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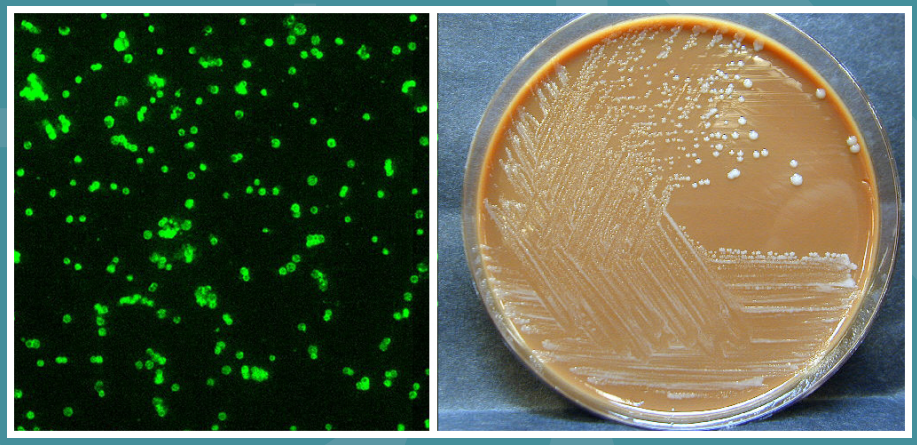
Protocol for Detection of *Francisella tularensis* in Environmental Samples During the Remediation Phase of a Tularemia Incident



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Protocol for Detection of *Francisella tularensis* in Environmental Samples During the Remediation Phase of a Tularemia Incident

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

Section 10 Figure 2: Left – *Francisella tularensis* colonies on CHOC agar (Source: Public Health Image Library); Right – *Francisella tularensis* colonies on CHAB agar (Source: Public Health Image Library)

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Acronyms

ABI	Applied Biosystems® Incorporated
BD	Becton, Dickinson and Company
BHI	brain heart infusion
BMBL	Biosafety in Microbiological and Biomedical Laboratories
BSC	Biological safety cabinet
BSL	Biosafety level
BVFH	Brain Heart Infusion/Vitox/Fildes/Histidine
°C	Degree(s) Centigrade
CDC	Centers for Disease Control and Prevention
CESER	Center for Environmental Solutions and Emergency Response
CFR	Code of Federal Regulations
CFU	Colony forming unit(s)
CHAB	Cystine heart agar with rabbit blood and antibiotics
CHOC	Chocolate agar
C_T	Threshold Cycle
C_T (T_0) or T_0 C_T	C_T value at time zero (pre-incubation)
C_T (T_f)	C_T value at time final (post-incubation)
C_T (T_{30}) or T_{30} C_T	C_T value after 30 hours incubation
ΔC_T	Delta threshold cycle
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
F. tularensis	*Francisella tularensis*
FAM	6-carboxyfluorescein
FBI	Federal Bureau of Investigation
FEM	Forum on Environmental Measurement
g	gram(s)
h	hour(s)
HSMMD	Homeland Security and Materials Management Division
HSRP	Homeland Security Research Program
ICLN	Integrated Consortium of Laboratory Networks
IEC	International Electrotechnical Commission

ISO	International Organization for Standardization
LRN	Laboratory Response Network
mg	milligram(s)
min	minute(s)
µL	microliter(s)
mL	milliliter(s)
mM	millimolar
NAD	Nicotinamide adenine dinucleotide (growth factor)
NG	No growth
NIST	National Institute of Standards and Technology
NTC	No template control
OSHA	Occupational Safety and Health Administration
ORD	Office of Research and Development
PBS	Phosphate buffered saline
PBST	PBS with 0.025% Tween 20
PC	Positive control
PCR	Polymerase chain reaction
pdpD	Pathogenicity determinant protein D
PMP	paramagnetic particles
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
rpm	Revolutions per minute
RV-PCR	Rapid viability–polymerase chain reaction
SDS	Safety data sheet
sec	second(s)
SWGFACT	Scientific Working Group on Forensic Analysis of Chemical Terrorism
T_0	Time 0, prior to incubation
T_{30}	Time after 30 h of incubation
Taq	*Thermus aquaticus* bacterium
T_f	Time final, after incubation
TE	Tris(hydroxymethyl)aminomethane-Hydrochloride-EDTA
TNTC	Too numerous to count
TSB	Trypticase™ soy broth
UNG	Uracil-N-glycosilase
UV	Ultraviolet
VBNC	Viable but non-culturable
WLA	Water Laboratory Alliance
1X	1-fold concentrated (no concentration)
2X	2-fold concentrated
6X	6-fold concentrated
10X	10-fold concentrated

Trademarked Products

Trademark	Holder	Location
Acrovent™	Pall Corporation	Ann Arbor, MI
AeraSeal™	Excel Scientific	Victorville, CA
Amicon®	EMD Millipore®	Billerica, MA
AmpErase®	ThermoFisher Scientific	Waltham, MA
AmpliTaq Gold®	Life Technologies	Carlsbad, CA
Bacto™	BD Biosciences	Franklin Lakes, NJ
BD Clay Adams™ Nutator Mixer	BD Diagnostics	Sparks, MD
Biopur® Safe-Lock®	Eppendorf	United States
Applied BioSystems®	Life Technologies™	Carlsbad, CA
BBL™	BD Diagnostics	Sparks, MD
Black Hole Quencher®	Biosearch Technologies, Inc.	Novato, CA
Costar®	Corning	Tewksbury, MA
Cole Parmer®	Cole Parmer®	Vernon Hills, IL
Dispatch®	Clorox Company	United States
Difco™	Becton, Dickinson & Co.	Franklin Lakes, NJ
Durapore®	Millipore Corporation	Billerica, MA
DynaMag™	Life Technologies	Carlsbad, CA
Epicentre®	Epicentre Technologies Corp.	Madison, WI
Fluoropore™	Merck KGaA	Darmstadt, Germany
Invitrogen®	Life Technologies™	Carlsbad, CA
IsoVitaleX™	BD Biosciences	Franklin Lakes, NJ
Kendall™	Covidien, Inc.	Mansfield, MA
Life Technologies™	Life Technologies™	Carlsbad, CA
Masterflex®	Cole Parmer®	Vernon Hills, IL
MagNA Pure®	Roche Diagnostics	Indianapolis, IN
MagneSil® Blood Genomic	Promega	Madison, WI
MaxQ™	Thermo Scientific Inc	Lenexa, KS
MicroFunnel™	Pall Corporation	Ann Arbor, MI
Microcon®	EMD Millipore	Billerica, MA
Millipore®	Merck KGaA	Darmstadt, Germany
Nalgene®	Nalge Nunc Corporation	Rochester, NY
Primer Express™	Applied Biosystems LLC.	San Francisco, CA
Remel™	Remel Inc.	Lenexa, KS
Stomacher®	Seward	United Kingdom
TaqMan®	Life Technologies™	Carlsbad, CA
Trypticase™ soy agar	BD Diagnostics	Sparks, MD
Trypticase™ soy broth	BD Diagnostics	Sparks, MD
Tween®	Sigma-Aldrich	St. Louis, MO
Ultracel®	EMD Millipore®	Billerica, MA

Trademark	Holder	Location
Ultrafree®	EMD Millipore	Billerica, MA
Versalon™	Covidien, Inc.	Mansfield, MA
Ziploc™	S.C. Johnson	Racine, WI

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Introduction

The core mission of the U.S. Environmental Protection Agency (EPA) is to protect human health and the environment. After the 2001 terrorist attacks and the anthrax bioterrorism incidents that resulted in human casualties as well as public and private facility closures, this core mission was expanded to address critical homeland security related needs. Specifically, EPA was designated as the primary federal agency responsible for the protection of indoor/outdoor structures and water infrastructure vulnerable to chemical, biological, and radiological terrorist attacks. EPA is working to meet the specific homeland security roles and responsibilities that are laid out in a series of statutes, presidential directives and national frameworks. To enhance and expand the EPA's preparedness for response to an intentional or unintentional incident or disaster that results in chemical, biological or radiological contamination, the Homeland Security Research Program (HSRP) within the EPA Office of Research and Development (ORD) conducts state-of-the art research and provides science and technology needed to effectively recover from such incidents.

Francisella tularensis (*F. tularensis*) is the bacterium that causes tularemia infection in humans and animals. Due to its high virulence and low infective dose (Reference 16.1), and its potential use as a biological weapon (References 16.1-16.3), *F. tularensis* is considered a high priority biothreat agent. Based on the realities of the 2001 anthrax bioterrorism incident, *F. tularensis* is also considered a high-priority bioterrorism agent for which response preparedness is extremely important. Once a tularemia incident has occurred, regardless of its origin as a natural, accidental or an intentional release, the response activities would need to include environmental assessment of contamination, decontamination, and clearance. Therefore, combining the homeland security and natural tularemia outbreak related responsibilities, EPA needs to have detailed analytical methods for detection of *F. tularensis* in environmental matrices, including water. To address this critical need, EPA-ORD's HSRP has generated this protocol for detection of *F. tularensis* in environmental samples for use by the EPA's Environmental Response Laboratory Network (ERLN) and Water Laboratory Alliance (WLA).

F. tularensis is a zoonotic pathogen with an extremely wide host range that includes mammals, birds and amphibians. The primary hosts for *F. tularensis* are believed to be small rodents (e.g., hares, voles, muskrats). Humans usually acquire infection by direct contact with infected animals or by animal associated biting arthropods. Ticks, mosquitoes and biting flies have all been implicated as capable vectors. Although less common, contaminated soil, water, infected carcasses, and aerosolized particles have been implicated as sources of infection (Reference16.2). *F. tularensis* type A strain has been isolated from natural waters and mud contaminated by muskrats and beavers, and the organisms may be capable of multiplication in these environments. *F. tularensis* type B strain has also been isolated from surface waters, including drinking water supplies. This pathogen could remain viable for more than 100 days in water and enter a viable but non-culturable (VBNC) state (Reference 16.4). In a weaponized form, *F. tularensis* can persist in the environment for a long time and the aerosolized form can cause wide-spread outbreak (Reference 16.2).

To complement an effective sample collection strategy during a suspected *F. tularensis* 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 the following analytical approaches for the detection of *F. tularensis* in various environmental samples:

1. Real-time polymerase chain reaction (PCR) to detect the presence of the DNA of *F. tularensis*.
2. Traditional microbiological culture to detect the presence of viable *F. tularensis*.
3. Rapid Viability-PCR (RV-PCR) method to detect the presence of viable *F. tularensis* in water samples (16.5).

