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Optimization of a Rapid Viability Polymerase Chain Reaction (RV-PCR) Protocol for Detection of *Francisella tularensis* in Water Samples

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FINAL REPORT

Optimization of a Rapid Viability Polymerase Chain Reaction (RV-PCR) Protocol for Detection of *Francisella tularensis* in Water Samples

by

Sanjiv R. Shah, Ph.D.

U.S. Environmental Protection Agency
Cincinnati, OH 45268

and

Staci Kane, Ph.D.
Teneile Alfaro

Lawrence Livermore National Laboratory
United States Department of Energy
Livermore, CA 94551

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U.S. Environmental Protection Agency

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Questions concerning this document, or its application should be addressed to:

Sanjiv R. Shah, Ph.D.
Disaster Characterization Branch
Homeland Security and Materials Management Division
Center for Environmental Solutions and Emergency Response
Office of Research and Development
U.S. Environmental Protection Agency
1300 Pennsylvania Avenue, NW
USEPA-8801RR
Washington, DC 20460
(202) 564-9522
shah.sanjiv@epa.gov

If you have difficulty accessing these PDF documents, please contact McCall.Amelia@epa.gov for assistance.

Acknowledgments

Research Team

Staci Kane and Teneile Alfaro (Lawrence Livermore National Laboratory)

EPA Technical Lead

Sanjiv Shah [U.S. EPA Homeland Security & Materials Management Division (HSMMD), Center for Environmental Solutions & Emergency Response (CESER])

Technical Reviewers

Worth Calfee (U.S. EPA. HSMMD, CESER)

David Bright (U.S. EPA Office of Land and Emergency Management – Office of Emergency Management – Consequence Management Advisory Division)

Latisha Mapp (U.S. EPA Office of Water – Water Security Division)

External Peer-Reviewers

Paul Morin (U.S. Food and Drug Administration)

Laura Rose (Centers for Disease Control and Prevention)

Quality Assurance Reviewer

Ramona Sherman (U.S. EPA. HSMMD, CESER)

Edit Reviewer

Marti Sinclair, (U.S. EPA. HSMMD, CESER: General Dynamics IT, EPA Contract HHSN316201200050W)

List of Abbreviations and Acronyms

ABI ..... Applied BioSystems, Inc.
ATD ..... Arizona Test Dust
Avg..... average
B. anthracis.....*Bacillus anthracis*
BHI ..... brain heart infusion
BHQ.....Black Hole Quencher
bp .....base pair
BSC .....biosafety cabinet
BSL-3.....biosafety level-3
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
CFU ..... colony forming unit(s)
C_T ..... cycle threshold
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_{24}) or T_{24} C_T ..... C_T value after 24 hours incubation
C_T (T_{30}) or T_{30} C_T ..... C_T value after 30 hours incubation
C_T (T_{36}) or T_{36} C_T ..... C_T value after 36 hours incubation
ΔC_T..... delta cycle threshold
DD..... distilled, deionized
DNA ..... deoxyribonucleic acid
dsDNA ..... double-stranded DNA
ERLN .....Environmental Response Laboratory Network
EPA .....U.S. Environmental Protection Agency
F. tularensis..... *Francisella tularensis*
FAM.....6-carboxyfluorescein
fg/µL .....femtogram(s) per microliter
g ..... gram(s)
h ..... hour(s)
HSMMD……..... Homeland Security and Materials Management Division
HSRP .....Homeland Security Research Program
ISO..... International Organization for Standardization
kb..... kilobase
LLNL ..... Lawrence Livermore National Laboratory
LRN ..... Laboratory Response Network
µg .....microgram(s)
µg/L..... microgram(s) per liter
µg/mL.....microgram(s) per milliliter
µm .....micrometer(s)
Mb.....mega base pairs
mg .....milligram(s)

min ..... minute(s)
µL ..... microliter(s)
mL ..... milliliter(s)
mg/L ..... milligram(s) per liter
MH2+ ..... 1X Mueller Hinton Broth 2 with supplements
mm ..... millimeter(s)
mM ..... millimolar
NA ..... not applicable
NDT ..... non-detect
ng/µL ..... nanogram(s) per microliter
NIST ..... National Institute of Standards and Technology
nm ..... nanometer(s)
NTC ..... No-Template Control
OD_{600} ..... optical density at 600 nm
ORD ..... Office of Research and Development
PBS ..... phosphate buffered saline
PCR ..... polymerase chain reaction
pg ..... picogram(s)
Plat. ..... Platinum
PMP ..... paramagnetic particles
ppm ..... part(s) per million
RCF ..... relative centrifugal force
RNA ..... ribonucleic acid
ROX 6-carboxyl-X-rhodamine
rpm ..... revolution(s) per minute
RV ..... rapid viability
RV-PCR ..... rapid viability–polymerase chain reaction
SD ..... standard deviation
sec ..... second(s)
T_0 ..... time 0, prior to incubation
T_{24} ..... after 24 h of incubation
T_{30} ..... after 30 h of incubation
T_{36} ..... after 36 h of incubation
Taq ..... Taq DNA Polymerase (from *Thermus aquaticus)*
T_f ..... time final, after incubation
TNTC ..... too numerous to count
UNG ..... uracil-N-glycosilase
VBNC ..... viable but not culturable
WLA ..... Water Laboratory Alliance
Y. pestis ..... *Yersinia pestis*
YPEB ..... *Yersinia pestis* Enrichment Broth
1X ..... 1-fold concentrated (no concentration)
6X ..... 6-fold concentrated
10X ..... 10-fold concentrated

Trademarked Products

Trademark	Holder	Location
ABI Gold™	Life Technologies	Carlsbad, CA
AB Applied BioSystems™	Life Technologies	Carlsbad, CA
AeraSeal™	Excel Scientific	Victorville, CA
AmpErase®	Thermo Fisher Scientific	Waltham, MA
AmpliTaq Gold®	Life Technologies	Carlsbad, CA
Bacto™	BD Biosciences	Franklin Lakes, NJ
BBL™	BD Biosciences	Franklin Lakes, NJ
BD BBL™	BD Biosciences	Franklin Lakes, NJ
Black Hole Quencher®	Biosearch Technologies	Petaluma, CA
Difco™	Becton, Dickinson & Co.	Franklin Lakes, NJ
Dynamag™	Life Technologies	Carlsbad, CA
Epicentre®	Epicentre Technologies Corp.	Madison, WI
Excel®	Microsoft® Corporation	Redmond, WA
Invitrogen®	Life Technologies	Carlsbad, CA
IsoVitaleX™	BD Biosciences	Franklin Lakes, NJ
Life Technologies™	Life Technologies	Carlsbad, CA
MagNA Pure®	Roche Diagnostics	Indianapolis, IN
MagneSil® Blood Genomic	Promega	Madison, WI
MasterPure®	Epicentre Technologies Corp.	Madison, WI
Millipore®, Milli-Q™	Millipore Corp.	Billerica, MA
PicoGreen®	Life Technologies	Carlsbad, CA
Platinum™ Taq Polymerase	Invitrogen	Carlsbad, CA
QIAamp®	Qiagen	Hilden, Germany
Quant-iT™	Life Technologies	Carlsbad, CA
Qubit®	Life Technologies	Carlsbad, CA
Remel™	Remel	Lenexa, KS
TaqMan®	Life Technologies	Carlsbad, CA

Executive Summary

Francisella tularensis (*F. tularensis*), the pathogen that causes tularemia in humans and animals, could be introduced into water infrastructure due to a natural outbreak, laboratory accident, or intentional contamination, and it can remain viable and infectious for some time in water. It is a category A select agent because it can readily be weaponized, has a low infective dose (1 – 10 cells), and can cause high morbidity and mortality. Due to these characteristics, national security concerns have been raised regarding this biothreat agent.

Designated as the sector-specific agency for water and wastewater systems, the U.S. Environmental Protection Agency (EPA) and, specifically, EPA's Office of Water would need timely and reliable water sample analysis results during response to and recovery from a waterborne tularemia outbreak. Regardless of the cause of contamination, there is a need for a rapid and sensitive analytical method to detect viable *F. tularensis*.

A rapid viability polymerase chain reaction (RV-PCR) method could offer advantages over the current plate culture-based method for viable *F. tularensis* detection, which requires incubation in liquid or on solid media for 3-5 days followed by confirmation of culture growth or presumptive pathogen colonies, respectively. For environmental samples with indigenous microbes, culture analysis can be challenged by overgrowth of non-target microbes, which can inhibit or mask growth from the slower-growing, fastidious *F. tularensis* cells. The RV-PCR method combines short-incubation broth culture (relative to the plate culture-based method), and sensitive, specific real-time polymerase chain reaction (PCR) analysis before and after sample incubation to assess viability. The RV-PCR method can afford rapid and high-sensitivity detection of viable biothreat agents even in backgrounds of high levels of debris, non-target microbial cells/spores, and dead target cells.

In a partnership between scientists at EPA's Homeland Security Research Program (HSRP) within the Office of Research and Development (ORD) and those at the Lawrence Livermore National Laboratory (LLNL), the RV-PCR method was developed and optimized to meet the need for a rapid analytical method for viable *F. tularensis* detection. In particular, the method was designed for use by the Water Laboratory Alliance, a network of laboratories established by EPA's Office of Water for water sample analysis.

In this research effort, the RV-PCR method was developed and evaluated for *F. tularensis* cells with the following key features: 24-hour (h) to 36-h incubation (dependent on incident-specific parameters), which was shortened from a 48-h incubation from previous efforts; sample incubation in 48-well plates for high throughput culture and sample handling; 10^1-cell/sample level (10-99 cells) sensitivity of detection; and a robust DNA extraction procedure that accommodates complex sample backgrounds. By optimizing the cell processing procedure and incorporating an automated DNA extraction/purification procedure (based on Roche MagNA Pure® reagents), the method performance was further improved. Furthermore, the PCR assay performance was significantly improved with addition of Platinum™ Taq Polymerase to the PCR reagent mix. While the platforms and reagents evaluated here showed good DNA yield and quality, other manual or automated platforms and associated reagents could be used for RV-PCR analysis as well.

In this effort, previous work on *F. tularensis* growth optimization was leveraged and adapted for development of concentrated growth medium. Further improvements in the broth culturing step enabled reproducible growth even at low inoculum levels (10–20 cells per 3 mL water sample). Addressing a range of potential "real world" complex sample types, additional recommendations were made based on challenge testing with potential soluble and insoluble chemical interferences, and with live, non-target or dead target biological interferences. Recommendations included extending the incubation time to 36 hours to further reduce the method's false negative rate for samples with low target cell numbers and high non-target microbial backgrounds.

While outside the scope of this effort, RV-PCR is expected to be compatible with ultrafiltration for cell concentration since a 10^2-cell level (100-999 cells) method sensitivity was observed even in complex samples containing chemical and biological interferences. The *F. tularensis* RV-PCR method will help enhance the Water Laboratory Alliance capability for rapid, reliable, and high-throughput water sample analysis in case of a man-made or natural tularemia outbreak.

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1. Introduction

The U.S. Environmental Protection Agency (EPA) is designated as the lead federal agency during the remediation phase of a response to a bioterrorism attack. The EPA is also designated as the sector-specific agency for water and wastewater systems for this response phase. In this role, EPA's decision makers will need timely and reliable water sample analysis results during response to a microbial contamination incident and subsequent recovery efforts.

Francisella tularensis (*F. tularensis*), the pathogen that causes tularemia in humans and animals, could be introduced into water infrastructure due to a natural outbreak, laboratory accident, or intentional contamination. It is known that *F. tularensis* and several other vegetative bacterial pathogens can remain viable and infectious for some time in certain environments including water (Anda et al., 2001; Berrada et al., 2011). As reviewed by Rice (2015), drinking water outbreaks of tularemia have been reported in the U.S. and several countries including Bulgaria, Georgia, Germany, Italy, Kosovo, Norway, Russia, Sweden, and Turkey.

F. tularensis is a category A select agent because it can readily be weaponized, has a low infective dose (1 – 10 cells; Jones et al., 2005; Saslaw et al., 1961), and high morbidity and mortality. Therefore, national security concerns have been raised regarding this bacterium. As a result, consensus-based recommendations have been developed for civilian defense if *F. tularensis* is used as a biological weapon (Dennis et al., 2001).

F. tularensis is a Gram-negative coccobacillus whose main transmission vectors are arthropods (ticks, deer flies), while small mammals (e.g., rabbits, muskrats) serve as reservoir hosts. Protozoa have also been suggested as an important environmental reservoir (Abd et al., 2003). A security concern surrounding this bacterium is its potential persistence in the environment. Due to its historical usage as a biological weapon (Gürcan, 2014) and the occurrence of natural tularemia outbreaks, there is a need for rapid and sensitive analytical methods for detection of viable *F. tularensis* in environmental samples.

Within EPA, the Office of Water is responsible for protecting and managing water resources. The Office of Water has established the Water Laboratory Alliance (WLA), a network of laboratories for water sample analysis. The WLA is also a significant component of the EPA's Environmental Response Laboratory Network (ERLN), established as a national network of laboratories. The WLA needs rapid and reliable sample analysis methods to assess the presence of live *F. tularensis*. To help meet the need for more rapid and accurate detection of viable *F. tularensis* in environmental samples, the EPA-HSRP, in collaboration with the Lawrence Livermore National Laboratory (LLNL) of the Department of Energy, has developed and optimized the rapid viability polymerase chain reaction (RV-PCR) method for rapid detection of viable *F. tularensis*. This method as well as that developed for detection of viable *Yersinia pestis* (which causes plague; US EPA, 2016) can serve as a template for the detection of other vegetative bacterial pathogens, where modifications to the method can be made to accommodate differences in growth requirements and characteristics.

