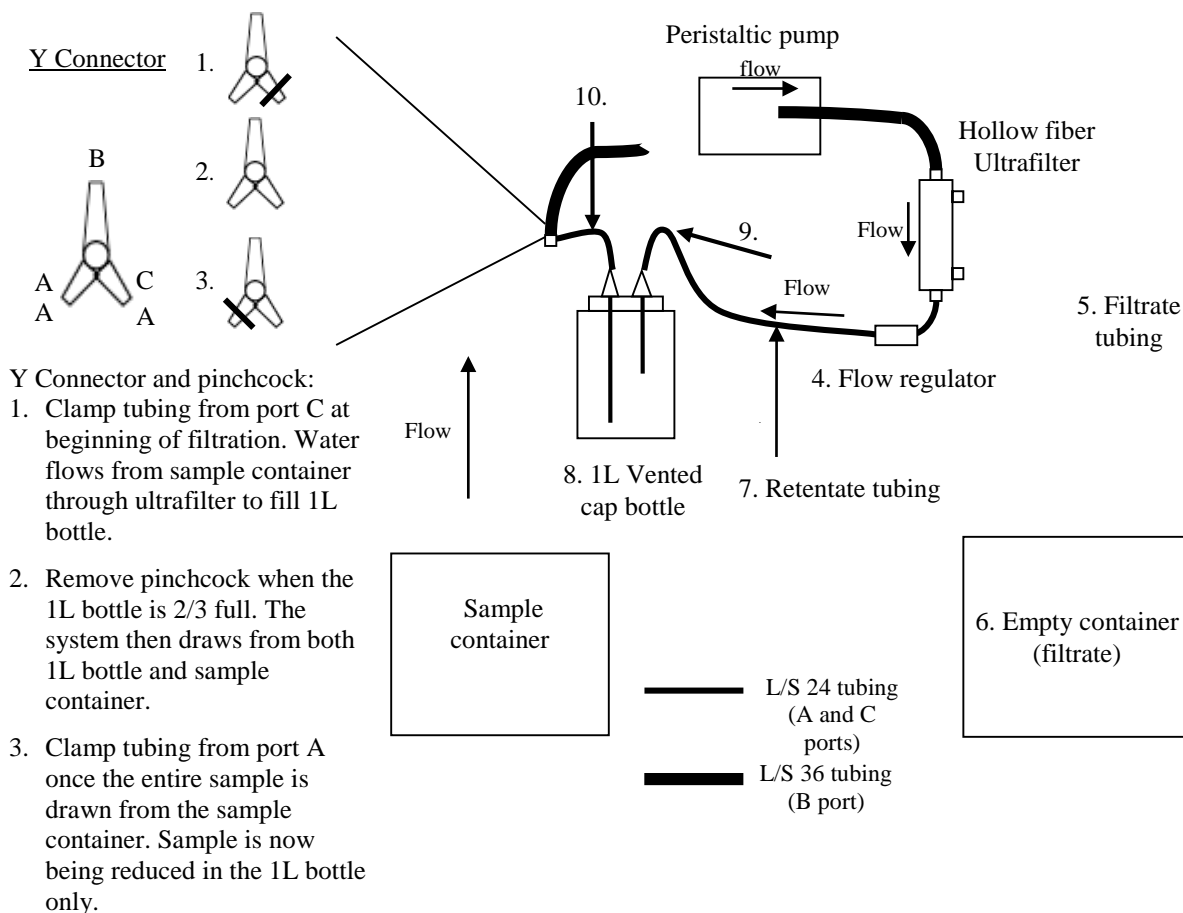
**2.3** Once the 1 L retentate bottle is ~2/3 of the way full, quickly close the open port of the vented cap with the rubber cap and remove the pinchcock from the tubing (Figures 7a and 7c, #2). The pump will now be drawing water from both the sample container and the 1 L vented cap (retentate) bottle. Make sure the water level in the 1 L retentate bottle does not continue to rise. If the water level in the 1 L bottle does rise, remove the rubber cap and apply pinchcock to the tubing from port A (Figures 7a and 7c, #3). Once the water level in the 1 L bottle is 2/3 of the way full, close port with rubber cap and remove the pinchcock (Figures 7a and 7c, #2).