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 *F. tularensis*. It should be noted that the Laboratory Response Network [LRN] laboratories providing support to EPA for environmental sample analyses may use LRN protocols.

Sample processing procedures are also provided for respective analytical methods for all environmental sample types including aerosol, particulate (surface swabs, wipes, and Sponge-Sticks™) and drinking water. 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 and updated as improved sample processing procedures and real-time PCR assays become available.

For drinking water samples, large volume samples may need to be analyzed after concentration to detect low concentrations of *F. tularensis*. For post- decontamination phase sample analysis using the culture method, selected isolated colonies need to be analyzed using real-time PCR to confirm the identity of *F. tularensis*, as opposed to traditional biochemical and serological testing.

CAUTION: At the time of publication, this protocol has not been validated. During any *F. tularensis* 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.*[5] The responsible federal agency may also refer to the *Validation Guidelines for Laboratories Performing*

Forensic Analysis of Chemical Terrorism[6] 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.

[5] U.S. Department of Homeland Security, Integrated Consortium of Laboratory Networks (ICLN), *ICLN Guidelines for Comparison of Validation Levels between Networks*, Original Version, https://web-icln.s3-fips-us-gov-west-1.amazonaws.com/default/assets/File/ICLN-Validation%20Levels%20Between%20Networks_003_01(1).pdf

[6] 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:

https://www.epa.gov/sites/production/files/2016-11/documents/emergency_response_validity_policy_reaffirmed_nov2016.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.[1] 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: https://www.epa.gov/emergency-response/environmental-response-laboratory-network; project-specific requirements also are included in individual Analytical Service Requests.

[1]Information regarding EPA's DQO process, considerations and planning is available at: https://www.epa.gov/quality/guidance-data-quality-objectives-process-epa-qag-4-august-2000

Protocol for Detection of *Francisella tularensis* in Environmental Samples During the Remediation Phase of a Tularemia Incident

1.0 Scope and Application

The purpose of this protocol is to provide multiple procedures and methods that can be used to detect *Francisella tularensis* (*F. tularensis*) in environmental samples. To detect the presence of the DNA of *F. tularensis,* this protocol includes a real-time polymerase chain reaction (PCR) based method. Since the PCR methods cannot determine viability of *F. tularensis*, this protocol includes 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 samples (16.5). During an actual incident validated assays from other sources (e.g., Defense Biological Products Assurance Office of the Department of Defense or Laboratory Response Network [LRN]) may be used.

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 *F. tularensis*.

2.0 Summary of Methods

2.1 Sample Analysis for Detection of *F. tularensis* 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® Incorporated (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 both the PCR assays included in Section 6.9.

2.2 Sample Analyses for Detection of Viable *F. tularensis*:

After samples have been appropriately processed, samples are plated on chocolate agar (CHOC) with IsoVitaleX™ and cysteine heart agar with rabbit blood and antibiotics (CHAB) (Reference 16.6) or inoculating into nutrient rich broth (e.g. Enrichment Culture and RV-PCR procedures). Both of these methods include specific real-time PCR assay-based confirmation of the identity of *F. tularensis* in the sample.

2.2.1 Culture Procedure

The culture option includes sample processing and plating serial dilutions of the processed sample and membrane filters on CHAB medium followed by rapid confirmation of morphologically typical isolated colonies using *F. tularensis* specific real-time PCR. Unless advised otherwise, real-time PCR based analysis should be performed using a Type strain-specific or both the PCR assays included in Section 6.9, 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 ΔC_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** (in Section 11.1) 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 both the PCR assays included in Section 6.9.

3.0 Interferences and Contamination

3.1 Poor recoveries of *F. tularensis* 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 also inhibit PCR reactions. Water samples suspected of containing iron or rust particles should be placed on a magnetic rack 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 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 the Centers for Disease Control and Prevention (CDC) and the Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition, (Reference 16.7), 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 *F. tularensis* infection. Due to the infectious nature of this organism, all samples should be handled and analyzed using biosafety requirements as dictated by BMBL (Reference 16.7) or by the most recent version and/or organizational health and safety plans. The CDC requires BSL-3 (biosafety level-3) handling of this organism.

4.2 Additional Recommended Safety Precautions

4.2.1 Disposable materials (e.g., pipets, loops) are recommended 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 *F. tularensis*

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 (https://www.osha.gov/laws-regs/regulations/standardnumber/1910/1910.120AppB)). In addition to OSHA guidance, CDC developed recommendations for PPE based on biosafety level (BSL) (Reference 16.7, 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 into a biohazard autoclave 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, 5th Edition, CDC 2009 (Reference 16.7) 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 Ziploc® 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 biohazard bag(s) 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 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. UFC510096 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 Edge seals for 96-well PCR plates (Adhesive Plate Sealers, 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 (If a medium is to be prepared in a laboratory)

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/work station

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); multi-tube adapter (Scientific Industries, Inc. Cat. No. SI-V525 or equivalent) optional

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 35ºC − 37ºC

5.5.14 Autoclave or steam sterilizer, capable of achieving 121°C (15 psi) for 30 minutes

5.5.15 Cold block (4ºC) 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)

5.5.20 DynaMag™ magnetic stand (ThermoFisher Scientific Cat. No. 12321D or equivalent)

5.5.21 MagNA Pure® Compact instrument (Roche Diagnostics)

6.0 Reagents and Standards

6.1 Reagent-grade chemicals must be used in all for all analysis/assays. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (Reference 16.8). 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 16.9); and *United States Pharmacopeia and National Formulary 24*, United States Pharmacopeial Convention, Md. (Reference 16.10).

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 0.025% Tween® 20 (PBST), pH 7.4 [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 10X phosphate buffered saline (PBS), pH 7.4, (Teknova Cat. No. P0196 or equivalent)

6.7 TE buffer (1X Tris [10 mM] -HCl-EDTA [1 mM Ethylenediaminetetraacetic acid]) buffer, pH 8.0 (Fisher Scientific Cat. No. BP2473-500 or equivalent)

6.8 TaqMan® 2X Universal PCR Master Mix (Life Technologies, Cat. No. 4304437)

6.9 PCR Assays

Ft1 (For *F. tularensis* Type A strain) targeting pdpD, pathogenicity determinant protein D, hypothetical protein of *F. tularensis* (Reference 16.11)

- Forward Primer (Ft1-F) – 5'- GAGACATCAATTAAAAGAAGCAATACCTT -3'
- Reverse Primer (Ft1-R) – 5'- CCAAGAGTACTATTTCCGGTTGGT -3'
- Probe (Ft1-Pr) – 5'-6FAM- AAAATTCTGCTCAGCAGGATTTTGATTTGGTT -BH Q1-3'

Ft2 (For *F. tularensis* Type B strain) targeting a junction between IS*Ftu2* and a flanking 3′ region of *F. tularensis* (Reference 16.11)

- Forward Primer (Ft2-F) – 5'- CTTGTACTTTTATTTGGCTACTGAGAAACT -3'
- Reverse Primer (Ft2-R) – 5'- CTTGCTTGGTTTGTAAATATAGTGGAA -3'
- Probe (Ft2-Pr) – 5'-6FAM- ACCTAGTTCAACCTCAAGACTTTTAGTAATGGGAATGTCA -BHQ1-3'

6.9.1 Preparation of concentrated 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 µM (0.1 nmoles/µL) and 40 µM (0.04 nmoles/µL) solutions of primers and probes, respectively. These primary (concentrated) stocks will be used to prepare working stock solutions that will then be used to prepare PCR assay mixes (Section 9.7) on the day of use. Example 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
REV Primer	35	350	0.1	100
Probe	17	425	0.04	40

FWD, forward; REV, reverse

Table 2. Example Working Stock Preparation

Concentrated Stock (µL)		PCR grade water (µL)	Dilution	Concentration	
				nmoles/µL	µM
FWD Primer	20	180	0.1	0.01	10
REV Primer	20	180	0.1	0.01	10
Probe	20	180	0.1	0.004	4

FWD, forward; REV, reverse

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

6.10 Positive Control (PC) –DNA isolated from an appropriate virulent *F. tularensis* strain containing all of the plasmids. For culture analyses, *F. tularensis* LVS strain (BSL-2 organism) or other

avirulent strains may be used as a PC to meet the laboratory's BSL.

6.11 Chocolate agar (CHOC) with IsoVitaleX, a selective *F. tularensis* mediumThe use of commercially prepared plates is recommended (BD Cat. No. 254060 or equivalent). The formulation of the medium is only provided to allow laboratories to ensure that their medium is equivalent to the medium included in the protocol.

6.11.2 Medium Composition:

Hemoglobin	10 g
Pancreatic Digest of Casein	7.5 g
Selected Meat Peptone	7.5 g
Sodium Chloride	5 g
Dipotassium Phosphate	4 g
Cornstarch	1 g
Monopotassium Phosphate	1 g
Agar	12 g
Hemoglobin	10 g
IsoVitaleX Enrichment	12 mL
Pyridoxal	0.01 g
Growth Factors	0.5 g
Reagent-grade Water	1000 mL

6.11.3 BD IsoVitaleX

Glucose	100 g
Cysteine Hydrochloride	25.9 g
L-Glutamine	10 g
L-Cystine	1.1 g
Adenine	1 g
NAD (nicotinamide adenine)	
dinucleotide (growth factor)	0.25 g
Thiamine Pyrophosphate	0.1 g
Guanine Hydrochloride	0.03 g
Ferric Nitrate	0.02g
P-Aminobenzoic Acid	0.013 g
Vitamin B-12	0.01 g
Thiamine Hydrochloride	0.003 g
Reagent-grade Water	1000 mL

6.12 Cystine Heart Agar with Rabbit Blood (CHAB), a selective *F. tularensis* medium

6.12.1. The use of commercially prepared media is recommended (Thermo Scientific Cat. No. R01346 or equivalent). The formulation of the medium is only provided to allow laboratories to ensure that their medium is equivalent to the medium included in the protocol.

6.12.2. Medium Composition:

Casein Peptone	13 g
Dextrose	10 g
Sodium chloride	5 g
Yeast Extract	5 g
Beef Heart Infusion	2 g
L-Cystine	1 g
Penicillin	100,000 U
Polymyxin B	100,000 U
Rabbit Blood	5 %
Agar	15 g
Reagent-grade water	1000 mL

6.13 Tryptic Soy broth with IsoVitaleX

6.13.1. The use of commercially prepared dehydrated medium (Fisher Cat. No. DF0370-17-3 or equivalent) and IsoVitaleX (Fisher Cat. No. B11875 or equivalent) is recommended. If commercially prepared medium is not available, prepare medium using procedures in 6.13.2–6.13.4.