Methods to more rapidly determine pathogen presence and viability are needed as part of EPA's capabilities to ensure public safety and to help mitigate impacts of facility and infrastructure closures following a biological agent release. The current plate culture-based methods used for detection of *F. tularensis* and other biothreat agents are labor-intensive and have a low throughput (~30–40 samples processed per laboratory shift), with confirmed results obtained only after several days. A review of methods for soil and water sample processing and analysis for *F. tularensis* described challenges with using traditional culture with these complex sample types (US EPA, 2015). In fact, multiple instances were reported where culture identification was unsuccessful.

While PCR methods typically have fewer deficiencies, it is well understood that rapid detection methods such as real-time PCR cannot distinguish between live (potentially infectious) and dead pathogens. However, features of real-time PCR were leveraged for development of RV-PCR, which combines short-incubation broth culture (relative to the plate culture-based method) to enable growth, with sensitive, specific real-time PCR analysis before and after sample incubation, to detect low concentrations of viable bacterial threat agents. Specifically, the method uses the change in real-time PCR response characteristic of cell growth, referred to as the change in cycle threshold (C_T) or ΔC_T, between the initial (before sample incubation) C_T at time 0, pre-incubation (C_T T_0) and the C_T at time final, after incubation (C_T T_f). Example PCR response curves are shown in **Figure 1** along with the criteria for positive detection, namely, $\Delta C_T \geq 6$ (which represents an increase in specific DNA content of ~2-log during incubation). The method allows detection of viable target biothreat agent even in backgrounds of high levels of debris, non-target microbial cells/spores, and dead target agent (as demonstrated for *Bacillus anthracis* spores [Létant et al., 2011] and *Y. pestis* cells [S. Kane, personal communication]). Furthermore, it is likely that viable but not culturable (VBNC) cells could be more readily detected from liquid culture used in RV-PCR analysis compared with solid media used in traditional plate culture analysis (Wai et al., 2000).

In addition, the RV-PCR method for *F. tularensis* detection can provide a higher throughput capability as compared to the traditional culture-based methods, and hence, can increase the laboratory capacity for sample analysis. In place of multiple sample dilutions, solid agar plates, and enrichment cultures per sample used by the culture method, the RV-PCR method uses a single well per sample on a 48-well plate (**Figure 2**).

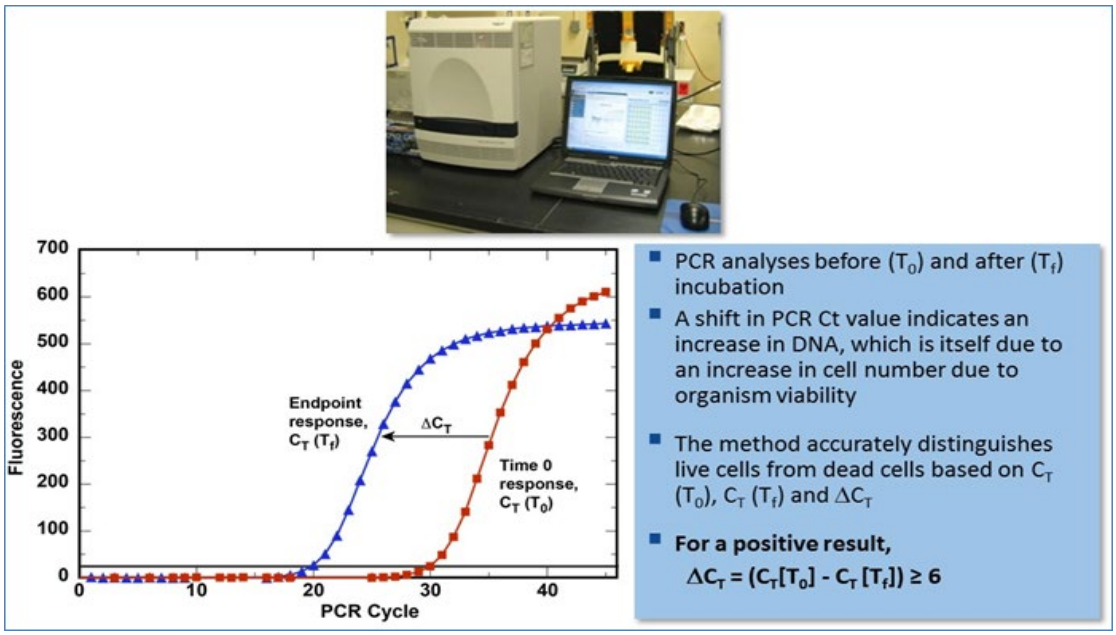

Figure 1. Example real-time PCR response curves showing parameters (C_T [T_0], C_T [T_f], and ΔC_T) used in determining presence or absence of viable cells in the original sample. A significant shift in PCR response curve indicates an increase in DNA, and thus, cell number. A curve is shown for the Time 0 (T_0) response. However, if no PCR response is observed, there would be a flat line, and the C_T would be set to the total number of cycles used (e.g., 45) to calculate a ΔC_T value.

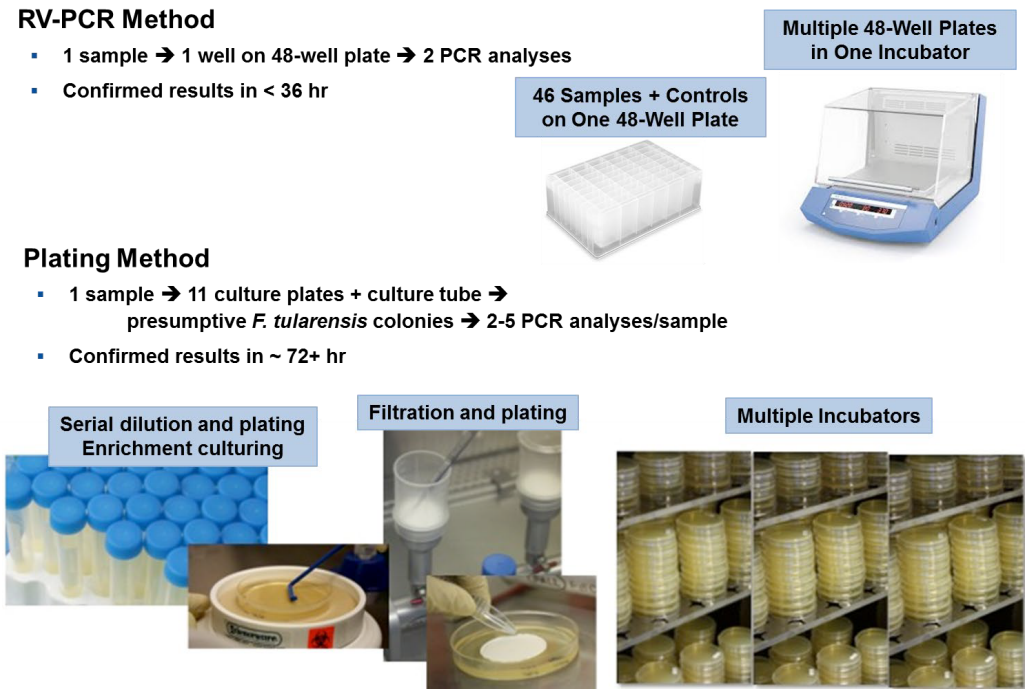

Figure 2. Comparison of the RV-PCR method to the traditional culture method for *F. tularensis.*

The effort reported here significantly expanded a previous effort where LLNL and EPA-ORD scientists developed preliminary RV-PCR protocols for *F. tularensis* and *Y. pestis*. The project work, conducted under earlier effort, led to protocols for *Y. pestis* and *F. tularensis* cells from wet wipes and buffered water samples (S. Létant, personal communication). Results from the previous effort showed the potential for higher throughput sample processing in 48-well plates and shorter incubation periods for confirmation of viable pathogen presence compared to current traditional culture-based methods; these efforts were the starting point for the work reported here. In addition, the EPA-sponsored work by Morris et al. (2017) to develop an optimal growth medium for *F. tularensis* Type A strains was leveraged for this effort.

This project focused on detection of *F. tularensis* in a water matrix with chemical and microbial challenges. Chemical challenges included ferrous sulfate, humic acids, and metal oxides present in Arizona Test Dust (ATD). These materials could negatively affect cell growth and cell recovery from water samples and/or interfere with subsequent analysis. Microbial challenges included microorganisms present in non-autoclaved ATD including *Bacillus* spp. and other non-target bacteria as well as fungal species as identified by Rose et al. (2011). The virulent *F. tularensis* Schu S4 reference strain was used throughout, added at different inoculum levels per water sample. In this effort, automated DNA extraction, concentration and purification steps were incorporated to further shorten the timeline. Features of real-time PCR analysis that benefit rapid analysis include low detection limits (typically <10 DNA copies per reaction), several order-of-magnitude concentration range (~8 logs), and the ability to detect low numbers of target organisms in the presence of high populations of non-target organisms; whereas, traditional culture methods are challenged with environmental backgrounds where target bacteria may be outcompeted by indigenous microorganisms.

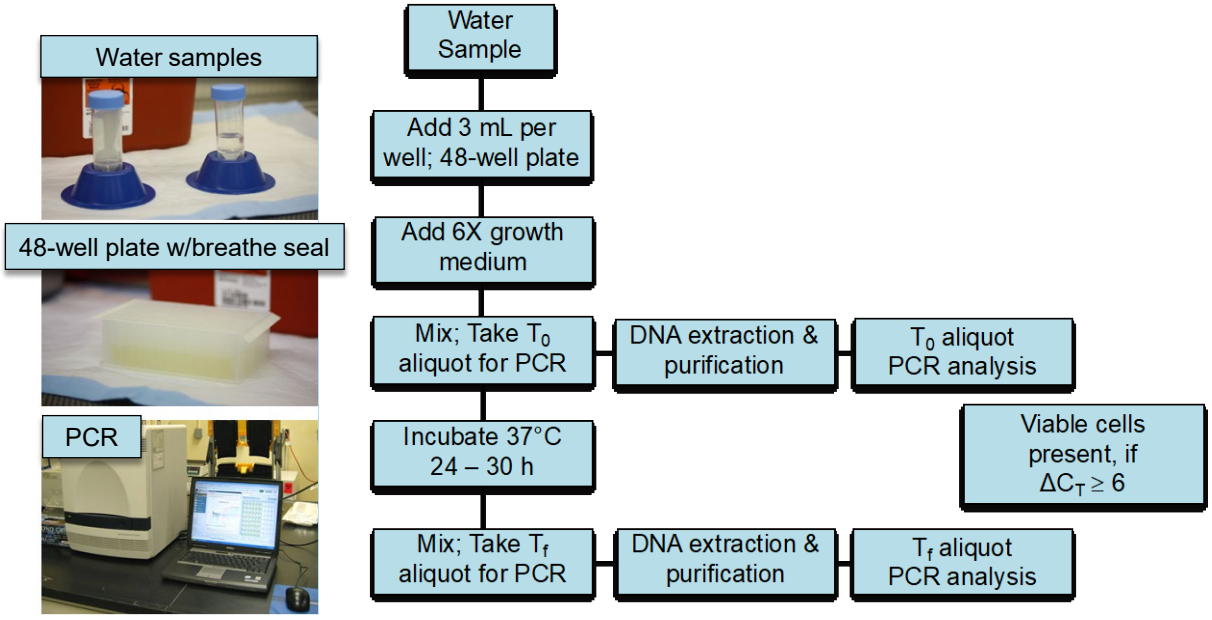

Figure 3. Flow chart for RV-PCR analysis of *F. tularensis* cells from water samples. Using a 48-well plate, 3.0 milliliter (mL) water sample was added with 0.6 mL 6X broth per 5-mL well.

The RV-PCR protocol steps and some of the equipment for *F. tularensis* are shown in **Figure 3**. In this study, manual DNA extraction was compared with automated extraction protocols to enable higher throughput analysis, using the same robotic platform available in some laboratories in the Centers for Disease Control and Prevention (CDC) Laboratory Response Network (LRN).

This report describes experiments and results focused on three major tasks:

- **Task 1. Optimization of *F. tularensis* growth in concentrated medium for RV-PCR analysis**
- **Task 2. Evaluation of manual and automated DNA extraction-purification procedures for *F. tularensis***
- **Task 3. Evaluation and optimization of an RV-PCR protocol for *F. tularensis* detection in water samples with relevant challenges.**

Tasks 1 and 2 included development and optimization of procedures for *F. tularensis* culturing and DNA recovery within the RV-PCR protocol, and. Task 3 used the optimized procedures for evaluation of the entire RV-PCR protocol. Challenge testing for Task 3 included the following additions: (1) humic acid and ferrous sulfate as potential chemical interferents, and (2) live (native) ATD as a source of potential growth-competing microorganisms (non-target cells/spores) and metal oxides.