- 2.4** Tighten the flow regulator by rolling the knob to the right until the “K” in the “KT” lettering on the front face of the flow regulator is directly in the middle of the adjustment knob (Figure 6). The back pressure provided by the flow regulator should produce a filtrate rate between 1000 – 1400 mL/min.

**Note:** *The flow rate does not need to be measured.*



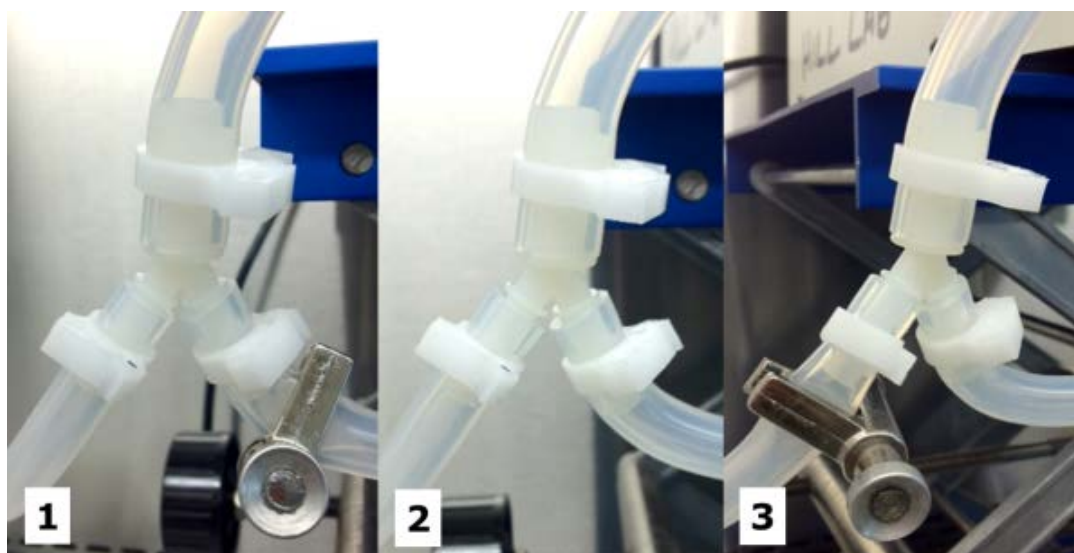
**Figure 6.** Position flow regulator rolling clamp over “K” in “KT”.



**Figure 7a.** Recirculating ultrafiltration assembly.



**Figure 7b. Photograph of the ultrafiltration system set up inside a BSC.**



**Figure 7c. Pinchcock positions.**

- 2.5** When the sample container is empty, apply the pinchcock to clamp the tubing from port A (Figures 7a and 7c, #3), loosen the flow regulator and turn off the pump.

**Note:** *Whenever the pump needs to be stopped, apply the pinchcock to clamp the tubing from port A so that the water containing microbes cannot flow back into the sample tank, and then quickly press the stop button.*

- 2.6** If this is the only sample container, skip to step 2.13.



- 2.7** Remove the sample tubing set from the empty sample container and carefully place it in a sterile 1 L beaker. Remove the filtrate pipet in the same manner and place it in a second sterile 1 L beaker.

**Note:** *To prevent drips, ensure the pipet is free of any remaining sample while still inside the sample container. Labs may consider placing the pipet back into the plastic sleeve prior to placing it into the 1 L beaker. Alternatively, if sample container is disposable, the tubing may be cut and the pipet can be disposed of inside the sample container, which should then go into an autoclavable biohazard bag.*

- 2.8** Remove the empty sample container and replace with the next sample container to be filtered. Also replace the full filtrate container with an empty filtrate container (containing 1% bleach as directed in Section 1.3.5).

**Note:** *Follow Biosafety in Microbiological and Biomedical Laboratories (BMBL) and lab-specific safety practices for BSL-3 working conditions while slowly moving containers into and out of the BSC. Let airflow re-establish for a minimum of 15 minutes each time the sash is lifted.*

- 2.9** Place the sample tubing set into the new sample container and the filtrate tubing set into the new, empty filtrate container.

- 2.10** Turn the pump back on and remove the pinchcock from the tubing from port A (Figures 7a and 7c, #2) so that the water is once again being drawn from both the container and the 1 L bottle.