6.13.2. Medium Composition

Tryptone H	15 g
Soytone	5 g
Sodium chloride	5 g
IsoVitaleX	20 mL
Reagent-grade water	1000 mL

6.13.3. Add reagents except IsoVitaleX to 950 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Warm slightly and stir to dissolve the powder completely. Adjust pH to 7.3 ± 0.2 with 1.0 N HCl or 1.0 N NaOH and bring to 1000 mL with reagent-grade water. Autoclave at 121°C (15 psi) for 15 minutes. Do not overheat. Cool to room temperature.

6.13.4. Aseptically add 20 mL of IsoVitaleX to the cooled medium and swirl to mix. Store at 4°C in screw cap bottles for a maximum of three months.

6.14 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)

6.15 MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Cat. No. 03730 964001)

6.16 MagNA Pure LC Total Nucleic Acid Isolation Kit-Additional Lysis/Binding Buffer (Roche Cat. No. 03246779001)

6.17 6X BVFH - Brain Heart Infusion (BHI) Broth with 20% (v/v) Vitox Supplement (final 2% Vitox in 1X), 60% (v/v) Fildes (final 10% Fildes in 1X), and 1% (w/v) L-histidine (BVFH)

- Weigh 22.2 g BactoTM Brain Heart Infusion Broth Base powder into 500 mL flask or bottle.
- Weigh 0.6 g L-histidine into the same 500 mL flask or bottle.
- Add 28 mL MilliQ® H_2O or equivalent.
- Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
- Allow broth to cool down to room temperature under a BSC before storing at 4°C.
- Check pH and adjust to 7.2 ± 0.2.
- For addition of Oxoid® Vitox Supplement, reconstitute Vitox supplement in buffer provided by vendor and add 12 mL of Vitox supplement to give a concentration of 12% in the sterilized 6X BHI+histidine broth (2% when diluted to 1X).
- For addition of Remel™ Fildes Enrichment supplement, add 60 mL Fildes to give a concentration of 60% in the sterilized 6X BHI+histidine+Vitox broth (10% when diluted to 1X).
- Test samples of the 6X BVFH product for sterility by incubating a 0.6 mL aliquot into 5.4 mL buffer in a 50-mL conical tube and incubating at 37°C for 3 days. Confirm sterility before use in the RV-PCR assay.

6.18 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.

6.19 100% Ethanol (200-proof) for preparation of 70% ethanol by dilution with PCR-grade water.

7.0 Calibration and Standardization

7.1 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.

7.2 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.

7.3 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.

7.4 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.

7.5 Calibrate pH meter following manufacturer's instructions 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.

7.6 Verify balance calibration every two months with reference weights (e.g., ASTM Class 2).

7.7 Micropipettors should be calibrated at least annually and tested for accuracy on a weekly basis.

7.8 Follow manufacturer instructions for calibration of real-time PCR instruments.

7.9 Re-certify BSCs annually. Re-certification must be performed by a qualified technician.

7.10 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.

7.11 Refrigerated centrifuges should be checked to confirm temperature and revolutions per minute (rpm) on a quarterly basis. Record the data in a log book.

7.12 Vacuum pressure (e.g., pumps, in house system) should be checked on a regular basis to ensure that the pressure is 5–10 psi. Higher or lower vacuum pressure could negatively impact recoveries.

7.13 Sample integrity — Samples should be checked for loss of 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 – data are flagged and marked as exceeding temperature), so that a decision can be made regarding whether the data should be considered valid/invalid.

7.14 Analyst qualifications — Only those analysts who have been trained and have demonstrated proficiency with these analytical techniques should perform this procedure.

7.15 Proficiency testing (PT) — The laboratory should have analysts analyze test 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.

8.0 Quality Control (QC)

8.1 Quality assurance and quality control are closely related. However, this section will describe them separately to highlight QC considerations specific to this protocol.

8.2 Media sterility check — The laboratory should test media sterility by incubating a single unit (tube or Petri dish) from each batch of medium (CHOC with IsoVitaleX and CHAB) at 35ºC − 37ºC for 24 ± 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.

8.3 Culture: Positive control (PC) — The laboratory should analyze PCs (known quantity of cells) to ensure that all media and reagents are performing properly. *F. tularensis* LVS 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.

8.4 PCR: Positive control (PC) — DNA isolated from an appropriate virulent *F. tularensis* strain containing all the plasmids 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 *F. tularensis* total DNA per 5 µL of PCR-grade water. All PCs should result in a cycle threshold (C_T) ≤ 40 and replicates should be within ± 1 C_T of each other.

8.5 External inhibition control (EIC, also referred to as sample matrix control) of 50 pg genomic DNA from *F. tularensis* — 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 *F. tularensis* DNA per 1 µL of PCR-grade water. Using a 10 µL pipettor, carefully add 1 µL of the DNA to the EIC wells on a PCR plate and then add 5 µL of sample DNA extract to each well and mix thoroughly. The PCR results from the PC and EICs (both containing 50 pg of *F. tularensis* 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.***

8.6 No template control (NTC, also referred to as reagent blank) — The laboratory should analyze NTCs (5 µL 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.

8.7 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 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***

8.8 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. Refer to **Table 3** for appropriate PNC.

8.9 For RV-PCR based analysis, the T_0 and T_{30} or T_f 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_0 and T_{30} or T_f extracts.

Table 3. Sample Processing Negative Controls (PNCs)

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 *F. tularensis* in samples based simply on the presence of the target 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 *F. tularensis*.

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 (e.g., gloves, lab coat). The CDC requires BSL-3 handling of this organism. All wastes should be handled according to the CDC and the BMBL handling 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.

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 a biohazard autoclave 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 a bag containing a sample into the Stomacher® (Section 5.5.19) so the Sponge-Stick sponge/wipe rests evenly between the homogenizer paddles and stomach 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 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 elution suspension for each sample as described above.

9.1.11 Place the 50 mL tubes from step 9.1.9 into sealing centrifuge buckets and decontaminate the outside of the centrifuge buckets before removing them from the BSC.

9.1.12 Centrifuge tubes at maximum speed (~3200 × 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 vortex mixed for two minutes in 10 second bursts, if possible use a vortexer with a multi-tube adapter to reduce processing time for multiple samples.

9.1.16 Remove 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 × 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 the tube for 1 minute in 10 second bursts.

9.1.20 Repeat steps 9.1.1 through 9.1.19 for each sponge-stick or wipe 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 the CDC and the 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 3 mL of sterile PBS for larger swabs 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 minimum 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 9.2.1 through 9.2.4 for each swab sample.

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 the CDC and the 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 2 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 using sterile forceps into a biohazard autoclave bag.

9.3.4. Repeat steps 9.3.1 through 9.3.3 for each air filter 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)

It is anticipated that the large volume water sample has undergone primary and secondary concentration and the resultant concentrated sample has been filtered through a membrane filter. Therefore, this sub-section describes the procedure for processing of such a membrane filter received in a 50 mL screw cap tube or other appropriate container.

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 the CDC and the 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 filter and vortex at the highest setting for 2 minutes with 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 9.4.1 through 9.4.3 for each membrane 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)

Note: All subsequent procedures involving manipulation of water samples 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 the CDC and the BMBL waste management and disposal requirements.

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 × g, with the brake off, for 15 minutes at 4°C.

9.5.4 Remove approximately 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 Repeat steps 9.5.1 through 9.5.4 for each water sample.

9.5.6 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 (~100 mg) of the 106 µm glass beads and two level capfuls (~100 mg) of the 425–600 µm 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 6.18) or bleach wipes (Section 5.1.3). Store the remaining suspension at 4ºC. 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 3 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 (4ºC) for 2 minutes (or until cool to touch). If tubes leak during bead-beating, wipe tubes and bead-beater thoroughly with a 10% pH amended bleach solution (Section 6.18) 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 0.22 µm Ultrafree®-MC filter units (Section 5.1.29; Millipore® Cat. No. UFC30GV0S), two Amicon Ultra filter inserts, and six Amicon Ultra collection tubes (Section 5.1.28; Millipore® Cat. No. UFC510096) with sample ID for each bead-beating tube (Section 9.6.4); and one 0.1 µm Ultrafree®-MC centrifugal filter device (Section 5.1.30; Millipore® Cat. No. UFC30VV00).

Note: It may not be necessary to label all the collection tubes as long as the Amicon Ultra filter insert is clearly labeled.

- In a BSC, centrifuge the bead-beating tubes (Section 9.6.4) at 7000 rpm for 2 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 0.22 µm yellow top filter units (Section 5.1.29; Millipore® 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 3 minutes at 4ºC.

Note: Ensure that the supernatant has been filtered. Centrifuge for an additional 2 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 the insert and forceps in an autoclavable biohazard bag. Transfer 0.5 mL of the filtrate from the collection tube to Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC510096). 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® Cat. No. UFC50VL96). Cap the filter units.
- Centrifuge (Section 5.5.6; Eppendorf 5415R/5424R) at 7000 rpm for 2 minutes at 4ºC.
- Open the filter units. Remove the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC510096) with disposable forceps (gripping only the sides) and transfer to new collection tubes (Section 5.1.28; Millipore® 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 the second yellow top filter units to the corresponding Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC510096). Do not transfer any particulate matter that may be evident at bottom of tubes. Cap the filter units.
- Centrifuge at 7000 rpm for 3 minutes at 4ºC.
- Open the filter units. Remove the Amicon® Ultra filter inserts using disposable forceps (gripping only the sides) and transfer to new collection tubes (Section 5.1.28; Millipore® 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 6.7) to the filter.
- Centrifuge at 7000 rpm for 2 minutes at 4ºC.
- Open the filter units. Carefully remove the retentate from the top of the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC510096), 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. UFC510096) 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. UFC510096) 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 6.7) to the Amicon® Ultra filters. Cap the filter units.
- Centrifuge at 7000 rpm for 3 minutes at 4ºC.
- Open the filter units. Transfer the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC510096) 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 6.7) to the Amicon® Ultra filters. Cap the filter units.
- Centrifuge at 7000 rpm for 3 minutes at 4ºC.
- Open the filter units. Transfer the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC510096) 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 4ºC.
- Check fluid level in the Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC510096). 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. UFC510096) 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 *F. tularensis* 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 0.22 µm Amicon® Ultra filter inserts (Section 5.1.28; Millipore® Cat. No. UFC510096) 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. UFC510096) with collection tubes (Section 5.1.28; Millipore® Cat. No. UFC50VL96) into a biohazard autoclave bag.
- Repeat the above step for all the samples/retentates.
- Place the 0.1 µm Ultrafree®-MC filter devices (Section 5.1.30; Millipore® Cat. No. UFC30VV00) into a centrifuge (Section 5.4.6; Eppendorf 5415R/5424R).
- Centrifuge at 8000 × g (approximately, 9200 rpm) for 2 minutes at 4ºC (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) and the forceps in an autoclavable biohazard bag. Place the collection tubes in a cold block (4ºC).