As mentioned, previous growth medium development efforts (Morris et al., 2017) were leveraged for Task 1. In this effort, different approaches to concentrate the growth medium were evaluated to enable addition of a larger volume water sample, ultimately yielding a 1X concentration for RV-PCR analysis. A 10X concentrated growth medium was evaluated initially based on successful development of a *Y. pestis* RV-PCR method that used 0.3 mL 10X *Y. pestis* Enrichment Broth (US EPA, 2016; p. 12) with 2.7 mL water sample containing *Y. pestis* cells. Based on initial poor results using 10X concentrated medium for *F. tularensis* growth, a 6X medium was also evaluated in this effort, showing improved results.

Previously, a low-throughput manual DNA extraction method with a modified Promega MagneSil bead-based protocol was used (US EPA, 2016). In Task 2 of this effort, an automated platform, Roche MagNA Pure® Compact instrument, was evaluated for inclusion in the *F. tularensis* RV-PCR protocol. This platform as well as a higher throughput platform (e.g., MagNA Pure® LC) could be used to increase throughput for RV-PCR analysis. In addition, automated processing would involve less handling of samples and therefore less risk of exposure to the pathogen. Combined with automated DNA extraction, the RV-PCR protocol could further shorten the timeline for detection of viable *F. tularensis* compared with traditional culture approaches, or even enable detection from complex environmental samples where current methods are limited (e.g., indigenous microbes could mask and/or inhibit growth of *F. tularensis*). From previous efforts with *B. anthracis* Sterne cells, the Roche MagNA Pure® Compact with associated extraction kits and protocols has been shown to provide good DNA yields, with extracts showing on average 3.3 lower C_T values compared with the Promega MagneSil® reagents (S. Kane, personal communication).

This project focused on method optimization to detect the lowest possible number of viable *F. tularensis* cells in water, targeting the 10^1-cell level (10-99 cells), using RV-PCR. In addition, efforts to make the method more operational and to provide higher throughput capability were conducted, followed by evaluation of the optimized method with reference challenge materials relevant to water samples (Task 3). Chemical challenges included ferric sulfate and humic acid, since these compounds have the potential to negatively affect cell growth, cell recovery from water samples, and/or interfere with subsequent analysis (Schrader et al., 2012; Sidstedt et al., 2015). In addition, microbial challenges included indigenous microorganisms present in native reference dust (e.g., Arizona Test Dust ISO 12103-1; Powder Technology, Inc., Arden Hills, MN). Controls were included to ensure that sample-processing protocols did not negatively impact cell viability.

2. Materials and Methods

The following provides general information about the materials and methods used throughout the study with more specific information provided with the individual experiments in the Results and Discussion section.

2.1 Bacterial Strains, Growth Media, and Incubation Conditions

The virulent *F. tularensis* strain Schu S4 from the LLNL strain collection was used for all experiments. The *F. tularensis* culture was grown in Brain Heart Infusion (BHI)/Vitox/Fildes/Histidine (BVFH) medium or Mueller Hinton 2 medium (cation-adjusted) with added glucose, ferric pyrophosphate, and IsoVitaleX™, referred to as 1X Mueller Hinton Broth 2 with supplements (MH2+) medium. The MH2+ medium was prepared as follows: (1) 11 g BBL™ Mueller Hinton Broth 2 (Cation-Adjusted) powder was added to 480 mL Milli-Q™ H_2O, which was mixed and heated to boiling; (2) the mixture was autoclaved at 121°C for 25 minutes; (3) after allowing the broth to cool to room temperature, 5 mL 10% sterile glucose solution, 5 mL sterile 2.5% ferric pyrophosphate, and 10 mL sterile IsoVitaleX (BD BBL™ Biosciences, Cat. No. 211876) were added aseptically; and (4) the medium was mixed and aliquoted into 50 mL tubes and refrigerated until use. The BVFH medium was prepared as described by Morris et al. (2017) for a 1X concentration. The 1X BVFH medium contained: 3.7% weight (w)/volume (v) Bacto™ BHI (BD Biosciences, Franklin Lakes, NJ, Cat. No. 237500); 0.1% (w/v) L-histidine (Sigma-Aldrich, St. Louis, MO, Cat. No. H8000-100G); and 2% (v/v) Vitox Supplement (Oxoid, Lenexa, KS; Cat. No. SR0090A); and 10% (v/v) Remel™ Fildes Enrichment (Remel, Lenexa, KS; Cat. No. R45037).

For the RV-PCR method, use of concentrated growth medium was required so that a larger volume water sample (up to 3 mL) could be analyzed. The 10X BVFH medium was prepared, however, it was not possible to make one of the components, the Remel™ Fildes Enrichment, at a 10X concentration because it comes as a solution and is required at 10% in 1X BVFH medium. However, it was possible to accommodate the Fildes Enrichment at 5X. Briefly, the 10X BVFH medium was prepared by (1) dissolving Bacto™ BHI powder and L-histidine into 30 mL Millipore Milli-Q™ (Millipore Corp., Billerica, CA) purified water followed by autoclaving and cooling the solution, (2) adding 20 mL of sterile Vitox Supplement, and (3) adding 50 mL of sterile Fildes Enrichment. The 10X BVFH medium was subsequently diluted to a 1X

concentration with phosphate-buffered saline (PBS; Teknova, Hollister, CA; Cat. No. P0261), as a surrogate for a water sample. As mentioned, the 1X medium had a final concentration of 5% Fildes.

Since it was observed that some medium components precipitated when the 10X BVFH medium was prepared as described above, filter-sterilization (0.2 µm) was also evaluated. In this case, the BHI powder and L-histidine were dissolved in 30 mL MilliQ® H_2O and brought to boiling for 1 minute, (min) followed by cooling the solution. After cooling, 20 mL of sterile Vitox Supplement and 50 mL of sterile Fildes Enrichment were added, and the solution was mixed. The solution was then filter-sterilized with a disposable filtration system. The 10X solution was stored at 4°C until use.

To optimize *F. tularensis* growth, different diluents were also evaluated for dilution of 10X BVFH to 1X. The diluents included sterile PBS (pH 7.4), sterile Butterfield's buffer, or sterile distilled, deionized (DD) H_2O. The Butterfield's buffer was prepared by dissolving 34 g KH_2PO_4 in 500 mL Milli-Q™ H_2O, adjusting the pH to 7.2, bringing the volume to 1 L with Milli-Q™ H_2O, and autoclaving the solution for 15 min at 121°C. These media prepared with different diluents all had a final 5% Fildes concentration after dilution to 1X. For comparison, *F. tularensis* growth was also evaluated in 1X BVFH which either had a final concentration of 5% or 10% Fildes Enrichment. Prior to autoclaving or filter-sterilizing, the pH was adjusted with pellets of NaOH if the starting pH was below 7.

Later experiments used a 6X BVFH medium to address the issues of reduced Fildes Enrichment concentration and the observed precipitation of medium components when the medium was prepared at a 10X concentration. The 6X BVFH medium was prepared as follows: (1) BHI powder and L-histidine were dissolved in 28 mL Millipore water and brought to boiling for 1 minute followed by cooling the solution, (2) 12 mL of sterile Vitox Supplement was added, and (3) 60 mL of sterile Fildes Enrichment was added, and (4) the solution was filter-sterilized with a disposable filtration system for a final concentration of 10% Fildes Enrichment when diluted to 1X. The 6X solution was stored at 4°C until use. There was no evidence of any insoluble material or precipitate formed during storage of the 6X medium or during incubation/cell growth for the 6X solution diluted to 1X.

2.2 *Francisella* tularensis Schu S4 Cell Suspension Preparation

F. tularensis Schu S4 cell stocks were prepared in BVFH or MH2+ growth medium and grown to mid-to late-log phase and then supplemented with 15% (v/v) glycerol for storage at -80°C. Frozen stocks were used to start cultures on Chocolate Agar plates (Hardy Diagnostics, Santa Maria, CA; Cat. No. E14) for experiments. Liquid growth medium was inoculated from cells on 3–-day old streak plates. Initially, overnight 5-mL cultures were started from 2-3 colonies each (from a streak plate) and were incubated with shaking (170 or 180 rpm) at 37°C. Later, to provide more reproducible inocula, three selected colonies were suspended in 200 µL or 500 µL PBS, which was mixed well, and 20 µL were used to inoculate replicate 5-mL cultures. The inoculum was also serially-diluted and plated to determine the number of cells added to the culture. This 5-mL overnight culture was incubated for 15 to 24 hour (h), as specified below for individual experiments. Typically, the overnight culture was prepared in 1X BVFH and grown to an OD_{600} (optical density at 600 nm) of ~ 1.5 which was diluted 10-fold in 1X BVFH (~1 ×

10^9colony colony-forming units ([CFU)/]/mL), followed by centrifugation (3,000 × *g* for 10 min at 4°C), washing one time in PBS buffer, and centrifugation was repeated. Then, the cell pellet was suspended in sterile PBS buffer, and diluted in PBS to obtain the desired cell density.

In some cases, as specified below, the resulting culture suspension was then acclimated to low nutrient conditions by storage in buffer at 4°C for 24 h prior to use (which also mimicked storage at 4°C prior to sample analysis). In other cases, as described below, cells were used directly after washing and resuspension, and compared to cells that were held for 24 h. The cells used directly after washing and resuspension were also serially-diluted and plated to determine the number of cells before storage at 4°C. The cells were also plated after the 24-h hold at 4°C to determine any loss in cell viability.

2.3 Sample Matrix Used in This Study

As per EPA protocol (US EPA, 2017), a large volume water sample (1 – 2 L) is typically collected and concentrated onto filter media, after which bacterial contaminants are recovered from the filter by washing with PBS for subsequent analysis. Considering such use of PBS for recovering and suspending the pathogens from the water samples, PBS (Teknova, Inc., Hollister, CA; Cat. No. P0261) was used as a substitute for water samples because it maintained cell viability and represented a reproducible matrix in terms of pH and chemical composition to facilitate consistent experimental results during the RV-PCR method development. Throughout the report, the term "sample" refers to *F. tularensis* Schu S4 cell suspensions prepared in PBS. Materials were added to this buffer including: i (1) iron sulfate heptahydrate (Sigma-Aldrich, Cat. No. 215422) and humic acid (Sigma-Aldrich, Cat. No. 53680-10G) to represent chemical interferences and (2) ATD (Section 2.4) to represent chemical, biological (live, nontarget microorganisms), and physical challenges.

2.4 Addition of Dust Background

ATD (International Organization for Standardization (ISO) 12103-1, A3 Medium Test Dust; Powder Technology, Arden Hills, MN) was used to evaluate biological and chemical inhibition effects on *F. tularensis* growth and PCR. Chemical composition analysis performed by the manufacturer indicated the material consisted of: SiO_2 (68–76%), Al_2O_3 (10–15%), Fe_2O_3 (2–5%), Na_2O (2–4%), CaO (2–5%), MgO (1–2%), TiO_2 (0.5–1.0%), and K_2O (2–5%). Dust was added at 4 mg/mL. While the microbial component is likely variable, the dust was shown to contain ~5 x 10^4 CFU/10 mg background microbes including fungi and bacterial spores in previous studies (Rose et al., 2011). Dust was nonsterilized, made into a slurry, and added to samples at a final concentration of 4 mg/mL.

2.5 Preparation of $FeSO_4$ and Humic Acid Solutions as Challenge Materials

Iron sulfate hexahydrate and humic acid were added to samples to test for chemical interferences affecting *F. tularensis* growth and PCR. Humic acid was used as a surrogate for natural organic matter. An $FeSO_4$ solution was prepared in sterile distilled, deionized (DD) water and added to samples at a final concentration of 10 micrograms (µg)/mL Fe^{2+}. A humic acid solution was also prepared in sterile DD water and added to samples at a final concentration of 50 µg/mL. These concentrations were at the upper end of the range of values expected for drinking water samples (NRC, 1979; WHO, 1996; US EPA, 2005).

2.6 Rapid-Viability PCR Method

An outline of the RV-PCR protocol steps is shown in Figure 3, including pictures of some of the equipment used in sample processing and analysis. The method was like the method developed for *Y. pestis* (US EPA, 2016), although 6X concentrated media were used. In contrast to the RV-PCR protocol for *B. anthracis* spores (US EPA, 2017), multiple vacuum filtration steps for concentration and buffer washes could not be used to concentrate vegetative cells and reliably maintain their viability. Therefore, the water sample (PBS) was not vacuum filtered but rather was prepared using 6X-concentrated broth, in the proper ratio, to use as much of the sample as possible and still provide optimal growth conditions. The wells of the 48-well plates accommodate 5 mL such that up to 3.0 mL water sample and 0.6 mL 6X broth were used.

After mixing by pipettor, a 0.5-mL aliquot removed from the total 3.6mL in each sample well before incubation (time 0, prior to incubation (T_0) aliquot), transferred to 2-mL Eppendorf tubes, and centrifuged at 20,800 relative centrifugal force (RCF) for 10 min at 4°C, after which 300 µL were removed and discarded. The cell pellets in the remaining 0.2 mL were then frozen prior to deoxyribonucleic acid (DNA) extraction and PCR analysis following the protocol detailed below. During method development, in some cases (as specified in this report), 0.25 mL aliquots were removed and processed as described above except that only 50 µL was removed, leaving 0.2 mL pellets. The 48-well plate was sealed with a sterile AeraSeal™ breathable adhesive seal (Excel Scientific, Victorville, CA; Cat. No. BS-25) and incubated for different time periods from 18 to 40 h at 37°C with shaking at 180 revolutions per minute (rpm) prior to removal of the 0.5-mL aliquots (or 0.25-mL aliquots as noted) for the different time points. Aliquots were typically stored at -20°C and processed 1–2 days after receipt. However, samples could be processed immediately if staff were working in shifts (e.g., three, eight-hour shifts per day) to address sample volume and decrease time to results. While manual processing was used in this study, automated DNA extraction protocols could also be used.