- 2.11** Tighten the flow regulator to the same position described in Section 2.4 and continue filtration.

- 2.12** Repeat steps 2.7 – 2.11 for the rest of the containers.

- 2.13** When the last container has been emptied, apply the pinchcock to clamp the tubing from port A (Figures 7a and 7c, #3) so that the sample is only being re-circulated in the 1 L bottle, loosen the flow regulator, and remove the rubber cap from the 1 L bottle.

- 2.14** When the sample in the 1 L bottle draws down to about 1.5” from the bottom, move the pinchcock to clamp the tubing from port C (Figures 7a and 7c, #1), so that only air is being drawn into the filtration system by the pump.

**Note:** *Continue flushing until all the retentate is out of the tubing and the filter. The retentate tubing may be lifted two to three times to help flush the water that has settled in the retentate tubing into the 1 L bottle.*

- 2.15** Turn off the pump when all of the retentate has been flushed from the tubing and the filter. The final volume should be approximately 200 mL – 250 mL.

- 2.16** Carefully unscrew the steel clamps from the ports on the vented cap bottle and remove the tubing connections from the top ports (containing the concentrated sample). Place them onto the ports of the second vented cap bottle containing the elution solution and secure the clamps (remove the closed cap from the elution solution bottle first).

- 2.17** Unscrew the vented cap from the first bottle containing the sample. Lift and securely hold the cap with one hand (tubing remains inside the bottle) while using a 25 mL or 50 mL pipet in the other hand to measure and transfer the sample retentate into a sterile, 1 L plastic bottle. Record the retentate volume.

- 2.18** Secure the second vented cap bottle containing the elution solution with the 3-pronged clamp from the first vented cap bottle. Check that the flow regulator is fully opened, the pinchcock is clamped to the tubing from port A (Figures 7a and 7c, #3) and the rubber cap is removed from the 1 L vented cap bottle. The assembly should now be ready for elution of the ultrafilter.

- 2.19** Turn on the pump, allowing the eluent volume to gradually reduce until the level draws down to 1.5" from the bottom of the bottle. Move the pinchcock to clamp the tubing from port C (Figure 7a and 7c, #1) to flush the rest of the eluent from the tubing and filter. Turn off the pump.
- 2.20** Unscrew the vented cap assembly from the second bottle containing the concentrated eluent. Lift and securely hold the cap with one hand (tubing remains inside the bottle) while using a 25 mL or 50 mL pipet in the other hand to measure and transfer the eluent to the 1 L plastic sample bottle containing the retentate. The total volume of the final UF concentrate should be 400 mL – 500 mL.

### 3.0 Secondary Water Concentration

**Note:** *Water samples ranging from  $\geq 50$  mL to  $<10$  L (Section 9.4) or 10 mL to  $<10$  L (Section 10.4) should be concentrated using membrane filtration as described below, prior to analyses.*

- 3.1** Inside of a BSC, assemble the membrane filtration setup shown in Figure 8, ensuring the HEPA filter (Vacushield™) is attached with #24 tubing (or alternative vacuum tubing) to the house vacuum and the side arm of the backup flask. Connect the side arm of the main flask to the stopper of the backup flask with vacuum tubing. This tubing length should be long enough such that the tubing rests on the surface of the BSC to help stabilize the flasks. Attach an additional piece of vacuum tubing to the side arm of the main flask for connection to the disposable filter units.

- 3.1.1** Use the piece of #24 tubing from the side arm of the main filter flask to attach the quick disconnect side arm on the filter unit (Figure 8, #5) and use sterile forceps to place a new filter onto the base of each filter unit (Figure 8, #3).