- Carefully wipe the outside of the collection tubes containing sample DNA extract with a 10% pH amended bleach solution (Section 6.18) or bleach wipes (Section 5.1.3).
- Using clean gloves, place the cold block (4ºC) 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

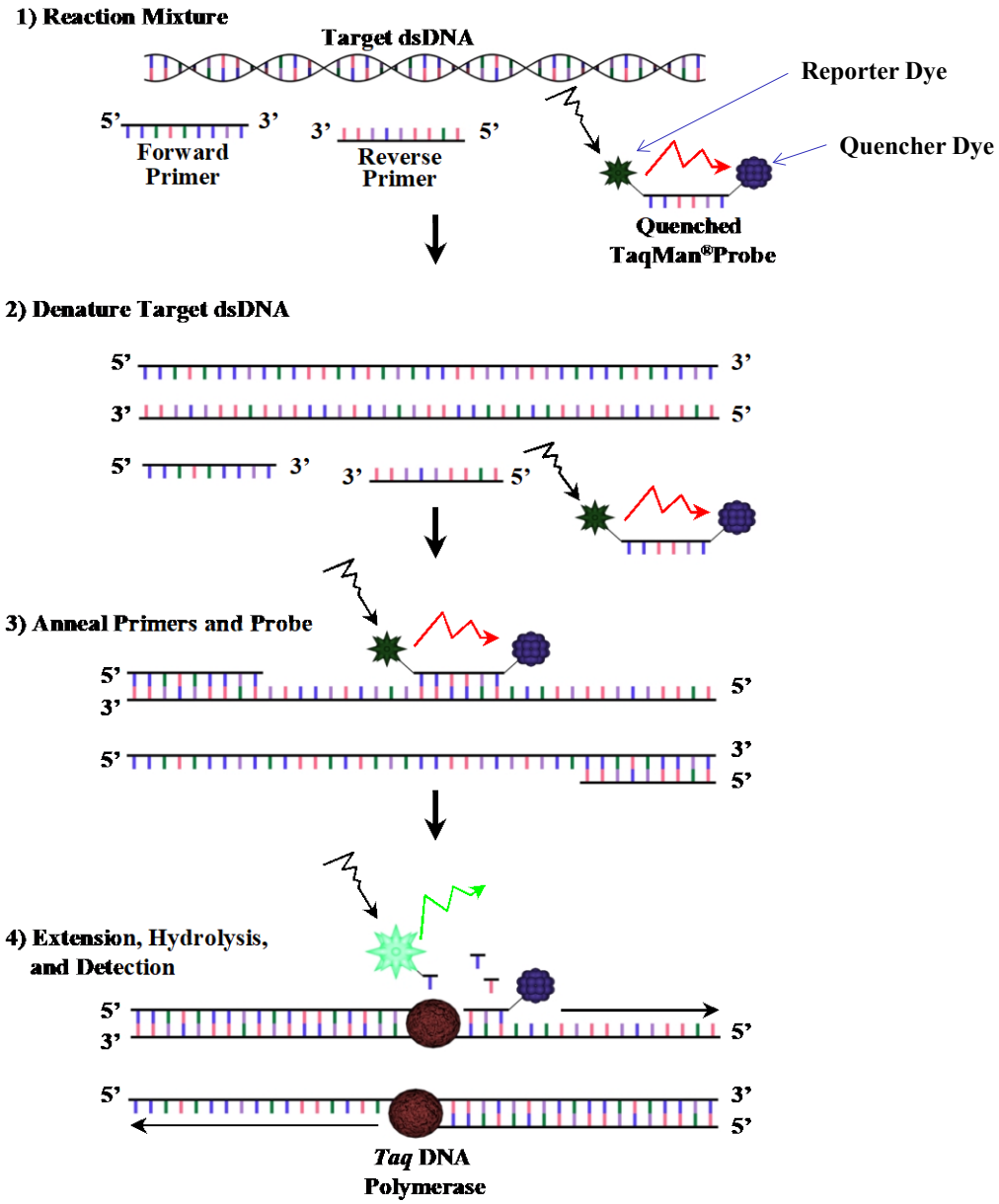


Figure 1. Real-time PCR Amplification.

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 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**). 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, two single-plex PCR assays are included. The Ft1 and Ft2 PCR assays target the pathogenicity determinant protein D (pdpD), hypothetical protein and a junction between ISFtu2 and a flanking 3′ region, respectively.

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 6.18) 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 8.6), PC (Section 8.3) and three replicates of the PNC (Section 8.8) per run. In addition, include three reactions for each sample including field blanks (Section 8.7) and two reactions for the EIC (Section 8.5) 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 *F. tularensis* Single-plex PCR Assay Master Mix Preparation for 70 Reactions

Reagent	Volume per reaction (µL)	Total Volume (µL)	Final Concentration
TaqMan® 2X Universal Master Mix	12.5	875	1X
Platinum® *Taq* DNA Polymerase	0.25 (1.25 Units)	17.5	1.25 Units
Forward primer, 10 µM	0.5	35	0.20 µM
Reverse primer, 10 µM	0.5	35	0.20 µM
Probe, 4 µM	0.4	28	0.064 µM
PCR-grade water	5.85	409.5	N/A
Total Volume	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 (*F. tularensis* 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 4ºC, 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 thermal cycling conditions on the ABI 7500 Fast are provided in **Table 5**. Fluorescence is automatically measured at the end of the 60ºC annealing-extension combined step.

Table 5. PCR Thermal Cycling Conditions[a, b]

Steps	UNG[c] Incubation	AmpliTaq Gold Activation	PCR, 45 Cycles[d]	
	Hold	Hold	Denaturation	Annealing/Extension
Temperature	50ºC	95ºC	95ºC	60ºC
Time	2 minutes	10 minutes	5 seconds	20 seconds[e]

[a] Run Mode: Fast 7500
[b] Reaction volume 25 µL
[c] Uracil-DNA glycosylase
[d] Fast Ramp: 3.5°C/s up and 3.5°C/s down
[e] 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 µL 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 µL 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 µL of the PC (*F. tularensis* DNA [10 pg/µL]) 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 µL of the PNC extract to the three PNC wells.

9.7.16 Add 5 µL of each sample DNA extract to the respective sample wells and EIC wells.

9.7.17 Add 1 µL of the PC (*F. tularensis* DNA [50 pg/µL]) 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 clear Edge Seal for good contact. Change gloves.

9.7.19 Centrifuge sealed PCR plate for 1 minute at 2000 rpm and 4ºC, 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 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 clean-up procedures

- Dispose of all biological materials in biohazard autoclave 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 6.18) or bleach wipes (Section 5.1.3), followed by 70% isopropyl and a deionized 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.

Media sterility checks (Section 8.2) and positive controls (Section 8.4) 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 the CDC and the 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.
- 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 into a biohazard autoclave 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 a bag containing a 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 sponge/wipe from the outside of the bag with your gloved 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 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. 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 maximum speed (~3200 × 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 approximately 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 vortex mixed for two minutes in 10 second bursts using a multi-tube vortexer.***

- 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 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 tube 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) through (b) for each sample.

10.1.3 Culture Cell Suspensions on CHOC with IsoVitaleX and CHAB

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 CHOC with IsoVitaleX plate and a pre-dried CHAB plate (labeled 10^{-1}).

b. After vortexing tubes, pipet 0.1 mL of 10^{-1} suspension onto surface of pre-dried CHOC with IsoVitaleX plate and a pre-dried CHAB plate (labeled 10^{-2}).

c. After vortexing tubes, pipet 0.1 mL of 10^{-2} suspension onto surface of pre-dried CHOC with IsoVitaleX plate and a pre-dried CHAB 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. 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 Colonies

Invert the CHOC with IsoVitaleX and CHAB plates and incubate them at 35ºC − 37ºC for a maximum of 7 days. Plates should be examined at 24-hour intervals for a maximum of 7 days, if necessary.

- CHOC with IsoVitaleX Plates

a. *F. tularensis* produces gray-white, opaque colonies, usually too small to be seen as individual colonies at 24 hours. After incubation for 48 hours or more, colonies are approximately 1-2 mm in diameter, white to gray to bluish-gray, opaque, flat, with an entire edge, smooth and have a shiny surface (**Figure 2**).

b. Count the number of *F. tularensis* colonies on each plate and record results.

- CHAB Plates

a. *F. tularensis* produces smooth, entire edge, greenish-white, and butyrous (buttery) with opalescent sheen colonies at 48 to 72 hours. Colonies are usually 2 to 4 mm after incubation for 48 to 72 hours (Figure 2).

b. Count the number of *F. tularensis* colonies on each plate and record results.

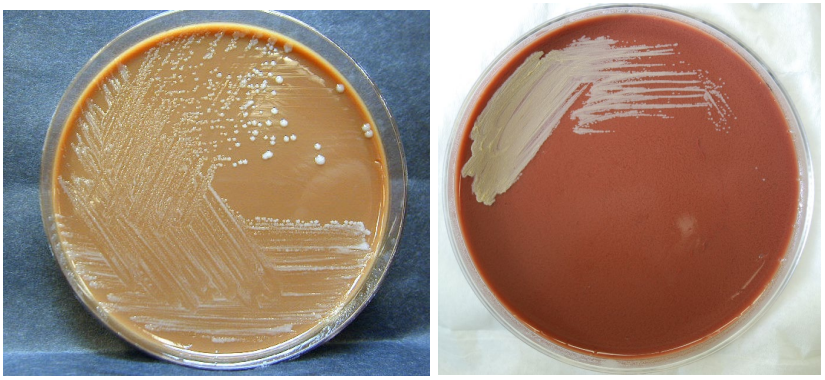


Figure 2. *Francisella tularensis* colonies on CHOC (left) and CHAB (right) agar after 48 hours.
(Source: Public Health Image Library, Centers for Disease Control and Prevention)

- Plate Counts

  a. If the number of colonies is ≤ 250/plate, record 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 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 10.1.1) 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 cup in an autoclavable biohazard bag. Remove the membranes with sterile forceps and place them grid-side up on labeled CHOC with IsoVitaleX and CHAB 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.