Each 0.2 mL suspension (re-suspended pellet) was processed for DNA extraction and purification using the Promega paramagnetic particle (PMP)-based kit (MagneSil® Blood Genomic, Max Yield System; Promega, Madison, WI; Cat. No. MD1360). This kit enables DNA recovery from multiple complex samples simultaneously using a magnetic bead-based cleanup method. The method used was the same as the method developed for *Y. pestis* DNA extraction (US EPA, 2016), which was modified from the method developed for *B. anthracis* cells (Létant et al., 2011). When used with the appropriate buffers, the PMPs bind and later release DNA with appropriate buffers resulting in DNA concentration and purification.

Briefly, the cell pellet in the remaining 200-µL aliquot was thawed and 800-µL Lysis Buffer were added. The mixture was vortex mixed and incubated for 5 min. Next, 600-µL of paramagnetic particle (PMP) mix were added and mixed by vortexing. The tubes were placed on a magnetic rack, the PMPs were adhered to the side of the tube next to the magnet, and the supernatant was removed by pipetting. One lysis wash step with 360 µL of Lysis Buffer was included, followed by vortex-mixing, placing on the magnetic rack, and subsequent supernatant removal. Two washes with 360 µL of Salt Wash were then performed, in each case followed by mixing by vortexing and removal of the supernatant. Finally, two washes with 500 µL of Alcohol Wash solution were performed with mixing by vortexing and supernatant removal. A

final wash with 70% ethanol was included to enhance PMP drying. PMPs were air-dried for 2 min and then dried at 80°C for 20 minutes.

DNA was then eluted by addition of Elution Buffer followed by cycles of vortexing and heating at 80°C. Samples were cooled for 5 min prior to mixing and transferring to the magnetic rack. Typically, 170–200 µL were recovered and transferred into a clean 1.5 mL Eppendorf tube. If particles remained, the sample was subsequently centrifuged at 20,800 RCF for 5 min at 4°C, and the supernatant was transferred to a clean Eppendorf tube. The DNA extracts were stored at -20°C until they could be analyzed by real-time PCR. Both undiluted and 10-fold-diluted DNA extracts (prepared in PCR water) for both T_0 and later time points were also analyzed by PCR to check for PCR inhibition (i.e., if the difference between C_T values for 10-fold diluted and undiluted extracts is negative and/or significantly less than three).

2.7 Automated DNA Extraction-Purification

The protocol for DNA extraction of *F. tularensis* cells on the automated Roche MagNA Pure Compact instrument was based on the vendor's protocol using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, Indianapolis, IN; Cat. No. 03730964001), but modified by adding a heat lysis step, as described in section 2.8. The sample pellets taken at T_0 and T_f were thawed and treated by heat lysis at 70°C for 10 min prior to the Roche chemical lysis protocol. After heat lysis, samples were cooled for three minutes and 300 µL Roche chemical lysis buffer was added, followed by vortexing tubes for 10 seconds (sec) at 1,800-2,000 rpm, and incubation at room temperature (22–25 °C) for 30 min. Samples were briefly vortexed every 5 min during the 30-min incubation. Samples were processed on MagNA Pure® Compact robot with DNA-Bacteria Purification Protocol (Appendix A).

2.8 Protocol for *Francisella tularensis* Schu S4 Inactivation for Automated DNA Extraction-Purification in the Biosafety Level-3 (BSL-3) Laboratory

The protocol for inactivation of *F. tularensis* cells for subsequent DNA extraction on the automated Roche MagNA Pure® Compact instrument was developed based on an LRN protocol (CDC, personal communication). The protocol was required since *F. tularensis* cell lysates needed to be removed from the biosafety cabinet (BSC) to complete the DNA extraction process on the robot outside the BSC; this procedure constituted a removal from containment (per the Centers for Disease Control and Prevention [CDC]) although the robot is in the same laboratory.

For approval of the inactivation procedure by the LLNL Biosafety Office, triplicates of both inactivated samples and positive controls (not inactivated) were to be tested in three separate experiments. In these tests, 100% of the inactivated material (or controls) were plated, with plates incubated under optimal growth conditions for 96 h. Further guidance was provided from CDC via the Select Agent Facility staff that an additional experiment was required to assess whether the chemicals used to inactivate cells were sufficiently washed from the samples (by repeated centrifugation and resuspension) prior to culture analysis. In this case, the inactivated samples were split in half after wash steps, with half plated directly and the other half spiked with a known amount of *F. tularensis* cells prior to plating (where growth would indicate sufficient removal of the inactivating chemicals). After approval of the procedure, cell lysates could be removed from the BSC and processed directly on the Roche platform.

The inactivation procedure was reviewed by the Select Agent Facility staff including the LLNL responsible official, and approved after some iteration. In addition, a checklist was generated to document all the conditions of the experiments, including that starting cell densities were within specified ranges and that all materials were within expiry dates. The checklist was also reviewed and approved by the responsible official and Select Agent Facility staff for meeting adequate biosafety procedures in the laboratory.

After reviewing preliminary data on cell inactivation with the initial chemical lysis (30 minutes (min) at room temperature with mixing every 5 min), the EPA technical lead suggested adding a step to perform heat lysis at 70°C for 10 min prior to conducting the Roche chemical lysis protocol. Changes were made to the Inactivation Protocol and Checklist as appropriate, and the modified procedure was submitted for review and approval by the LLNL responsible official.

2.9 *F. tularensis* DNA Standards for Real-Time PCR

F. tularensis DNA standards were generated from harvested cells from overnight incubation of 5 mL MH2+ cultures inoculated from 2-3 individual colonies from Chocolate Agar plates. A MasterPure™ Complete DNA and RNA (ribonucleic acid) Purification Kit (Epicentre® Technologies Corp., Madison, WI; Cat. No. MC85200) was used to extract genomic DNA from the pure culture following the manufacturer's protocol. This kit is designed for producing genomic DNA from a small number of larger volume cultures to generate higher quantities of DNA, whereas the Promega MagneSil kit, optimized for use in RV-PCR (Section 2.8), is designed for many small sample volumes (0.2 mL after concentration via centrifugation). The resulting genomic DNA was measured using the high sensitivity Quant-iT™ DNA assay (Invitrogen, Carlsbad, CA; Cat. No. Q32854) with a Qubit™ fluorometer (Cat. No. Q33216). Standard concentrations prepared in PCR-grade water ranged from 1 femtogram (fg)/microliter (µL) to 1 nanogram (ng)/µL. Each PCR plate contained seven 10-fold dilutions, ranging from 5 fg per 25-µL PCR to 5 ng per 25-µL PCR.

2.10 Real-Time PCR Analysis

The foundation for RV-PCR assay development is sensitive and specific real-time PCR assays. In a previous study, high-quality signatures using computational tools for primer and TaqMan® probe design, which were developed by Dr. Sanjiv Shah (while at Edgewood Chemical and Biological Center of the U.S. Department of Defense) and LLNL, were used to design *F. tularensis* real-time PCR assays (S. Lètant, personal communication). In addition, *in silico* analysis (i.e., performed via computer simulation) and rigorous wet-chemistry screening approaches were used to further narrow the selection of candidate signatures, by screening against an extensive panel of environmental extracts, bacteria, eukaryotes, near-neighbors, and target strain DNAs. Furthermore, the selected assays were tested against target DNA templates to yield sensitive assays. Two assays with the best sensitivity were selected for this effort to optimize the RV-PCR protocol. Both assays target the *pdp*D, pathogenicity determinant protein D unique to Type A *F. tularensis* with the F4 assay designed at LLNL and the F5 assay designed at the Centers for Disease Control and Prevention (Kugeler et al., 2006).

An Applied Biosystems® 7500 Fast Real-Time PCR System (Foster City, CA) was used to perform real-time PCR. Each well of a 96-well PCR plate contained five-microliter (µL) sample aliquots added to 20 µL of PCR mix. The PCR mix (Appendix A, Page 64) contained TaqMan® 2X Universal PCR Master Mix (Life Technologies, Carlsbad, CA; Cat. No. 4304437), which included AmpliTaq Gold® DNA polymerase, deoxynucleotide triphosphates, a 6-Carboxyl-X-Rhodamine (ROX) passive reference dye (for signal normalization), and AmpErase® UNG (uraciluracil-N-glycosilase), which prevented carry-over contamination (from PCR products). The mix also contained forward and reverse primers, and a probe labeled at the 5' end with FAM (6-carboxyfluorescein) for the reporter dye and labeled at the 3' end with Black Hole Quencher® (BHQ-1) for the quencher dye. Finally, Platinum™ Taq DNA Polymerase (Invitrogen, Cat. No. 10966026) was included at 0.25 µL per reaction (1.25 U) since it was shown to improve assay sensitivity. The assay primer and probe sequences are listed in **Table 1**. PCR-grade water (Teknova, Cat. No. W3350) was used to make the mix volume up to 20 µL per reaction, and 5 µL of sample was added to bring the total volume to 25 µL. The following cycling conditions were used: 2 min at 50ºC for UNG incubation, 10 min at 95ºC for DNA polymerase activation, and 45 amplification cycles (5 sec at 95ºC for denaturation and 20 sec at 60ºC for annealing/extension). Three replicate samples were analyzed per experimental condition, and three replicate PCR analyses were conducted per sample replicate. DNA extracts from different time points from the same samples were analyzed on the same plate to standardize the analysis conditions. The ROX dye in the Applied Biosystems, Inc. (ABI) Universal Master Mix was used to normalize the fluorescent reporter signal. Automatic baseline and threshold settings were used throughout.

Table 1. Nucleotide Sequences of the Primer/Probe Sets Used for *F. tularensis* RV-PCR Analysis

Assay	Forward Primer*	Reverse Primer*	Probe*	Amplicon Length (bp)
F4	TTGCTCCAGTAGCTGC AAGATT	CCAAGTGCTT GGTGGTGGTA	FAM-TGCTGCCGAGATGTT TTCATTATTAACTGA TGC-BHQ	125
F5	GAGACATCAATTAAAA GAAGCAATACCTT	CCAAGAGTAC TATTTCCGGTT GGT	FAM-AAAATTCTGCTC AGCAGGATTTTGAT TTGGTT-BHQ	105

* Sequences are listed in 5' to 3' orientation. Acronyms: Bp, base pair; FAM, fluorescein; BHQ, Black Hole Quencher

2.11 Interpretation of RV-PCR Results

As a starting point for RV-PCR detection of viable *F. tularensis* cells, the criteria developed for *B. anthracis* and *Y. pestis* were employed. Specifically, for positive detection, the incubation endpoint PCR C_T or C_T value at time final (post-incubation), C_T (T_f) ≤ 39 and the ΔC_T (C_T [T_0] – C_T [T_f]) ≥ 6 (where f = final) were required. For initial optimization, most of the work was

conducted with 24-h or 30-h incubation, such that $T_f = T_{24}$ or T_{30}. For cases where no PCR response was obtained (non-detect [NDT] results), the C_T values were set to 45 (since 45 PCR cycles were used), to calculate ΔC_T. A $\Delta C_T \geq 6$ represented an increase in DNA concentration of approximately 2-log (100-fold) because of the presence of viable cells in the original sample that propagated during incubation. Depending on end user requirements, a higher ΔC_T (C_T [T_0] – C_T [T_f]) ≥ 9 (approximately three log-increase [1000-fold] in DNA concentration), and a corresponding lower endpoint (C_T of ≤ 36) could be used. For individual replicates within an experiment, the RV-PCR result was considered positive when at least two of three replicates met the algorithm requirement. Specifically, if two or three of three PCR replicates were NDT, then a C_T value of 45 was assigned as the average T_0 C_T (C_T value at time zero [pre-incubation]) or T_f C_T (as appropriate) to calculate ΔC_T; whereas, if two or three of three replicates showed positive C_T values, the average of the positive C_T values was used to calculate ΔC_T.

The RV-PCR method sensitivity of detection was equivalent to the *F. tularensis* cell level where 100% of the spiked samples had $\Delta C_T \geq 6$. This value was essentially an analytical sensitivity of detection for the RV-PCR method and did not account for any losses that could occur from sampling and sample handling prior to RV-PCR analysis.

2.12 Data Analysis and Presentation

The criteria for positive/negative detection was based on both ΔC_T and the time final after incubation (T_f) C_T. Data tables show both individual PCR replicates as well as averages and standard deviations calculated in Microsoft Excel®. As mentioned above, if a single PCR replicate were positive and the other two replicates were non-detect, the sample was considered NDT and the sample T_0 or T_f C_T value was set to 45 to calculate ΔC_T. Single replicate positive high C_T values (e.g., 39–44) were likely due to cross contamination. For two or three positives of three PCR replicates per sample, the average and standard deviation were calculated.