**Note:** *Disposable filter units (Section 1.1.8) come with cellulose nitrate membrane filters, which must be replaced with the mixed ester cellulose filters (Section 1.1.10). DO NOT remove the cellulose support pad on the base of the unit when replacing membrane filters. It is recommended to keep a small autoclave bag inside the BSC during processing for safe disposal of filter funnels.*

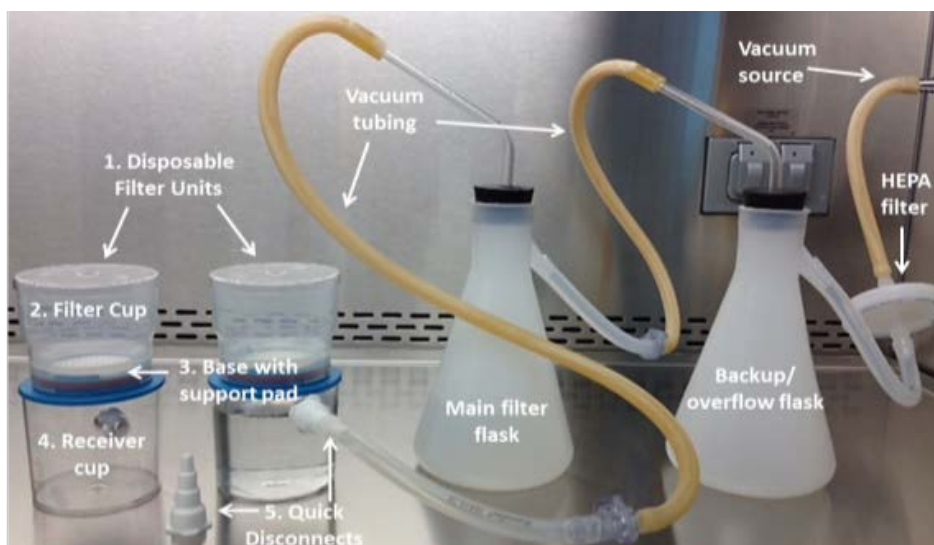


Figure 8. Membrane filtration setup.



- 3.2** Run a negative control before filtering any sample concentrate. Add 20 mL PBS to the filter cup (Figure 8, #2) and turn on the vacuum. Once the PBS has finished filtering, turn off the vacuum and remove the cup from the base of the filter unit (Figure 8, #3). Retrieve the membrane filter with sterile forceps and place onto a CIN plate. Incubate at 25°C–28°C for a maximum of 5 days. The use of SBA for the negative control may be a more stringent control compared to CIN, but it may be susceptible to contamination by non-target organisms; growth on CIN would be indicative of *Yersinia*.

**Note:** *If it is anticipated that there is a high concentration of Y. pestis and other background organisms in the sample, it may be necessary to filter aliquots of the retentate. In this case, split the retentate into aliquots (2.0, 20, and 2 × 200 mL) and filter each aliquot as described below. A single filter unit may be used to filter all of the aliquots from the same sample as long as the aliquots are filtered in the order of smallest to largest volume (e.g., 2.0 mL → 20 mL → 200 mL).*

- 3.3** Use sterile forceps to place a new 0.45 µm filter onto the support pad on the base of either a new filter unit or the one used for the filtration blank. Reattach the filter cup to the base.
- 3.4** Add the retentate slowly to the filter unit (Figure 8, #2) and turn on the vacuum (not all the retentate will fit at once). Continue to add retentate to the filter unit, taking care to avoid clogging the filter. Once the sample has finished filtering, rinse the filter cup 3 times with PBS in a squirt bottle. Turn off the vacuum; remove the filter cup from the bottom portion (Figure 8, #3) of the filter unit and set it on a sterile surface.

**Note:** *If the final retentate is particularly cloudy or has noticeable sediment, it might be necessary to split the retentate volume between two or more filter units.*

- 3.5** Using sterile disposable forceps, grab the edge of the membrane at the filter unit base and fold it toward the other end. While holding the 2 edges together, take the forceps and place the folded membrane into the bottom half of a 50 mL conical tube, avoiding the conical portion. Close the tube.
- 3.6** Repeat steps 3.1 – 3.5 for the remaining samples/aliquots.

**Note:** *If multiple filtrations were run due to excess sediment, process all of the membrane filters.*

- 3.7** For polymerase chain reaction (PCR) analysis, proceed to Section 9.4 of the protocol.
- 3.8** For culture analysis, proceed to Section 10.4 of the protocol.

## **4.0 Decontamination and Disposal and/or Reuse**

- 4.1** Filtrates should be collected in a container containing sufficient bleach such that the final concentration is at least 1% (e.g., 200 mL bleach in a 20-L container). After 30-minute contact time, the treated filtrate can be poured down the drain.