  Invert and incubate CHOC with IsoVitaleX and CHAB plates at 35ºC − 37ºC for a maximum of 7 days. Plates should be examined daily for growth. Count the number of colonies and record results.

- Plate Counts

  a. If the number of colonies is ≤ 80/plate, record 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 colony forming units (CFUs) per sample, as described in Section 12.2.2.

  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 trypticase™ soy broth (TSB) with IsoVitaleX.***

However, if a problem of background microorganisms overgrowth on a filter is anticipated, the enrichment culture would be required.

10.1.6 Enrich in TSB with IsoVitaleX

- Add the remainder of the undiluted suspension to the specimen cup containing the corresponding sponge/wipe. Add 40 mL TSB with IsoVitaleX to the tube. Repeat for each sample. Incubate the specimen cups at 35ºC − 37ºC. *F. tularensis* grows slowly; the inoculated broth should be incubated and observed for at least 10 days.
- Observe the TSB with IsoVitaleX Enrichment
  - **a.** If broth is not cloudy, record as no growth (NG) and incubate for an additional 24 hours.
  - **b.** If broth is cloudy, record as positive growth (G+) and proceed to next step.
  - **c.** Cap the cup tightly and mix TSB with IsoVitaleX with growth for 30 seconds. Use a sterile loop to remove a loopful of broth with a 10 µL loop and streak on a CHAB plate for isolation. Repeat two times for a total of three CHAB isolation plates.
  - **d.** Incubate the CHAB isolation plates and TSB with IsoVitaleX with growth for a maximum of 10 days at 35ºC − 37ºC.
  - **e.** Examine plates for *F. tularensis*-suspect colonies. If any colonies are isolated, proceed to PCR confirmation (Section 10.5).
  - **f.** If no suspect colonies are observed, perform PCR on TSB with IsoVitaleX 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 the CDC and the 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 2 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 TSB with IsoVitaleX and set aside.
- Repeat steps described above for each swab sample.

10.2.2 Serially Dilute the Cell 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 tube 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) through (b) for each sample.

10.2.3 Culture Cell Suspensions on CHOC with IsoVitaleX and CHAB

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 CHOC with IsoVitaleX plate and a pre-dried CHAB plate (labeled 10^{-1}).
- **b.** After vortexing tubes, pipet 0.1 mL of 10^{-1} suspension onto surface of pre-dried CHOC with IsoVitaleX plate and a pre-dried CHAB plate (labeled 10^{-2}).
- **c.** After vortexing tubes, pipet 0.1 mL of 10^{-2} suspension onto surface of pre-dried CHOC with IsoVitaleX plate and a pre-dried CHAB 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. 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 Colonies

Invert the CHOC with IsoVitaleX and CHAB plates and incubate them at 35ºC − 37ºC for a maximum of 7 days. Plates should be examined at 24-hour intervals for a maximum of 7 days, if necessary.

- <u>CHOC with IsoVitaleX</u> <u>Plates</u>
  - **a.** *F. tularensis* produces gray-white, opaque colonies, usually too small to be seen as individual colonies at 24 hours. After incubation for 48 hours or more, colonies are approximately 1-2 mm in diameter, white to gray to bluish-gray, opaque, flat, with an entire edge, smooth and have a shiny surface (Figure 2).
  - **b.** Count the number of *F. tularensis* colonies on each plate and record results.

- CHAB Plates

  a. *F. tularensis* produces smooth, entire edge, greenish-white, and butyrous with opalescent sheen colonies at 48 to 72 hours. Colonies are usually 2 to 4 mm after incubation for 48 to 72 hours (Figure 2).

  b. Count the number of *F. tularensis* colonies on each plate and record results.

- Plate Counts

  a. If the number of colonies is ≤ 250/plate, record 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 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 10.2.2 [c]) 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.
- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard cup in an autoclavable biohazard bag. Remove the membranes with sterile forceps and place them grid-side up on labeled CHOC with IsoVitaleX and CHAB plates. Ensure 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 10.2.5) described above for each sample.
- Invert and incubate CHOC with IsoVitaleX and CHAB plates at 35ºC − 37ºC for a maximum of 7 days. Count the number of colonies and record results.
- Plate Counts

  a. If the number of colonies is ≤ 80/plate, record 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 in Section 12.2.2.

  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 TSB with IsoVitaleX. **However, if a problem of background microorganisms overgrowth on a filter is anticipated, the enrichment culture would be required.**

10.2.6 Enrich in TSB with IsoVitaleX

- Add the remainder of the undiluted suspension to the specimen cup containing the corresponding sponge/wipe. Add 40 mL TSB with IsoVitaleX to the tube. Repeat for each sample. Incubate the specimen cups at 35ºC − 37ºC. *F. tularensis* grows slowly; the inoculated broth should be incubated and observed for at least 10 days.
- Observe the TSB Enrichment
  - **a.** If broth is not cloudy, record as no growth (NG) and incubate for an additional 24 hours.
  - **b.** If broth is cloudy, record as positive growth (G+) and proceed to next step.
  - **c.** Cap the cup tightly and mix TSB with IsoVitaleX with positive growth for 30 seconds. Use a sterile loop to remove a loopful of broth with a 10 µL loop and streak on a CHAB plate for isolation. Repeat two times for a total of three CHAB isolation plates.
  - **d.** Incubate the CHAB isolation plates and TSB with IsoVitaleX with growth at 35ºC − 37ºC for a maximum of 10 days.
  - **e.** Examine plates for *F. tularensis*-suspect colonies. If any colonies are isolated, proceed to PCR confirmation (Section 10.5).
  - **f.** If no suspect colonies are observed, perform PCR on TSB with IsoVitaleX 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 the CDC and the 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 into a leak-proof, 50 mL conical tube containing an air filter and vortex on high for 2 minutes.
- Transfer liquid to a sterile, labeled 50 mL tube.
- Retain air filter in the initial 50 mL conical tube 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 tube 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 tube 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 stock 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 CHOC with IsoVitaleX and CHAB

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 CHOC with IsoVitaleX plate and a pre-dried CHAB plate (labeled 10^{-1}).

b. After vortexing tubes, pipet 0.1 mL of 10^{-1} suspension onto surface of pre-dried CHOC with IsoVitaleX plate and a pre-dried CHAB plate (labeled 10^{-2}).

c. After vortexing tubes, pipet 0.1 mL of 10^{-2} suspension onto surface of pre-dried CHOC with IsoVitaleX plate and a pre-dried CHAB 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^{-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. 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.

10.3.4 Incubate and Enumerate Colonies

Invert the CHOC with IsoVitaleX and CHAB plates and incubate them at 35ºC − 37ºC for a maximum of 7 days. Plates should be examined at 24-hour intervals for a maximum of 7 days, if necessary.

- CHOC with IsoVitaleX Plates

a. *F. tularensis* produces gray-white, opaque colonies, usually too small to be seen as individual colonies at 24 hours. After incubation for 48 hours or more, colonies are

approximately 1-2 mm in diameter, white to gray to bluish-gray, opaque, flat, with an entire edge, smooth and have a shiny surface (Figure 2).

b. Count the number of *F. tularensis* colonies on each plate and record results.

- CHAB Plates

  a. *F. tularensis* produces smooth, entire edge, greenish-white, and butyrous with opalescent sheen colonies at 48 to 72 hours. Colonies are usually 2 to 4 mm after incubation for 48 to 72 hours (Figure 2).

  b. Count the number of *F. tularensis* colonies on each plate and record results.

  c. Count the number of colonies on each plate and record results.

- Plate Counts

  a. If the number of colonies is ≤ 250/plate, record 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 cup in an autoclavable biohazard bag. Remove the membranes with sterile forceps and place them grid-side up on labeled CHOC with IsoVitaleX and CHAB plates. Ensure 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.
- Invert and incubate CHOC with IsoVitaleX and CHAB plates at 35ºC − 37ºC for a maximum of 7 days. Plates should be examined daily for growth. Count the number of colonies and record results.
- Plate Counts

  a. If the number of colonies is ≤ 80/plate, record 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 in Section 12.2.2.

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 TSB with IsoVitaleX.*** **However, if a problem of background microorganisms overgrowth on a filter is anticipated, the enrichment culture would be required.**

10.3.6 Enrich in TSB with IsoVitaleX

- Add the remainder of the undiluted suspension to the specimen cup containing the corresponding sponge/wipe. Add 40 mL TSB with IsoVitaleX to the tube. Repeat for each sample. Incubate the specimen cups at 35ºC − 37ºC. *F. tularensis* grows slowly; the inoculated broth should be incubated and observed for at least 10 days.
- Observe the TSB with IsoVitaleX Enrichment

a. If broth is not cloudy, record as no growth (NG) and incubate for an additional 24 hours.

b. If broth is cloudy, record as positive growth (G+) and proceed to next step.

c. Cap the cup tightly and mix TSB with IsoVitaleX with positive growth for 30 seconds. Use a sterile loop to remove a loopful of broth with a 10 µL loop and streak on a CHAB plate for isolation. Repeat two times for a total of three CHAB isolation plates.

d. Incubate the CHAB isolation plates and TSB with IsoVitaleX with growth at 35ºC − 37ºC for a maximum of 10 days.

e. Examine plates for *F. tularensis*-suspect colonies. If any colonies are isolated, proceed to PCR confirmation (Section 10.5).

f. If no suspect colonies are observed, perform PCR on TSB with IsoVitaleX with

10.4 Sample Processing and Plating for Water Samples

It is anticipated that the large volume water sample has undergone primary and secondary concentration and the resultant concentrated sample has been filtered through a membrane filter. Therefore, this sub-section describes the procedure for processing of such a membrane filter received in a 50 mL screw cap tube or other appropriate container.

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 the CDC and the BMBL waste management and disposal requirements.