The overall SD from all sample replicates was calculated using the following equation,

$$\text{Overall or joint SD} = \sqrt{\{[(n_1 - 1){s_1}^2 + (n_2 - 1){s_2}^2 + (n_3 - 1){s_3}^2 + (n_1 \times [X_1 - \bar{X}]^2) + (n_2 \times [X_2 - \bar{X}]^2) + (n_3 \times [X_3 - \bar{X}]^2)]/(n_1 + n_2 + n_3 - 1)\}}$$

where n_1, n_2, and n_3 = the number of PCR analyses per sample for sample replicates 1, 2, and 3; s_1, s_2, and s_3 = the standard deviation (SD) of the C_T values for the individual samples; X_1, X_2, and X_3 = the average C_T values for the individual samples; $\bar{X}$ = the overall average C_T value for the samples. The overall SD equation was modified accordingly for either two or four replicate samples or positive controls. In cases where three replicate PCR analyses per sample (or control) were conducted, the overall average for the replicate samples (or controls) was simply the average of the individual sample (or control) averages. Culture data are shown in CFU/mL or CFU/sample (corrected for dilution) based on the average and SD of triplicate plates with colony counts within the range of 25–250 CFU/plate.

3. Quality Assurance and Quality Control

3.1 Laboratory Inspections

Monthly laboratory inspections were conducted by the project principal investigator to comply with Department of Energy and CDC safety and security policies. In addition, the LLNL responsible official and/or biosafety officer conducted annual laboratory inspections. Inspections included the following:

- Documenting laboratory cleanliness;
- Certifying laboratory safety equipment, including the BSC, robotic enclosure, and autoclave;
- Reviewing waste handling procedures;
- Taking inventory of select agents (in addition, 25% inventory conducted quarterly); and
- Reviewing personnel training.

3.2 Calibration

The ABI 7500 Fast PCR instrument was calibrated and underwent preventive maintenance conducted annually. Micropipettors were inspected and calibrated by the vendor annually; in addition, quarterly in-house pipettor calibration was conducted gravimetrically. Balances were calibrated annually using National Institute of Standards and Technology (NIST)-traceable standard weights. Records from these calibration activities were documented and reviewed by the project principal investigator.

3.3 Storage Conditions

An alarm system was used for refrigerators and freezers to ensure storage conditions were within acceptable ranges. In addition, NIST-traceable temperature-recording devices were included where PCR reagents and frozen cell pellets (for DNA processing) and DNA extracts were maintained. The temperature was recorded daily to ensure the proper range was maintained. NIST-traceable thermometers were placed in each incubator as well to provide temperature monitoring.

3.4 Spiking

Plating of the initial cell suspensions (or inoculum) and one or more negative samples (samples spiked with PBS) to test for cross-contamination was conducted for each experiment.

3.5 Real-time PCR Analysis

During the experiment, *F. tularensis* Schu S4 extracted DNA standards were analyzed on every PCR plate, along with the samples, as described in the Materials and Methods Section 2.10, to verify reagent quality and instrument performance. DNA standards were prepared from *F. tularensis* Schu S4 cells as described in the Materials and Methods section.

3.6 Replication

In general, for each treatment in an experiment, a minimum of three replicate samples were analyzed. Replicate samples were spiked at the same time using the same cell suspension dilution and processed at the same time following the same laboratory processes. Results are presented as average C_T values (for the RV-PCR method) or CFU for the spread plate method, with corresponding SD.

3.7 Controls

Negative controls included in the experiments used the same matrix as the test samples with no cells added. These controls served as a cross-contamination check and the experiment was to be repeated if negative controls showed positive results. A negative (No-Template Control, NTC) was also included with each PCR plate to check for PCR contamination. If the negative control showed positive PCR results, extra care was taken to decontaminate work surfaces and prepare new reagents followed by repeating the PCR analysis.

3.8 Data Quality Objectives/Data Quality Indicators

The objective of this research effort was to develop a qualitative, RV-PCR-based detection method for *F. tularensis*. Balance, pipettor, and PCR cycler instruments were calibrated at the following intervals—annually for the balance and cycler and quarterly for the pipettors. Calibrations were not found to be out of range (e.g., within 0.01%). For cases where the data quality was outside the acceptable range (i.e., if a negative control showed one of three positive PCR results due to potential PCR cross-contamination), the PCR analysis was repeated to ensure the expected result was obtained. Throughout the study, negative controls showed negative results across triplicate analyses. In addition, PCR standard curves compared between plates within an experiment were used to confirm variability between replicate DNA standards (within one C_T value of the average). For individual replicates within an experiment, the RV-PCR result was considered positive when at least two of three replicates met the algorithm requirement as described in Section 2.11. In general, replicate experiments showed consistent trends; any deviations as well as potential explanations for slight discrepancies are included in the report.

4. Results and Discussion

The following section presents results for the three project tasks and also provides some discussion of results. In addition, specific details relevant to the given experiment are included such as cell concentrations tested, broth used, and PCR assay employed, allowing the relevant information to be near the results to better understand the relationship between the experiment variables and the data. The relevant Materials and Methods sections provide general information whereas the paragraph(s) before the results description in this section provide specific information.

4.1 Task 1. Optimize *F. tularensis* growth in concentrated medium for RV-PCR analysis

4.1.1. Task Objectives

The objective of the first task was to optimize growth of *F. tularensis* Schu S4 using concentrated medium diluted to 1X to analyze larger volume water samples under optimal growth conditions. For high-throughput sample processing and analysis, the 48-well format was used. This format was also used for RV-PCR analysis of *Y. pestis*, which served as the model for this method. This task also included evaluation and optimization of real-time PCR assays and assessment of the concentrated growth medium via RV-PCR analysis.

4.1.2 Comparison and Selection of Growth Medium for F. tularensis Schu S4 Propagation

In a preliminary experiment, 1X BVFH medium was compared with 1X MH2+ medium to determine which produced higher Schu S4 cell densities determined spectrophotometrically. The experiment was used to confirm results from an earlier study in which the BVFH medium was shown to be superior to other growth media tested (Morris et al., 2017). MH2+ medium has been used by researchers at LLNL to propagate *F. tularensis*. Similar but not identical media to MH2+ were tested by Morris et al. (2017). Overnight 5-mL cultures using both media types were started from two colonies each (from a streak plate) and were incubated with shaking (170 rpm) at 37°C. The OD_{600} values after 24-h incubation are shown in **Table 2**. The second experiment likely had a slightly higher inoculum density since the final OD values were higher than the OD values for the first experiment. For two different experiments, growth on BVFH produced higher OD values compared to MH2+. Therefore, for subsequent method development, BVFH medium was used.

Table 2. Growth of *F. tularensis* Schu S4 on 1X BVFH and MH2+ medium

Growth Medium	OD_{600} after 24-h**		
	Expt. 1	Expt. 2	
	Undiluted	Undiluted	10-fold Diluted
1X BVFH	1.26	>2*	0.31
MH2+	0.92	1.4	0.16

* The UV-Vis spectrometer plateaued at 2.

** OD_{600} values are from duplicate measurements from a single aliquot (Expt. 1) or from single measurements (Expt. 2). Dilution was performed using the same growth medium as for culturing cells.

4.1.3 Evaluation of F. tularensis Schu S4 Growth on 10X BVFH Diluted to 1X Compared to 1X BVFH Medium

An experiment was conducted as described above although in this case, three *F. tularensis* Schu S4 colonies were suspended in 200 µL PBS, and then 20 µL was used to inoculate replicate 5 mL cultures of either 1X BVFH medium or 10X BVFH diluted to 1X using PBS as diluent. This inoculum preparation method was used for subsequent experiments as well. The starting cell

density in this case was determined to be ~2.7×10^7 CFU/mL. High numbers of cells were used as inoculum so that UV-Vis spectroscopy could be used to estimate cell growth and screen treatments for subsequent culture plating analysis. The 10X BVFH medium was prepared as described in Section 2.1 (and in Appendix A). In this experiment, the pH of the 10X diluted medium was not adjusted and when later tested, it was 6.62 ± 0.02 (for triplicate measurements) and the pH for 1X BVFH was measured at 7.16 ± 0.004. The lower pH could have negatively impacted growth. When BHI was prepared as 1X, the pH was ~7.4. However, when prepared as a 30 mL 10X stock, the pH was low (~6.6), and after autoclaving and adding the Fildes Enrichment (50 mL) and Vitox Supplement (20 mL) the pH was 6.8.

The results from the growth experiment (15 h) showed that OD_{600} values for 1X culture replicates ranged from 0.5 to 0.35, while there was no change in OD values (i.e., no growth) for the 10X diluted to 1X culture (0.02). The OD measurements were confirmed by culture plating showing ~ 2-log increase in growth on 1X BVFH and no increase in cell number for 10X BVFH diluted to 1X (data not shown). While the final concentration of Fildes Enrichment is 10% in the 1X BVFH medium and 5% in the 10X BVFH medium diluted to 1X, this factor was not thought to account for the complete lack of growth with the latter medium. Alternatively, the poor *F. tularensis* growth could have been due to the lower pH; therefore, a subsequent experiment was conducted in which the 10X BVFH diluted to 1X medium was pH-adjusted to 7.2 prior to testing. In addition, other formulations were tested to trouble-shoot the poor growth on 10X BVFH diluted to 1X since we noted that some 10X BVFH medium components may have precipitated, thereby effectively reducing the available (soluble) nutrient content. These other formulations included Butterfield's Buffer or sterile ddH_2O used as diluents instead of PBS. Furthermore, 10X BVFH medium that was filter-sterilized (0.2-micron filter) was tested in addition to one prepared by autoclaving the BHI/L-histidine stock prior to aseptic addition of supplements. In all cases, the pH was adjusted with addition of NaOH pellets if the starting pH was below 7. Additional information for media preparation is included in Section 2.1.

After medium preparation, 5-mL cultures were inoculated as described previously in this case, to a final cell density of 8.1×10^6 CFU/mL from plating analysis. The 5-mL cultures were incubated for 15 h at 37°C (180 rpm). In some cases, precipitates were observed for 10X medium as particles adhered to the glass container. Whether any precipitates re-dissolved could not be determined. However, this phenomenon was not ideal for standardized growth conditions, and the media with and without inoculum (negative control) showed similar OD values. Only the 1X BVFH medium supported *F. tularensis* growth, with an ~2-log increase over the 15-h incubation (data not shown). Based on these issues, a less concentrated medium was tested, that would still enable use of a 3-mL water sample for a better sensitivity of detection, as described below.

4.1.4 Evaluation of F. tularensis Schu S4 Growth on 6X BVFH Medium Diluted to 1X Compared to 1X BVFH Medium

Since the 10X BVFH medium diluted to 1X did not support Schu S4 growth, a 6X growth medium was tested (prepared as described in Section 2.1). This formulation also enabled a final Fildes concentration of 10% when diluted to 1X. PBS was used to dilute the 6X medium to 1X, and 5-mL cultures of each media type were inoculated as described (Section 2.2). Actual counts were 1.5×10^7 CFU/mL from plating analysis. Schu S4 growth after 18.5 hours is shown in

Table 3 as OD_{600} and CFU/mL from plating analysis. In addition, a p-value from a t-test comparing CFU/mL for the two different media formulations is listed. Growth of Schu S4 on 1X BVFH medium was superior to the growth of Schu S4 on 6X diluted to 1X ($p = 0.01$), although both media types supported more than 2-log increase in cell number over the incubation period.

Table 3. Growth of *F. tularensis* Schu S4 on 6X BVFH Diluted to 1X vs. 1X BVFH Medium

Medium*	Sample Replicate	OD_{600} after 18.5 h	Avg CFU/mL after 18.5 h	t-Test (p-value): CFU/mL
6X BVFH diluted to 1X	1	1.28	7.97×10^9	0.01
	2	1.35	8.60×10^9	
	3	1.31	7.97×10^9	
	Avg. (SD)*	**1.31 (0.04)**	**$8.18\ (0.37) \times 10^9$**	
1X BVFH	1	1.54	1.15×10^{10}	
	2	1.60	1.11×10^{10}	
	3	1.47	1.13×10^{10}	
	Avg. (SD)*	**1.54 (0.07)**	**$1.13\ (0.022) \times 10^{10}$**	

* Data are the average (Avg.) and standard deviation (SD) from triplicate samples.

A replicate experiment was conducted to compare the two media types. Five-mL cultures were inoculated to yield starting cell numbers of 9.7×10^6 CFU/mL (from plate count analysis). After incubation for 18.5 h, OD_{600} measurements were taken, and dilution series with plating was conducted. For this experiment, the culture results were more comparable between media types (**Table 4**). There was no significant difference between the 6X diluted to 1X medium and the 1X medium ($p \sim 0.18$). Therefore, the 6X BVFH medium diluted to 1X was used for the RV-PCR method optimization.

Table 4. Growth of *F. tularensis* Schu S4 on 6X BVFH Diluted to 1X vs. 1X BVFH Medium – Replicate Experiment

Medium	Sample Replicate	OD_{600} after 18.5 h	Avg. CFU/mL after 18.5 h	t-test (p-value): CFU/mL
6X BVFH diluted to 1X	1	1.18	3.90×10^9	0.18
	2	1.16	4.53×10^9	
	3	1.17	4.77×10^9	
	Avg. (SD)*	**1.17 (0.01)**	**4.40 (0.45)** $\times 10^9$	
1X BVFH	1	1.23	4.57×10^9	
	2	1.28	7.43×10^9	
	3	1.21	5.60×10^9	
	Avg. (SD)*	**1.24 (0.04)**	**5.87 (1.45)** $\times 10^9$	

Data are the average (Avg.) and standard deviation (SD) from triplicate samples.