### **4.2 Ultrafiltration Supplies**

- 4.2.1** Disposable sample containers (e.g., Cubitainers™ [Section 1.1.33]) can be flattened by slowly compressing the container while inside BSC. Secure the cap, decontaminate the surface and slowly remove from the BSC and place in an autoclavable biohazard waste container. Reusable containers should be capped, surface-decontaminated and removed from BSC for autoclaving.

**4.2.2** Prior to disconnecting the ultrafiltration assembly, flush 500 mL of 1% bleach solution through the entire assembly so that all tubing is in contact with the bleach.

4.2.2.1 Place sample pipet in 500 mL of 1% bleach solution and adjust pump speed to 50% (~1450 mL/minute).

4.2.2.2 Ensure rubber stopper is off the vented cap, tubing from port C is clamped with pinchcock (Fig. 7c, #1) and turn on pump.

4.2.2.3 Pump the bleach until approximately 100 mL remains in the 500 mL bleach bottle (tubing should be filled with solution, 1-L bottle should be ~1/2 to 2/3 full).

4.2.2.4 Move pinchcock to clamp the tubing from port A (Fig. 7c, #3) and turn off the pump.

4.2.2.5 Ensure a minimum contact time of 30 minutes. Once this is completed, flush all of the bleach solution out of the system so that it is collected in the 1-L vented cap bottle. Then place the tubing assembly except the pinchcock and stainless steel clamps and ultrafilter, into an autoclavable biohazard container.

**4.2.3** The vented cap bottle assemblies (including inner tubing and rubber caps), 1-L bottles and 1-L beakers should be autoclaved for a minimum of 30 minutes at 121°C at 15 psi. After sterilization, bottles should be washed and re-autoclaved prior to reuse.

**4.2.4** Wipe down the pinchcock with 10% bleach followed by 70% ethanol before removing from BSC.

**4.2.5** Soak steel clamps in a beaker of 0.1N NaOH solution for 15 minutes, followed by soaking in 10% bleach (made fresh) for 15 minutes and then rinse thoroughly with deionized (DI) water prior to reuse.

#### **4.3 Secondary Concentration**

**4.3.1** Filter units and Centricons should be autoclaved and disposed of in an autoclavable biohazard bag.

**4.3.2** Filter flasks/stopper set-ups for membrane filtration should be autoclaved and reused.

#### **4.4 Decontamination of Surfaces and/or Spills**

Surfaces and spills should be decontaminated by the following three-step process:

- Wet and wipe down with 0.1 N NaOH and let sit for 15 minutes.
- Wet and wipe down with 10% bleach and let sit for 15 minutes.
- Wipe with 70% ethanol

**Note:** *It is recommended to have these solutions readily available in squeeze bottles during processing for immediate surface decontamination.*

## **5.0 Limitations**

- 5.1** If the procedure is not performed correctly, it may result in false negative results.
- 5.2** Water from certain sources may contain higher levels of minerals, organic compounds or other substances which may affect the water concentration procedure and subsequent testing procedures for the detection of potential BT agents.
- 5.3** The presence of chlorine-based disinfectants in the water supply will inhibit or prevent the growth of most microorganisms. Sodium thiosulfate should be added as soon as possible to inactivate chlorine in the water. Addition of sodium thiosulfate at the time of collection of the water sample is recommended.
- 5.4** Even with the addition of sodium thiosulfate, some organisms may not survive the filtration process and may only be detected by real-time PCR.

## **6.0 Acronyms**

BMBL	Biosafety in Microbiological and Biomedical Laboratories
BSC	Biological safety cabinet
BSL	Biological safety level
BT	Bioterrorism threat
DI	Deionized
FBS	Fetal bovine serum
MF	Masterflex®
NaPP	Sodium poly-phosphate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SOP	Standard operating procedure
UF	Ultrafiltration

## **7.0 Reference**

- 7.1** U.S. Environmental Protection Agency and U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. 2011. Comparison of Ultrafiltration Techniques for Recovering Biothreat Agents in Water. EPA 600/R-11/103  
[http://cfpub.epa.gov/si/si\\_public\\_record\\_report.cfm?address=nhsr/&dirEntryId=238310](http://cfpub.epa.gov/si/si_public_record_report.cfm?address=nhsr/&dirEntryId=238310)



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