10.4.1 Recover Bacteria from the MicroFunnel™ Membrane

- If the membranes 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 into a screw cap, 50 mL conical tube containing a membrane filter and vortex on high for 2 minutes.
- Transfer liquid to a sterile, labeled 50 mL tube.
- Retain membrane filter in the initial 50 mL conical tube 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 tube 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 tube 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 3 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 CHOC with IsoVitaleX and CHAB

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 CHOC with IsoVitaleX plate and a pre-dried CHAB plate (labeled 10^{-1}).
- **b.** After vortexing tubes, pipet 0.1 mL of 10^{-1} suspension onto surface of pre-dried CHOC with IsoVitaleX plate and a pre-dried CHAB plate (labeled 10^{-2}).
- **c.** After vortexing tubes, pipet 0.1 mL of 10^{-2} suspension onto surface of pre-dried CHOC with IsoVitaleX plate and a pre-dried CHAB plate (labeled 10^{-3}).
- **d.** After vortexing tubes, pipet 0.1 mL of 10^{-3} suspension onto surface of pre-dried CHOC with IsoVitaleX plate and a pre-dried CHAB 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. 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 Colonies

Invert the CHOC with IsoVitaleX and CHAB plates and incubate them at 35ºC − 37ºC for a maximum of 7 days. Plates should be examined at 24-hour intervals for a maximum of 7 days, if necessary.

- CHOC with IsoVitaleX Plates

  a. *F. tularensis* produces gray-white, opaque colonies, usually too small to be seen as individual colonies at 24 hours. After incubation for 48 hours or more, colonies are approximately 1-2 mm in diameter, white to gray to bluish-gray, opaque, flat, with an entire edge, smooth and have a shiny surface (Figure 2).

  b. Count the number of *F. tularensis* colonies on each plate and record results.

- CHAB Plates

  a. *F. tularensis* produces smooth, entire edge, greenish-white, and butyrous with opalescent sheen colonies at 48 to 72 hours. Colonies are usually 2 to 4 mm after incubation for 48 to 72 hours (Figure 2).

  b. Count the number of *F. tularensis* colonies on each plate and record results.

- Plate Counts

  a. If the number of colonies is ≤ 250/plate, record 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 < 20 mm Hg.
- 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 10.4.2 [d]) 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.
- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard cup in an autoclavable biohazard bag. Remove the membranes with sterile forceps and place them grid-side up on labeled CHOC with

IsoVitaleX and CHAB plates. Ensure 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.
- Invert and incubate CHOC with IsoVitaleX and CHAB plates at 35ºC−37ºC for a maximum of 7 days. Plates should be examined daily for growth. Count the number of colonies and record results.
- Plate Counts
  - **a.** If the number of colonies is ≤ 80/plate, record 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 in Section 12.2.2.

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 TSB with IsoVitaleX.*** **However, if a problem of background microorganisms overgrowth on a filter is anticipated, the enrichment culture would be required.**

10.4.6 Enrich in TSB with IsoVitaleX

- Add the remainder of the undiluted suspension to the specimen cup containing the corresponding sponge/wipe. Add 40 mL TSB with IsoVitaleX to the tube. Repeat for each sample. Incubate the specimen cups at 35ºC − 37ºC. *F. tularensis* grows slowly; the inoculated broth should be incubated and observed for at least 10 days.
- Observe the TSB with IsoVitaleX Enrichment
  - **a.** If broth is not cloudy, record as no growth (NG) and incubate for an additional 24 hours.
  - **b.** If broth is cloudy, record as positive growth (G+) and proceed to next step.
  - **c.** Cap the cup tightly and mix TSB with IsoVitaleX with positive growth for 30 seconds. Use a sterile loop to remove a loopful of broth with a 10 µL loop and streak on a CHAB plate for isolation. Repeat two times for a total of three CHAB isolation plates.
  - **d.** Incubate the CHAB isolation plates and TSB with IsoVitaleX with growth at 35ºC − 37ºC for a maximum of 10 days.
  - **e.** Examine plates for *F. tularensis*-suspect colonies. If any colonies are isolated, proceed to PCR confirmation (Section 10.5).
  - **f.** If no suspect colonies are observed, perform PCR on TSB with IsoVitaleX with growth according to Section 10.5.

10.5 Confirm *F. tularensis* Colonies by Real-time PCR Analysis

10.5.1 DNA Preparation from Cultured Cells

- Cells grown on solid culture medium

  a. Pipet 100 µL of PCR-grade water into a 1.5 mL Eppendorf microcentrifuge tube (Section 5.1.20).

  b. Use a disposable 1 µL inoculating loop or pre-wetted swab to remove bacterial growth from a typical *F. tularensis* colony grown on CHOC with IsoVitaleX or CHAB plates.

Note: ***In some cases, it may be difficult to remove the bacterial growth with a loop. If this happens, use a sterile applicator swab. 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 the swab or the loop in an autoclavable biohazard bag. Proceed to Section 10.5.2.

- Cells grown in liquid culture medium

  a. Transfer 50 µL of broth with growth to a microcentrifuge tube.

  b. Place tube into a refrigerated microcentrifuge and spin at 12,000 × g for 2 minutes.

  c. Remove and discard the supernatant in an autoclavable biohazard bag. Add 100 µL 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 vortex mix for 3-5 secs.
- Place the capped tubes in a floating rack if using the water bath. Otherwise, place the capped tube in the heat block at 95°C–98°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 BSL3 laboratory.
- Remove the tubes from the water bath or heat block and place them directly in a cold block (4ºC). Chill 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 (Section 5.1.30; Millipore® Cat. No. UFC30VV00). following extraction of DNA will remove *F.*

tularensis cells that might 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).
- Centrifuge at 8000 × g (approximately, 9200 rpm) for 2 minutes at 4ºC.

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 and the forceps 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 6.18) 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 (4ºC) 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 1week.***

10.5.4 Use 5 µL of the lysate as the DNA template to run the PCR analysis in triplicate using the Ft1 and Ft2 assays.

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.

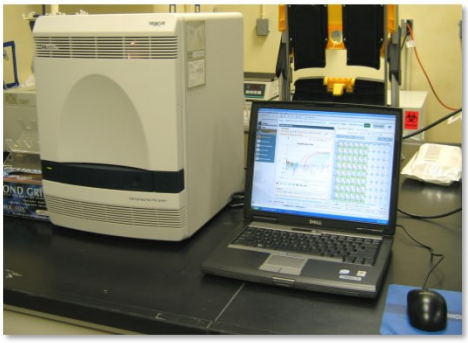
11.0 Rapid Viability-Polymerase Chain Reaction (RV-PCR) Method

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 *F. tularensis*. This section includes a RV-PCR method with appropriate sample processing procedures for detection of *F. tularensis* 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 (16.5) uses a single well on a 48-well plate per sample for *F. tularensis* (Figure 3).



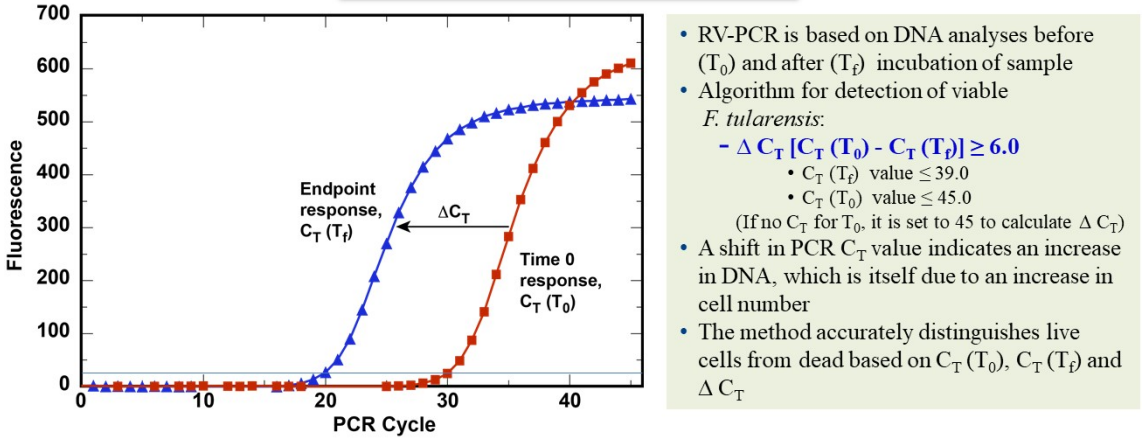


Figure 3. Example real-time PCR amplification curves for the initial T_0 aliquot and the T_f (final) endpoint aliquot.

The RV-PCR protocol steps and some of the equipment for *F. tularensis* 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 48-well plate at 37ºC for 30 hours. This is the

T_0 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 at 37ºC for 30 hours or more, another aliquot is withdrawn. This is the T_{30} or T_f aliquot. Both the T_0 and T_{30} or T_f aliquots are then processed to extract and purify *F. tularensis* total DNA. The T_0 and T_{30} or T_f DNA extracts are then analyzed, in triplicate, using real-time PCR to detect the presence of *F. tularensis* DNA. The C_T values for both the T_0 and T_{30} or T_f DNA extracts are recorded and compared. A change in C_T for the T_{30} or T_f aliquot relative to the C_T for the T_0 aliquot is calculated as follows: $\Delta C_T = (C_T [T_0] - C_T [T_{30}$ or $T_f])$. A $\Delta C_T \geq 6$ (i.e., the endpoint PCR C_T of ≤ 39 for the T_{30} or T_f DNA extract in a 45-cycle PCR) is set as a cut-off value for a positive detection of viable *F. tularensis* in the sample. The $\Delta C_T \geq 6$ criterion represents an approximate two log increase in DNA concentration at T_{30} or T_f relative to T_0. The increase in DNA concentration at T_{30} or T_f is as a result of the presence of viable *F. tularensis* bacteria in the sample that grew during the 30 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, two single-plex PCR assays (See Section 6.9, Ft1 and Ft2) are included. However, for sample analysis during a confirmed tularemia incident for which the *F. tularensis* strain has already been identified and characterized, only the Type A (Ft1) or B (Ft2) specific real-time PCR assay targeting a strain-specific gene may be performed.