4.1.5 Evaluation of F. tularensis Real-Time PCR Assays for RV-PCR Analysis

The *F. tularensis* F4 and F5 real-time PCR assays were tested with Schu S4 genomic DNA using Universal 2X PCR Master Mix and standard conditions with and without inclusion of Platinum (Plat) Taq DNA Polymerase (0.25 µL per reaction) as described in Section 2.10. The genomic DNA preparation measured by UV-Vis spectrometry showed 228 ng/µL and the 260/280 ratio was 2.05, showing good quality DNA. When measured by the Qubit HS dsDNA assay, the quantity was 110 ng/µL; the difference in quantities by methods was likely due to UV-Vis spectroscopy measuring RNA in addition to DNA. Appropriate dilutions were made based on the Qubit® measurement to test 500 picograms (pg) to 5 fg genomic DNA. The results are shown in **Table 5**. The data showed that addition of Platinum™ Taq enzyme resulted in an average of 9.8 and 8.9 lower C_T values for the F4 and F5 assays, respectively. Therefore, Platinum™ Taq polymerase was included in the PCR mix used for subsequent experiments.

Table 5. Real-time PCR Results for F4 and F5 assays for *F. tularensis*

DNA (pg)	Replicate	Avg. C_T (SD)*		C_T Difference	Avg. C_T (SD)*		C_T Difference
		F4	F4 + Taq		F5	F5 + Taq	
500	1	27.2 (0.4)	18.6 (0.2)	-8.6	27.6 (0.6)	19.6 (0.02)	-8.0
	2	27.1 (0.6)	18.6 (0.1)	-8.5	27.3 (0.5)	19.4 (0.05)	-7.8
	Avg. (SD)	**27.2 (0.4)**	**18.6 (0.1)**	**-8.6 (0.1)**	**27.5 (0.4)**	**19.5 (0.09)**	**-7.9 (0.1)**
50	1	30.7 (0.6)	22.2 (0.07)	-8.5	30.8 (0.3)	23.0 (0.09)	-7.8
	2	30.9 (0.7)	22.1 (0.1)	-8.8	31.2 (0.6)	22.9 (0.07)	-8.3
	Avg. (SD)	**30.8 (0.5)**	**22.2 (0.07)**	**-8.7 (0.2)**	**31.0 (0.4)**	**23.0 (0.07)**	**-8.1 (0.4)**
5	1	35.1 (0.8)	25.7 (0.2)	-9.4	35.0 (0.5)	26.4 (0.09)	-8.7
	2	35.7 (0.7)	25.7 (0.09)	-10.0	35.3 (0.8)	26.3 (0.1)	-9.0
	Avg. (SD)	**35.4 (0.6)**	**25.7 (0.1)**	**-9.7 (0.4)**	**35.2 (0.5)**	**26.4 (0.08)**	**-8.9 (0.2)**
0.5	1	39.8 (0.8)	29.2 (0.2)	-10.6	38.9 (0.3)	30.0 (0.06)	-9.0
	2	40.1 (0.7)	29.2 (0.1)	-10.9	39.7 (0.7)	29.2 (0.1)	-9.7
	Avg. (SD)	**40.0 (0.6)**	**29.2 (0.1)**	**-10.8 (0.2)**	**39.3 (0.5)**	**29.6 (0.4)**	**-9.4 (0.5)**
0.05	1	43.8 (1.0)	33.0 (0.5)	-10.9	43.0 (0.4)	33.2 (0.1)	-9.9
	2	44.2 (0.9)	32.8 (0.2)	-11.4	44.1 (0.09)	33.5 (0.1)	-10.6
	Avg. (SD)	**44.0 (0.7)**	**32.9 (0.3)**	**-11.2 (0.4)**	**43.6 (0.5)**	**33.4 (0.2)**	**-10.3 (0.5)**
0.005	1	NDT	36.3 (0.5)	NA	NDT	36.9 (0.3)	NA
	2	NDT	36.3 (0.8)	NA	NDT	37.3 (1.2)	NA
	Avg. (SD)	**NA**	**36.3 (0.5)**	**NA**	**NA**	**37.1 (0.6)**	**NA**
Overall C_T Difference Avg. (SD)				**-9.8 (1.1)**			**-8.9 (1.0)**

***** Data are averages (Avg.) and standard deviations (SD) from three replicates. Data were generated using manual threshold settings, and thresholds were the same for both assays with and without Plat Taq Polymerase (Taq).
NA, not applicable; NDT, non-detect.

4.1.6. Evaluation of F. tularensis Schu S4 Growth and RV-PCR Performance: Effect of Holding Cells at 4°C Prior to Use

An experiment was conducted to determine whether 24 h was sufficient to detect viable cells from water samples by RV-PCR using the established parameters including dilution of 6X BVFH medium to 1X with addition of the water sample (3 mL water sample plus 0.6 mL 6X

medium) and incubation in 48-well plates (37°C, 180 rpm). Cells for inoculum were prepared as described previously (Section 2.2). In addition, since cells may be held for up to 24 h prior to sample receipt and analysis, a treatment was included where cells were held at 4°C for 24 h after dilution in PBS to 10^4, 10^3, and 10^2 CFU per sample. This 4°C-hold treatment was compared with cells prepared in PBS and used directly for culture and RV-PCR analysis. The starting (inoculum) and ending (T_{24}) cell densities were determined by dilution plating onto Chocolate Agar, and 0.5 mL aliquots were analyzed at T_0 and T_{24} by RV-PCR for both types of cells, those used directly and those stored for 24 h.

Culture and RV-PCR results are shown in **Table 6** for cells used directly and in **Table 7** for cells held for 24 h. The cells used directly were approximately 0.1 log higher (log CFU/mL) than those that were held for 24 h. However, there was a greater log difference in growth when incubated for 24 h at 37°C, ~0.3 log higher for cells used directly on average. The log differences ranged from ~1.72 to 1.93 on average for cells used directly and 1.40 to 1.61 for cells held at 4°C for 24 h.

Table 6. RV-PCR and Culture Results for *F. tularensis* Schu S4: Cells Used Without 4°C Hold for 24 Hours

Starting Cell Density (CFU/mL)	Sample Replicate - PCR Replicate	F5 Assay C_T (SD)*		ΔC_T (T_0 – T_{24})	T_{24} Cell Density Avg. CFU/mL	Cell Density Log Change
		T_0	T_{24}			
7.0×10^3	1 – 1	32.7	23.1	9.6	6.7×10^5	1.98
	1 – 2	32.5	23.2			
	1 – 3	32.9	23.2			
	Avg. (SD)	**32.7 (0.2)**	**23.1 (0.06)**			
	2 – 1	32.1	23.7	8.3	7.2×10^5	2.01
	2 – 2	31.8	23.7			
	2 – 3	32.1	23.8			
	Avg. (SD)	**32.0 (0.2)**	**23.7 (0.05)**			
	3 – 1	31.3	24.2	7.2	4.4×10^5	1.80
	3 – 2	31.4	24.3			
	3 – 3	31.6	24.2			
	Avg. (SD)	**31.4 (0.2)**	**24.2 (0.06)**			
	Overall Avg.	**NA**	**NA**	**8.4**	**$6.1\ (1.5) \times 10^5$**	**1.93**
7.0×10^2	1 – 1	35.2	27.3	7.6	3.3×10^4	1.67
	1 – 2	35.4	27.3			
	1 – 3	34.2	27.3			
	Avg. (SD)	**34.9 (0.6)**	**27.3 (0.04)**			
	2 – 1	35.5	26.5	9.5	3.4×10^4	1.68
	2 – 2	35.7	26.5			
	2 – 3	36.8	26.4			
	Avg. (SD)	**36.0 (0.7)**	**26.5 (0.04)**			
	3 – 1	35.0	27.2	7.6	4.6×10^4	1.82
	3 – 2	35.0	27.4			
	3 – 3	34.7	27.4			

	Avg. (SD)	**34.9 (0.2)**	**27.3 (0.1)**			
	Overall Avg.	**NA**	**NA**	**8.2**	**3.7 (0.7) × 10^4**	**1.72**
7.0 × 10^1	1 – 1	36.1	30.2	6.3	4.9 × 10^3	1.85
	1 – 2	36.2	30.2			
	1 – 3	37.0	30.1			
	Avg. (SD)	**36.5 (0.5)**	**30.2 (0.1)**			
	2 – 1	39.2	30.7	8.0	4.7 × 10^3	1.82
	2 – 2	NDT	30.8			
	2 – 3	38.1	30.7			
	Avg. (SD)	**38.7 (0.8)**	**30.7 (0.07)**			
	3 – 1	NDT	30.9	8.4	5.4 × 10^3	1.89
	3 – 2	39.1	30.7			
	3 – 3	39.4	30.8			
	Avg. (SD)	**39.2 (0.2)**	**30.8 (0.1)**			
	Overall Avg.	**NA**	**NA**	**7.6**	**5.0 (0.4) × 10^3**	**1.85**

* Average (Avg.) and standard deviation (SD) for triplicate samples and triplicate PCR analyses per sample.
Acronyms: CFU, colony-forming units; C_T, cycle threshold; NA, not applicable; NDT, non-detect.

Table 7. RV-PCR and Culture Results for *F. tularensis* Schu S4: Cells Held for 24 Hours at 4°C

Starting Cell Density (CFU/mL)	Sample Replicate - PCR Replicate	F5 Assay C_T (SD)*		ΔC_T ($T_0 - T_{24}$)	T_{24} Cell Density Avg. CFU/mL	Cell Density Log Change
		T_0	T_{24}			
5.5 × 10^3	1 – 1	33.4	25.3	7.9	1.5 × 10^5	1.42
	1 – 2	33.6	25.7			
	1 – 3	33.2	25.5			
	Avg. (SD)	**33.4 (0.2)**	**25.5 (0.2)**			
	2 – 1	32.1	25.6	6.5	1.7 × 10^5	1.41
	2 – 2	31.8	25.4			
	2 – 3	32.3	25.7			
	Avg. (SD)	**32.1 (0.3)**	**25.5 (0.1)**			
	3 – 1	33.0	25.5	7.1	1.4 × 10^5	1.35
	3 – 2	32.7	25.6			
	3 – 3	32.3	25.7			
	Avg. (SD)	**32.6 (0.4)**	**25.6 (0.07)**			
	Overall Avg.	**NA**	**NA**	**7.2**	**1.5 (0.2) × 10^5**	**1.40 (0.04)**
5.5 × 10^2	1 – 1	35.0	28.7	6.5	1.8 × 10^4	1.52
	1 – 2	35.4	28.7			
	1 – 3	35.2	28.7			
	Avg. (SD)	**35.2 (0.2)**	**28.7 (0.02)**			
	2 – 1	34.5	28.2	6.4	2.1 × 10^4	1.60
	2 – 2	34.8	28.4			
	2 – 3	34.9	28.4			
	Avg. (SD)	**34.7 (0.2)**	**28.3 (0.1)**			
	3 – 1	37.2	28.3	7.7	2.2 × 10^4	1.71
	3 – 2	35.2	28.3			
	3 – 3	35.6	28.3			

	Avg. (SD)	**36.0 (1.1)**	**28.3 (0.01)**			
	Overall Avg.	**NA**	**NA**	**6.9**	**2.0 (0.2) × 10^4**	**1.61 (0.09)**
5.5 × 10^1	1 – 1	37.3	32.7	5.2	1.8 × 10^3	1.61
	1 – 2	NDT	32.5			
	1 – 3	38.2	32.3			
	Avg. (SD)	**37.7 (0.7)**	**32.5 (0.2)**			
	2 – 1	NDT	31.9	12.9	1.9 × 10^3	1.45
	2 – 2	NDT	32.2			
	2 – 3	38.2	32.2			
	Avg. (SD)	**NDT**	**32.1 (0.2)**			
	3 – 1	38.4	32.6	5.7	1.3 × 10^3	1.53
	3 – 2	38.4	32.1			
	3 – 3	37.4	32.3			
	Avg. (SD)	**38.0 (0.6)**	**32.3 (0.2)**			
	Overall Avg.	**NA**	**NA**	**7.9**	**1.7 (0.3) × 10^3**	**1.53 (0.08)**

* Average (Avg.) and standard deviation (SD) for triplicate samples and triplicate PCR analyses per sample.
Acronyms: CFU, colony-forming units; C_T, cycle threshold; NA, not applicable; NDT, non-detect.

These data suggested that the cells held for 24 h had a lag in growth compared to cells used directly. Regardless, the ΔC_T values were typically greater than 6 for individual replicates, for all but two replicates at the lowest cell density after the 24 h, showing ΔC_T values of 5.2 and 5.7 (the third replicate had $\Delta C_T = 12.9$). Individual ΔC_T values ranged from 6.3 to 9.6 for cells used directly and from 5.2 to 12.9 for cells used after being held for 24 h.

A replicate experiment was conducted as described with results shown in **Table 8** for cells used directly and **Table 9** for cells used after holding at 4°C for 24 h. In this case, similar results were obtained although the log differences from T_0 to T_{24} were slightly higher for cells used directly (compared to the first experiment), ranging from ~1.86 to 2.13 on average, and ranging from ~1.46 to 1.69 on average for cells held at 4°C for 24 h. For this experiment, all had $\Delta C_T \geq 6$, with individual ΔC_T values ranging from 6.1 to 9.4 for cells used directly and from 6.4 to 7.7 for cells used after being held for 24 h.

As a summary, the average log cell increase and ΔC_T values (using 24-h incubation) for each starting cell density and experiment are shown in Table 10. For the replicate experiments, the average log CFU/mL increased ~1.83–1.97 for cells used directly and ~1.51–1.56 for cells held first at 4°C for 24-h (**Table 10**). The corresponding average ΔC_T values were ~8.1 for cells used directly and ~6.6–7.1 for cells held first at 4°C for 24-h. Although the trend showed slightly lower ΔC_T values by holding cells for 24 h, there were no statistically significant differences ($p > 0.01$) between average ΔC_T values for individual starting cell densities or for the average of all three starting cell densities between treatments. However, for a few cases, the results were significant for the average log CFU/mL increase (**Table 10**). Furthermore, those data suggested that the RV-PCR method sensitivity of detection for viable *F. tularensis* cells using 24-h incubation was in the range of 135 (starting cell density of 4.5 CFU/mL) to 210 (starting cell density of 7.0 CFU/mL) cells per 3-mL water sample.

Improved method sensitivity would be expected with longer RV-PCR incubation periods. Therefore, follow-on testing was performed using 30- and/or 36-h incubation periods. The RV-PCR data from DNA extracts used the manual Promega protocol since the automated Roche

MagNA Pure® protocol was not yet approved for use in the Biosafety Level biosafety level-3 (BSL-3) laboratory. Based on results from comparison studies between manual and automated DNA extraction/purification protocols for *Bacillus anthracis* (*B. anthracis*), we thought that lower C_T values would be generated using the automated platform. However, in this case, the ΔC_T values could be similar if lower C_T values are noted for both T_0 and T_{24} (or later) time points.

Table 8. RV-PCR and Culture Results for *F. tularensis* Schu S4: Cells Used Without 4°C 24-Hours Hold – Replicate Experiment

Starting Cell Density (CFU/mL)	Sample Replicate - PCR Replicate	F5 Assay C_T (SD)*		ΔC_T (T_0 – T_{24})	T_{24} Cell Density (CFU/mL)	Cell Density Log Change
		T_0	T_{24}			
4.5×10^3	1 – 1	31.0	21.7	9.4	8.3×10^5	2.26
	1 – 2	31.2	21.9			
	1 – 3	31.4	21.8			
	Avg. (SD)	**31.2 (0.2)**	**21.8 (0.09)**			
	2 – 1	31.7	24.1	7.8	ND**	ND**
	2 – 2	31.8	24.1			
	2 – 3	32.0	23.9			
	Avg. (SD)	**31.8 (0.2)**	**24.1 (0.1)**			
	3 – 1	31.6	22.3	9.1	4.0×10^5	1.94
	3 – 2	31.2	22.3			
	3 – 3	31.4	22.4			
	Avg. (SD)	**31.4 (0.2)**	**22.3 (0.04)**			
	Overall Avg. (SD)	**NA**	**NA**	**8.7 (0.9)**	**$6.1 (2.2) \times 10^5$**	**2.13 (0.23)**
4.5×10^2	1 – 1	34.6	26.4	8.2	3.9×10^4	1.94
	1 – 2	35.0	26.6			
	1 – 3	34.6	26.4			
	Avg. (SD)	**34.7 (0.2)**	**26.5 (0.09)**			
	2 – 1	35.1	26.5	8.3	4.0×10^4	1.94
	2 – 2	34.7	26.5			
	2 – 3	34.5	26.5			
	Avg. (SD)	**34.8 (0.3)**	**26.5 (0.02)**			
	3 – 1	35.0	26.7	8.2	3.4×10^4	1.87
	3 – 2	34.6	26.8			
	3 – 3	35.7	27.0			
	Avg. (SD)	**35.1 (0.6)**	**26.9 (0.2)**			
	Overall Avg. (SD)	**NA**	**NA**	**8.2 (0.03)**	**$3.8 (0.9) \times 10^4$**	**1.92 (0.04)**
4.5×10^1	1 – 1	38.7	29.9	8.1	3.7×10^3	1.91
	1 – 2	38.4	29.9			
	1 – 3	37.3	30.2			
	Avg. (SD)	**38.1 (0.7)**	**30.0 (0.2)**			
	2 – 1	NDT	30.2	7.9	2.9×10^3	1.81
	2 – 2	38.2	30.3			
	2 – 3	38.3	30.5			
	Avg. (SD)	**38.2 (0.1)**	**30.3 (0.2)**			
	3 – 1	36.6	30.3	6.1	3.2×10^3	1.85
	3 – 2	36.2	30.4			

	3 – 3	NDT	30.2			
	Avg. (SD)	**36.4 (0.3)**	**30.3 (0.06)**			
	Overall Avg. (SD)	**NA**	**NA**	**7.4 (1.1)**	**3.3 (0.5) × 10^3**	**1.86 (0.05)**

* Average (Avg.) and standard deviation (SD) for triplicate samples and triplicate PCR analyses per sample.
** ND = Not determined. Sample replicate had contamination with non-*F. tularensis* colonies such that accurate Schu S4 colony counts could not be obtained. Acronyms: CFU, colony-forming units; C_T, cycle threshold; NA, not applicable; NDT, non-detect.

Table 9. RV-PCR and Culture Results for *F. tularensis* Schu S4: Cells Held for 24 Hours at 4°C – Replicate Experiment

Starting Cell Density (CFU/mL)	Sample Replicate - PCR Replicate	F5 Assay C_T*		ΔC_T (T_0 – T_{24})	T_{24} Cell Density Avg. CFU/mL	Cell Density Log Change
		T_0	T_{24}			
4.6 × 10^3	1 – 1	31.2	23.4	7.6	2.6 × 10^5	1.75
	1 – 2	31.0	23.5			
	1 – 3	31.1	23.5			
	Avg. (SD)	**31.1 (0.07)**	**23.5 (0.05)**			
	2 – 1	31.3	23.5	7.7	2.9 × 10^5	1.80
	2 – 2	31.3	23.5			
	2 – 3	31.2	23.6			
	Avg. (SD)	**31.3 (0.006)**	**23.5 (0.05)**			
	3 – 1	31.2	24.0	7.3	1.5 × 10^5	1.52
	3 – 2	31.2	23.8			
	3 – 3	31.2	23.7			
	Avg. (SD)	**31.2 (0.03)**	**23.9 (0.1)**			
	Overall Avg (SD)	**NA**	**NA**	**7.6 (0.2)**	**2.3 (0.7) × 10^5**	**1.69 (0.15)**
4.6 × 10^2	1 – 1	35.4	27.8	7.2	1.3 × 10^4	1.44
	1 – 2	34.8	27.9			
	1 – 3	34.7	27.7			
	Avg. (SD)	**35.0 (0.4)**	**27.8 (0.09)**			
	2 – 1	33.5	27.4	6.2	1.8 × 10^4	1.59
	2 – 2	34.5	27.7			
	2 – 3	33.5	27.6			
	Avg. (SD)	**33.8 (0.5)**	**27.6 (0.2)**			
	3 – 1	34.8	27.4	7.1	1.7 × 10^4	1.56
	3 – 2	34.4	27.5			
	3 – 3	34.6	27.6			
	Avg. (SD)	**34.6 (0.2)**	**27.5 (0.1)**			
	Overall Avg. (SD)	**NA**	**NA**	**6.8 (0.5)**	**1.6 (1.0) × 10^4**	**1.53 (0.08)**
4.6 × 10^1	1 – 1	NDT	31.0	7.2	1.5 × 10^3	1.51
	1 – 2	NDT	30.9			
	1 – 3	38.2	30.9			
	Avg. (SD)	**38.2**	**30.9 (0.01)**			
	2 – 1	37.4	30.9	7.4	1.4 × 10^3	1.48
	2 – 2	NDT	31.1			
	2 – 3	39.7	31.3			

	Avg. (SD)	**38.5 (1.6)**	**31.1 (0.2)**			
	3 – 1	38.9	32.1	6.4	1.2×10^3	1.40
	3 – 2	38.6	32.0			
	3 – 3	37.5	31.7			
	Avg. (SD)	**38.3 (0.7)**	**32.0 (0.2)**			
	Overall Avg. (SD)	**NA**	**NA**	**7.0 (0.5)**	**$1.3 (0.2) \times 10^3$**	**1.46 (0.06)**

* Average (Avg.) and standard deviation (SD) for triplicate samples and triplicate PCR analyses per sample.
Acronyms: CFU, colony-forming units; C_T, cycle threshold; NA, not applicable; NDT, non-detect.

Table 10. Summary of RV-PCR and Culture Results (with 24-h Incubation) for *F. tularensis* Schu S4 Cells With and Without a 24-Hour Hold at 4°C (Tables 6–9)*

Starting Cell Density	Avg. (SD)** Log CFU/mL Increase ($T_0 - T_{24}$)		Avg. (SD)** ΔC_T ($T_0 - T_{24}$)	
	No 24-H Hold	24-H Hold	No 24-H Hold	24-H Hold
Experiment #1				
10^1	1.85 (0.03)	1.53 (0.08)	7.6 (1.1)	5.7 (0.4)
10^2	1.72 (0.08)	1.61 (0.09)	8.2 (1.1)	6.9 (0.7)
10^3	1.93 (0.12)†	1.40 (0.04)	8.4 (1.2)	7.2 (0.7)
Experiment #2				
10^1	1.86 (0.05)†	1.46 (0.06)	7.4 (1.1)	7.0 (0.5)
10^2	1.92 (0.04)	1.53 (0.08)	8.2 (0.03)	6.8 (0.5)
10^3	2.13 (0.23)	1.69 (0.15)	8.7 (0.9)	7.6 (0.2)

* The average log cell increase and ΔC_T values from Tables 6–9 are summarized here.
**Average (Avg.) and standard deviation (SD) were calculated from triplicate samples and triplicate analyses per sample. The SD was calculated using the equation in Section 2.12.
†Denotes significantly greater value ($p < 0.01$; paired, two-tailed T-test) for comparisons between with and without 4°C hold for different starting cell densities or for all three cell densities (10^1–10^3 CFU/mL).
Acronyms: CFU, colony-forming units; C_T, cycle threshold; H, hour.

4.1.7 Evaluation of F. tularensis Schu S4 *Growth and RV-PCR Performance Using 30- or 36-h Incubation*

From the RV-PCR experiment using 24-h incubation, with cells held for 24 h at 4°C prior to use, the lowest cell level (~165 CFU/3-mL water sample) showed 2 of 3 replicates with a ΔC_T value of < 6 (5.2 and 5.7; the 3rd replicate had $\Delta C_T = 6.1$); therefore, we tested both 30-h and 36-h incubation to determine if the ΔC_T cut-off for positive detection could be achieved for all sample replicates. This test was important since more complex samples containing growth and/or PCR inhibitors could have even lower ΔC_T values. Like the previous experiment, 6X BVFH medium was diluted to 1X with addition of the water sample (3 mL PBS with Schu S4 cells plus 0.6 mL 6X medium), and the inoculum was prepared as described previously. Two separate 48-well plates were used for the two different incubation periods.

Table 11. RV-PCR and Culture Results for *F. tularensis* Schu S4 Cells Incubated for 30 Hours

Starting Cell Density (CFU/mL)	Sample Replicate*	ΔC_T (T_0–T_{30})	T_{30} Cell Density Avg. CFU/mL	Cell Density Log Change
4.1×10^3	1	11.9	2.1×10^6	**2.83**
	2	10.9	2.0×10^6	
	3	10.9	1.4×10^6	
	Avg. (SD)	**11.2 (0.5)**	**$1.81\ (0.4) \times 10^6$**	
4.1×10^2	1	9.7	1.3×10^5	**2.54**
	2	11.9	1.4×10^5	
	3	9.5	1.6×10^5	
	Avg. (SD)	**10.4 (1.3)**	**$1.44\ (0.14) \times 10^5$**	
4.1×10^1	1	13.3	1.3×10^4	**2.73**
	2	12.4	7.8×10^3	
	3	12.3	8.3×10^3	
	Avg. (SD)	**12.7 (0.6)**	**$9.54\ (2.60) \times 10^3$**	
4.1	1	9.5	1.2×10^3	**2.83**
	2	8.3	9.0×10^2	
	3	9.8	1.4×10^3	
	Avg. (SD)	**9.2 (0.8)**	**$1.19\ (0.27) \times 10^3$**	

* Average (Avg.) and standard deviation (SD) for triplicate samples. Data are from manual DNA extraction.
Acronyms: CFU, colony-forming units; C_T, cycle threshold.