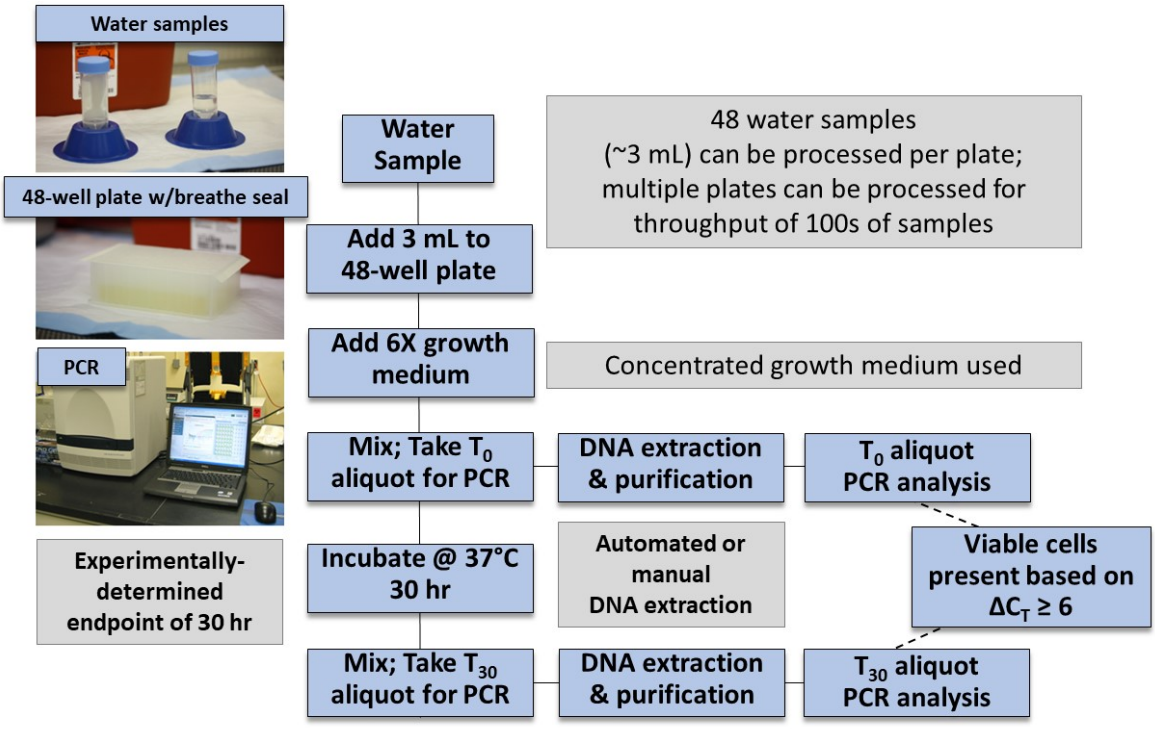


Figure 4. Flow Chart for RV-PCR Analysis of *Francisella tularensis* Cells from Water Samples.

11.2 RV-PCR: Sample Processing 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 the CDC and the 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.
- Ensure tubes are balanced and place tubes into sealing centrifuge buckets. Decontaminate centrifuge buckets with a 10% pH amended bleach solution (Section 6.18) or bleach wipes (Section 5.1.3) before removing them from the BSC.
- Centrifuge tubes at 3500 × 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.
- Vortex mix the remaining 3 mL and the pellet.
- 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 600 µL of 6X BVFH to each well of the 48-well plate using a 1000 µL pipettor. (Final BVFH ~ 1X). Mix the sample and growth medium well.
- Transfer 500 µL from each well of the 48-well plate to an appropriately labeled screw cap tube. This is a T_0 aliquot for each sample. Repeat for each sample.
- Store aliquots on ice or in cold block (4ºC).

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 bag.
- Incubate the 48-well plate at 37ºC for 30 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 4ºC.
- Remove 300 µL of supernatant and dispose to waste. Store pellets on ice or in cold block (4ºC). Alternatively, the pellet may be stored at -20ºC until it is ready to be processed for DNA extraction.

11.2.5 Process T_{30} 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 30-hour or longer incubation, transfer 500 µL from each well to an appropriately labeled 2-mL screw cap tube. Ensure that the T30 aliquot for each sample is taken from the same well from which the T0 aliquot for the corresponding sample was taken. This is a T_{30} or T_f aliquot for each sample.
- Centrifuge tubes at 14,000 rpm (20,800 RCF) for 10 minutes at 4ºC.
- Remove 300 µL of supernatant and dispose to waste. Store pellets on ice or in cold block (4ºC). Alternatively, the pellet may be stored at -20ºC until it is ready to be processed for DNA extraction.

11.3 RV-PCR: Manual DNA Extraction/Purification Procedure Using the Promega MagneSil® Kit Reagents

11.3.1 Thaw T_0 and T_{30} or T_f aliquots if they were stored at -20°C.

11.3.2 Add 800 µL of Lysis Buffer (VWR, Cat. No. PAMD1392 or equivalent) using a 1000 µL pipettor with a new tip for each sample. Cap the tubes and mix by vortexing on high (~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_0 and T_{30} lysate tubes hereafter referred to as "T_0 and T_{30} or T_f 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_0 and T_{30} or T_f 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_0 and T_{30} tubes (containing 1 mL sample-Lysis Buffer mix). 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_0 and T_{30} or T_f tubes, vortexing the PMPs suspension between each T_0 and T_{30} or T_f tube.

11.3.7 Vortex each T_0 and T_{30} or T_f tube for 5−10 seconds (high), incubate at room temperature for five minutes, briefly vortex, and then place on the DynaMag 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. Alternatively, tubes may be vortexed while in removable rack that interfaces with magnetic stand.

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 as necessary.

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_0 and T_{30} or T_f 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_0 and T_{30} or T_f 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_0 and T_{30} or T_f 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_0 and T_{30} tube one at a time and add 360 µL of Salt Wash solution (Section 6.12). Use a new tip for each T_0 and T_{30} or T_f 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_0 and T_{30} or T_f 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_0 and T_{30} tubes is not required. Use a new tip for each T_0 and T_{30} or T_f tube and discard used tips in a sharps container. Recap the tube. Repeat for all T_0 and T_{30} or T_f 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_0 and T_{30} or T_f 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_0 and T_{30} or T_f 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_0 and T_{30} or T_f tubes, vortex each tube for 5−10 seconds (low speed) and place on the magnetic stand. After all, T_0 and T_{30} or T_f 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_0 and T_{30} or T_f tubes is not required. Use a new tip for each T_0 and T_{30} or T_f 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. 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_0 and T_{30} or T_f 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_0 and T_{30} or T_f tubes and air dry for two minutes.

11.3.23 Heat the open T_0 and T_{30} or T_f 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_0 and T_{30} or T_f 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 4ºC for 1 minute. Place tube in 96-well tube rack.

11.3.30 Briefly vortex each tube (5−10 seconds) and place on the magnetic stand for at least 30 seconds. Bring the cold block (4ºC) to the BSC.

11.3.31 Collect elution liquid from each T_0 and T_{30} or T_f tube with a micropipettor and transfer to a clean, labeled, 1.5 mL tube (~80−90 µL) on a cold block at 4ºC (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_0 and T_{30} or T_f DNA extract tubes at 4ºC 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 -20ºC.

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 6.18), followed by 70% isopropyl alcohol, and a deionized water final rinse.

11.4 RV-PCR: Automated DNA Extraction/Purification Procedure (Roche MagNA Pure Compact kit)

11.4.1 When ready to proceed with inactivation, thaw pellet and heat lyse sample tubes by incubating at 70°C ± 2°C for 10 min. Allow sample tubes to cool briefly (2–3 min).

11.4.2 Add 300 µL lysis/binding buffer (MagNA Pure® LC Total Nucleic Acid Isolation Kit Lysis/Binding Buffer-Refill; Roche, Cat. No. 03 246 779 001) to each sample tube and close sample tube cap. Vortex on single tube vortexer for 5 sec at 1800-2000 rpm (VWR, IKA Model MV1 or equivalent).

11.4.3 Add 300 µL phosphate-buffered saline (PBS; 1X PBS, Teknova, Cat. No. P0261) to each PC Tube.

11.4.4 Vortex tubes for 10 sec on single tube vortexer at 1800-2000 rpm (VWR, IKA Model MV1 or equivalent).

11.4.5 Incubate tubes for 30 min at room temperature (in BSC). Every 5 min invert tubes 5 times to mix.

11.4.6 Adapt the Reagent Cartridge to room temperature (15 to 25°C) before use. The kit may not work well at temperatures outside the recommended range.

11.4.7 Use the MagNA Pure® Compact Nucleic Acid Isolation Kit I and select the DNA_Blood_external_lysis purification protocol (supplied with the MagNA Pure® Compact instrument). The sample and elution volumes must be chosen from the software menu.

11.4.8 Samples will be lysed and filtered manually using 0.2-micron Ultrafree-MC filter units (Millipore Cat. No. UFC30GV0S) following manufacturer's instructions, outside the MagNA Pure® Compact instrument. Filtered lysates are then transferred to the instrument and purification is carried out automatically. This procedure allows for physical

separation of the initial lysis/inactivation step(s) from the purification steps and enables use of inactivated sample material on the MagNA Pure® Compact instrument (e.g., when using potentially infectious sample material).

11.4.9 Turn on the instrument; ensure that the Tube Rack is seated correctly in the instrument.

11.4.10 Remove the Elution Tube Rack from the instrument.

11.4.11 Click the Run button on the Main Menu Screen to access Sample Ordering Screen 1.

11.4.12 Follow the software-guided workflow.

11.4.13 Remove a prefilled Reagent Cartridge from the blister pack. Handle each Reagent Cartridge as follows:

- Always wear gloves when handling the cartridge.
- Hold the cartridge only at the barcode imprinted area and the opposite side.
- Avoid touching the sealing foil covering the cartridge wells.
- Avoid touching the two single open wells and do not use them as handles.
- Avoid any foam formation and let the fluid within the cartridge wells settle again completely. If fluid remains under the sealing foil, knock the cartridge bottom gently on a flat lab bench surface. This is especially important for well 1 which contains a small volume of Proteinase K.

11.4.14 Check the cartridge integrity and filling volumes of the wells. Do not use cartridges that have a different pattern of filling or that are damaged.

11.4.15 Scan the cartridge barcode using the barcode scanner supplied with the instrument.

11.4.16 With the two isolated wells pointing away from you, insert all the wells on the Reagent Cartridge into the holes in the Cartridge Rack.

11.4.17 Use the guide slots on the rack to help position the cartridge.

11.4.18 Repeat the steps above for the desired numbers of samples (1 to 8).

11.4.19 Proceed to Sample Ordering Screen 2.

11.4.20 Select the appropriate purification protocol from the Protocol menu (DNA_Blood_external_lysis).

11.4.21 Select the elution volume (100 µL).

11.4.22 Optional: Select the Internal Control Volume (0 µL).

11.4.23 Insert the appropriate number of Tip Trays (one per sample) into the assigned position in the instrument Tip Rack.

11.4.24 Check if the Tip Tray holds a disposable tip or piercing tool in each position. Do not use tip trays that are not assembled accordingly.

11.4.25 Handle Tip Trays with care to prevent tips or piercing tool from falling out of the tray. Should this happen, discard the respective tip tray and tips. Use the Tip Tray Kit to replace missing Tip Trays.

11.4.26 Proceed to Sample Ordering Screen 3.

11.4.27 Scan the sample barcode from the primary sample tube or enter the sample ID.

11.4.28 One at a time, uncap and arrange the Sample Tubes in row 1 of the Tube Rack. Make sure, the brim of the tubes seats solidly on the rack. Discard caps to waste.

11.4.29 Scan the bar codes of the Elution Tubes.

11.4.30 Place the Elution Tubes into the Elution Tube Rack. Ensure the brim of the tubes seats solidly on the rack.

11.4.31 On the Confirmation Screen, check the information display.

11.4.32 If the information is correct, confirm it by touching the "Confirm Data" button, close the front cover, and start the run.

11.4.33 After the purification run has ended, the Result Screen appears showing the result of the isolation process for each channel.

11.4.34 The result will be PASS if the isolation run was completed without any warning or error.

11.4.35 The result will be FAIL if any interruption of the process or error occurred during the run. For each FAIL result, the result screen will show a brief error or warning messages to help you decide whether the error or warning can be ignored. Refer to the troubleshooting section of the MagNA Pure® Compact Operator's Manual.

11.4.36 Close the Elution Tubes with the supplied tube caps and remove the Elution Tube Rack or the Elution Tubes immediately after the end of the purification run.

11.4.37 If not proceeding directly to your downstream application, store DNA eluates at -20°C. DNA is stable for at least 6 to 12 months if stored properly.

11.4.38 Optional: Start the automated liquid waste discard.

11.4.39 Always empty the MagNA Pure® Compact Waste Tank after every purification run.

11.4.40 Treat liquid waste as potentially infectious (depending on sample material), and hazardous, since lysis buffers are present (see Safety Information in instrument manual and/or SDS for the lysis buffer).

11.4.41 Store DNA extract tubes "referred to as T0 or T30 DNA extracts" at 4ºC 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 -20ºC.

11.5 RV-PCR: Real-time PCR Analysis of T_0 and T_{30} or T_f DNA Extracts

Note: PCR Master Mix for 6 reactions per sample is required to accommodate the T_0 and T_{30} or T_f 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_0 and 3 for T_{30} or T_f DNA extracts). No EIC control is required for the samples.

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

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

11.5.3 Analyze T_0 and T_{30} or T_f DNA extracts on same PCR plate.

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

11.5.5 Perform 1:10 dilution of DNA extracts. Alternatively, only run samples undiluted (5 µL plus 20 µL PCR Master Mix).

11.5.6 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.

11.5.7 Mix DNA extracts by vortexing (5 seconds at low speed) and transfer 10 µL to the plate wells, following the plate layout.

11.5.8 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 optical seal using a clear Edge Seal.

11.5.9 Centrifuge sealed PCR plate for 1 minute at 2000 rpm.

11.5.10 Open the centrifuge safety cup in BSC, place plate on photo-tray, change gloves, transfer PCR plate to ABI® thermocycler.

11.5.11 The PCR cycling conditions on the ABI® 7500 Fast are provided in **Table 7**. Fluorescence is automatically measured at the end of the 60ºC annealing-extension combined step.

11.5.12 After cycle completion, discard sealed PCR plate to waste and autoclave. PCR plates with amplified product are never to be opened in the laboratory.

11.5.13 Follow laboratory cleanup procedure (11.3.34).

11.5.14 10.5.6 Refer to Sections 12.3 for Data Analyses and Calculations.

Table 6. Master Mix for Ft1 and Ft2 *Francisella tularensis* PCR Assays

Reagent	Volume (µL)	Final Concentration
TaqMan® 2X Universal Master Mix	12.5	1X
Platinum® *Taq* DNA Polymerase	0.25	1.25 U
Forward primer, 10 µM	0.5	0.20 µM
Reverse primer, 10 µM	0.5	0.20 µM
Probe, 4 µM	0.4	0.064 µM
Molecular Biology grade water	5.85	N/A
Template DNA	5	Variable
TOTAL	25	

Table 7. PCR Thermal Cycling Conditions [a, b]

Steps	UNG[c] Incubation	AmpliTaq Gold Activation	PCR, 45 cycles[d]	
	Hold	Hold	Denaturation	Annealing/Extension
Temperature	50ºC	95ºC	95ºC	60ºC
Time	2 minutes	10 minutes	5 seconds	20 seconds[e]

[a] Run Mode: Fast 7500
[b] Reaction volume 25 µL
[c] Uracil-DNA glycosylase
[d] Fast Ramp: 3.5°C/s up and 3.5°C/s down
[e] 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. Most instruments will perform this calculation for the user, but an equation is provided below should manual calculation be needed.

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

$$where\ N\ is\ the\ number\ of replicate\ reactions$$

Example:

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

$$\frac{\sum_{y=1}^{3} C_T(1)+C_T(2)+C_T(3)}{3} = \frac{\sum 20+25+24}{3} = \frac{67}{3} = 23 = Average\ C_T$$

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 *F. tularensis* 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.

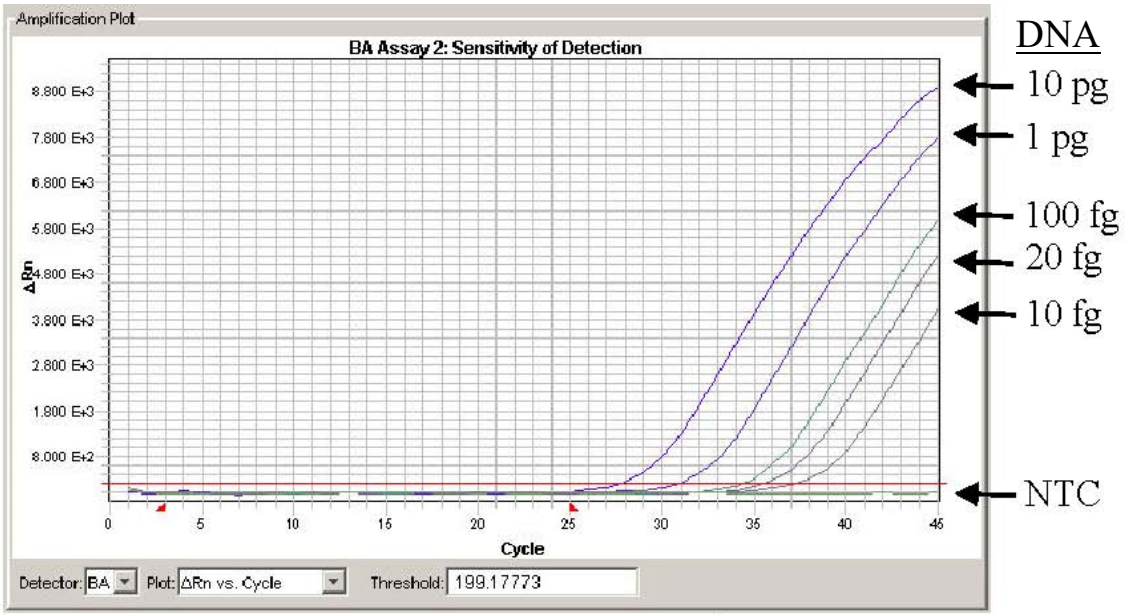


Figure 5. Example logarithmic curve for Fluorogenic PCR for *Bacillus anthracis.*

If the EIC for a sample results in a C_T value (≥ 3) compared to the C_T value for the positive control, there may be matrix inhibition. If the corresponding sample is negative ($C_T > 40$) for *F. tularensis*, 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 *F. tularensis* colonies.

Media sterility checks should not exhibit growth. Growth should also not be present on CHOC/CHAB 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 *F. tularensis.*

a. If the number of colonies is ≤ 250/plate, record 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, choose the plates with the counts closest to the acceptable range of 25-250 colonies. For example, if all plate counts are greater than 250 choose 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 number of colonies in the dilution series that produced ≤ 250 cells/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 (in this example, 5mL), 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:

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

12.2.2 MicroFunnel™ Filter Plating

Count the number of typical colonies (Figure 2) on each filter and record. Apply the following (a-c) when counting the colonies and report results based on the number of characteristic *F. tularensis* colonies.

Media sterility checks should not exhibit growth. Growth should also not be present on CHOC/CHAB 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 *F. tularensis.*

a. If the number of colonies is ≤ 80/plate, record 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, choose the plates with the counts closest to the acceptable range of 20-80 colonies. For example, if all plate counts are greater than 80 choose 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 by the average colony count per mL by the total suspension volume per sample, as in the following equation:

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

12.2.3 Enrichment in TSB with IsoVitaleX

Evaluate post-enrichment streaked CHOC/CHAB plates for the presence of *F. tularensis* colonies (Figure 2). If no suspect colonies are observed, broth should be evaluated for the presence of *F. tularensis*. Typical isolates or TSB with IsoVitaleX 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 TSB with IsoVitaleX 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 *F. tularensis.*

12.2.4 Confirmation of Colonies by Real-time PCR

Presence of *F. tularensis* typical colonies on the culture plate indicates the presence of

viable *F. tularensis* cells in the sample. A minimum of three typical colonies should be confirmed using real-time PCR and the either the Ft1 (For *F. tularensis* Type A strain) or Ft2 (For *F. tularensis* Type B strain) real-time PCR assay depending upon the type strain identified to be involved in an incident. Optionally, both Ft1 and Ft2 PCR assays can be used. The $C_T \leq 40$ and the presence of a logarithmic curve in the real time graph (Figure 5) for the sample indicates a positive result suggesting the presence of *F. tularensis* 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_{30} or T_f DNA extracts of each sample. Subtract the average C_T of the T_{30} 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 ΔC_T for the sample. The change (decrease) in the average C_T value from T_0 to T_{30} or T_f (ΔC_T) ≥ 6 indicates a positive result suggesting the presence of viable *F. tularensis* cell in the sample. A ΔC_T criterion of ≥ 6 (an approximate two log difference in DNA concentration) and a corresponding T_{30} or T_f C_T of ≤ 39, was set. If an incubation time longer than 30 hours was used for the RV-PCR, instead of T_{30}, appropriate T_f (incubation time) should be used (i.e., 36-40 hours for post-decontamination, field samples with high concentrations of dead *F. tularensis* cells). However, (ΔC_T) ≥ 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 ≤ 44 (in a 45-cycle PCR) to calculate the average T_0 C_T. A minimum of two out of three T_{30} or T_f PCR replicates must result in C_T values ≤ 39 to calculate the average C_T for a sample result to be considered positive. Negative controls (No Template 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

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

14.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be discarded in an autoclavable biohazard bag. 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 *F. tularensis* 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.

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