Table 12. RV-PCR and Culture Results for *F. tularensis* Schu S4 Cells Incubated for 36 Hours

Starting Cell Density (CFU/mL)	Sample Replicate*	ΔC_T (T_0–T_{36})	T_{36} Cell Density Avg. CFU/mL	Cell Density Log Change
4.1×10^3	1	16.0	9.0×10^6	**3.46**
	2	15.9	6.4×10^6	
	3	15.0	7.9×10^6	
	Avg. (SD)	**15.7 (0.6)**	**$7.76\ (1.3) \times 10^6$**	
4.1×10^2	1	12.8	4.9×10^5	**3.06**
	2	13.1	4.6×10^5	
	3	14.2	4.8×10^5	
	Avg. (SD)	**13.4 (0.7)**	**$4.77\ (0.14) \times 10^5$**	
4.1×10^1	1	16.4	6.0×10^4	**3.43**
	2	16.7	5.5×10^4	
	3	16.2	2.9×10^4	
	Avg. (SD)	**16.4 (0.2)**	**$4.79\ (1.6) \times 10^4$**	
4.1	1	12.7	8.2×10^3	**3.51**
	2	12.8	4.7×10^3	
	3	13.0	4.3×10^3	

Starting Cell Density (CFU/mL)	Sample Replicate*	ΔC_T (T_0–T_{36})	T_{36} Cell Density Avg. CFU/mL	Cell Density Log Change
	Avg. (SD)	**12.8 (0.2)**	**5.73 (2.2) × 10^3**	

* Average (Avg.) and standard deviation (SD) for triplicate samples. Data are from manual DNA extraction.
Acronyms: CFU, colony-forming units; C_T, cycle threshold.

incubation. For 30- (**Table 11**) or 36-hour (**Table 12**) incubation, the average ΔC_T did not appear to be a function of the starting cell density. The T_0 C_T values ranged from ~34–36.3 for the 10^3 CFU/mL level and ~36.7–39 for the 10^2 CFU/mL level, with non-detect PCR results for two lower starting cell densities (data not shown).

A replicate experiment was conducted following the same parameters including the same incubation periods. In this case, the starting cell densities ranged from ~5.7 to 5.7 × 10^3 per mL. The culture and RV-PCR results are shown in **Table 13** for the 30-h incubation and **Table 14** for the 36-h incubation. From culture analysis, the average log differences from T_0 to T_{30} and from T_0 to T_{36} ranged from ~2.50 to 2.79 and 2.86 to 3.25, respectively. The log change values were slightly lower than the first replicate experiment for the 36-h incubation, although values were higher than the 30-h incubation for this experiment, as expected. Results showed ΔC_T values ranging from 10.1 to 13.3 for the 30-h incubation and from 9.9 to 15.9 for the 36-h incubation. As in the previous experiment, the average ΔC_T did not vary as a function of the starting cell density. In this case, the T_0 C_T values ranged from ~31.5–33 for the 10^3 CFU/mL level, ~36–37 for the 10^2 CFU/mL level, 38–40 for the 10^1 CFU/mL level, and non-detect PCR results for the 10^0 CFU/mL level (data not shown). Together these data suggested that the 30-h incubation period would be suitable for testing potential PCR and growth inhibitors as part of Task 3, since differences in ΔC_T values compared with the control treatment could more easily be observed if there was an effect.

Table 13. RV-PCR and Culture Results for *F. tularensis* Schu S4 Cells Incubated for 30 Hours – Replicate Experiment

Starting Cell Density (CFU/mL)	Sample Replicate*	ΔC_T (T_0 – T_{30})	T_{30} Cell Density Avg. CFU/mL	Cell Density Log Change
5.7 × 10^3	1	11.8	3.7 × 10^6	**2.70**
	2	12.2	2.5 × 10^6	
	3	12.2	2.4 × 10^6	
	Avg. (SD)	**12.0 (0.2)**	**2.85 (0.73) × 10^6**	
5.7 × 10^2	1	11.0	1.9 × 10^5	**2.5**
	2	11.5	2.0 × 10^5	
	3	10.1	1.6 × 10^5	
	Avg. (SD)	**10.9 (0.7)**	**1.83 (0.22) × 10^5**	
5.7× 10^1	1	10.2	2.2 × 10^4	**2.61**
	2	11.5	2.5 × 10^4	
	3	10.3	2.3 × 10^4	
	Avg. (SD)	**10.7 (0.7)**	**2.35 (0.12) × 10^4**	
5.7	1	13.3	4.3 × 10^3	**2.79**

Starting Cell Density (CFU/mL)	Sample Replicate*	ΔC_T ($T_0 - T_{30}$)	T_{30} Cell Density Avg. CFU/mL	Cell Density Log Change
	2	12.5	3.5×10^3	
	3	12.4	2.8×10^3	
	Avg. (SD)	**12.7 (0.5)**	**$3.56\ (0.75) \times 10^3$**	

* Average (Avg.) and standard deviation (SD) for triplicate samples.
Acronyms: CFU, colony-forming units; C_T, cycle threshold.

Table 14. RV-PCR and Culture Results for *F. tularensis* Schu S4 Cells Incubated for 36 Hours – Replicate Experiment

Starting Cell Density (CFU/mL)	Sample Replicate*	ΔC_T (T_0–T_{36})	T_{36} Cell Density Avg. CFU/mL	Cell Density Log Change
5.7×10^3	1	13.5	1.2×10^7	**3.21**
	2	12.6	6.6×10^6	
	3	13.0	9.3×10^6	
	Avg. (SD)	**13.0 (0.5)**	**$9.20\ (2.5) \times 10^6$**	
5.7×10^2	1	13.0	1.0×10^6	**3.25**
	2	12.8	7.9×10^5	
	3	13.6	1.2×10^6	
	Avg. (SD)	**13.1 (0.4)**	**$1.02\ (0.22) \times 10^6$**	
5.7×10^1	1	9.9	4.6×10^4	**2.86**
	2	10.3	3.3×10^4	
	3	11.3	4.5×10^4	
	Avg. (SD)	**10.5 (0.7)**	**$4.12\ (0.71) \times 10^4$**	
5.7	1	14.5	4.7×10^3	**2.89**
	2	14.8	5.8×10^3	
	3	15.9	2.8×10^3	
	Avg. (SD)	**15.1 (0.7)**	**$4.43\ (1.5) \times 10^3$**	

* Average (Avg.) and standard deviation (SD) for triplicate samples.
Acronyms: CFU, colony-forming units; C_T, cycle threshold.

4.2 Task 2. Evaluate manual and automated DNA extraction-purification procedures for *F. tularensis*

4.2.1 Objectives

The objective for this task was to compare manual and automated DNA extraction protocols with the goal of using the latter for more rapid analysis. For this task, a second aliquot was taken during sample processing after 30- or 36-h incubation and frozen for subsequent automated DNA extraction. PCR results were then to be compared for rapidity, precision, lower C_T, and labor to the results from the first aliquot processed by manual extraction (reported for Task 1). These aliquots contained 10^3 to 10^4 cells per mL determined from culture analysis. To extract and analyze DNA from these aliquots containing virulent *F. tularensis* Schu S4 cells, equipment including a Roche MagNA Pure® Compact and ABI 750 Fast PCR instrument were moved into

the BSL3 laboratory. Since the Roche instrument was too large to be contained in a BSC, an inactivation protocol needed to be validated to move crude cell lysates outside the BSC to complete the DNA extraction process. According to LLNL biosafety officials, this protocol constituted a removal from containment (per the CDC) although the robot was in the same laboratory. The inactivation protocol was validated and documented prior to processing samples from experiments (see section 2.8).

4.2.2 Procedure for F. tularensis Schu S4 Inactivation for Automated DNA Extraction in the BSL3 Laboratory

For validation of the inactivation protocol prior to putting the samples on the automated DNA extraction platform (outside the BSC), triplicates of both inactivated samples and positive controls (not inactivated) were tested in three separate experiments. In these tests, 100% of the inactivated material (or controls) were plated, with plates incubated under optimal growth conditions for 96 h. An additional experiment was required by the CDC to assess whether the chemicals used to inactivate cells were sufficiently washed from the samples (by repeated centrifugation and resuspension) prior to culture analysis. In this case, the inactivated samples were split in half after wash steps, with half plated directly and the other half spiked with a known amount of *F. tularensis* cells prior to plating (where positive growth would indicate sufficient removal of the inactivating chemicals). The three experiments used aliquots containing ~ 3–8 × 10^8 CFU/mL, which was concentrated to 200 µL for inactivation.

After reviewing preliminary data on cell inactivation with the initial chemical lysis (30 min at room temperature with mixing every 5 min), a step was added to perform heat lysis at 70°C for 10 min prior to conducting the Roche chemical lysis protocol. Using the modified inactivation protocol and plating the entire treated and washed suspension showed no growth for triplicate inactivated samples for triplicate experiments. Positive controls showed expected results throughout. The testing showed that wash steps effectively removed the chemicals for inactivation since added *F. tularensis* cells showed growth, whereas directly plated inactivated (washed) cells had no growth.

4.2.3 Evaluation of Manual and Automated DNA Extraction-Purification Procedures Using 10^3 and 10^4 F. tularensis Schu S4 CFU/mL

For this task, aliquots taken from the RV-PCR experiments after either 30 or 36 h were extracted by two different DNA extraction methods followed by real-time PCR analysis. At the incubation endpoint, two 500 µL aliquots were taken, centrifuged to pellet the cells, and then 300 µL of the supernatant was removed prior to storing at -20°C. Specifically, aliquots that had between 10^3 and 10^4 cells based on culture plating analysis were selected to compare the manual Promega and automated Roche protocols. In this way, both methods could be evaluated using replicate aliquots with the same cell densities. The manual Promega protocol was used as previously described (US EPA, 2016), with one fewer lysis step and one fewer ethanol wash step compared to the *B. anthracis* protocol. For the Roche DNA extraction protocol, the same method used by the LRN on the MagNA Pure® Compact system was used. However, a 10-min heat lysis step (70°C) preceded the addition of bacterial lysis buffer and incubation for 30 min. The data for PCR analysis using the F5 *F. tularensis*-specific assay are shown in **Table 15** and **Table 17** for

30-h incubation and **Table 16** and **Table 18** for 36-h incubation. The tables list the initial and final cell densities in CFU/mL and PCR data in triplicate for both Promega and Roche DNA extracts, with averages and standard deviations.

The data showed that the Roche DNA extraction protocol recovered more amplifiable DNA than the Promega DNA extraction protocol, with lower C_T values evident for Roche DNA extracts. Therefore, the Roche DNA extraction method was more sensitive than the Promega DNA extraction method. For the first experiment (**Table 15**), the average difference between C_T values ranged from 2.9 ± 0.2 to 3.2 ± 1.0 for starting cell densities of 41 and 4.1 CFU/mL, respectively (for 30-h incubation). For 36-h incubation (Table 16), the average C_T differences ranged from 1.9 ± 0.3 to 1.7 ± 0.5 for starting cell densities of 41 and 4.1 CFU/mL, respectively. For the second experiment (**Table 17**), the average C_T differences were lower, ranging from 1.0 ± 0.3 to 1.6 ± 0.2 for 57 and 5.7 CFU/mL starting cell densities, respectively (for 30-h incubation). For 36-h incubation (Table 18), average C_T differences ranged from 1.2 ± 0.3 to 0.8 ± 0.8 for these starting cell densities. The large standard deviation of 0.8 was due to one replicate for the 5.7 CFU/mL sample set showing no difference in average C_T difference between methods. Overall, the Roche DNA extracts showed an average 1.8 ± 0.9 lower C_T values than the Promega DNA extracts over the four experiments shown in **Tables 15-18**. Only undiluted extracts were analyzed since the extracts were from clean samples (only cells and media components), and no PCR inhibition was expected. The data from undiluted extracts was deemed sufficient to compare the two extraction methods, without requiring additional analysis.

In addition to better performance, the Roche method is semi-automated and thus, was less labor-intensive, requiring initial manual processing for heat lysis and off-robot lysis (pipetting and incubation), followed by loading on the robot in batches of eight for automated processing. Centrifugation of the sample would be required if numerous particulates were present to avoid clogging or improper pipetting by the robot, potentially adding another step after heat lysis and initial chemical lysis. In this study, no centrifugation was needed since the highest particulate density was relatively low at 4 mg ATD/mL (Task 3).

Table 15. Comparison of Promega and Roche DNA Extraction-Purification Procedures for Detection of *F. tularensis* Schu S4 Cells by PCR After 30-Hour Incubation

Avg. Initial CFU/mL (from inoculum)	Avg. T_{30} CFU/mL (from plating)	Sample Replicate	PCR Replicate	Promega C_T T_{30}	Roche C_T T_{30}	C_T T_{30} Difference*
41	**1.3 (0.4) × 10^4**	**1**	1	31.5	28.9	**2.9**
			2	31.7	28.7	
			3	31.7	28.8	
			Avg. (SD)	**31.7 (0.1)**	**28.8 (0.1)**	
	7.8 (1.3) × 10^3	**2**	1	32.4	29.2	**3.1**
			2	33.0	29.6	
			3	32.3	29.6	
			Avg. (SD)	**32.6 (0.4)**	**29.5 (0.2)**	
	8.3 (0.4) × 10^3	**3**	1	32.6	30.0	**2.8**
			2	32.7	29.8	
			3	32.7	29.8	
			Avg. (SD)	**32.7 (0.08)**	**29.9 (0.1)**	
	9.5 (3.0) × 10^3	**Overall Avg. (SD)**		**32.3 (0.5)**	**29.4 (0.5)**	**2.9 (0.2)**
4.1	**1.2 (0.3) × 10^3**	**1**	1	36.2	32.8	**2.9**
			2	35.2	32.5	
			3	35.2	32.6	
			Avg. (SD)	**35.5 (0.6)**	**32.6 (0.2)**	
	9.0 (3.0) × 10^2	**2**	1	36.2	32.8	**4.2**
			2	37.0	32.0	
			3	37.0	32.6	
			Avg. (SD)	**36.7 (0.4)**	**32.5 (0.4)**	
	1.4 (0.1) × 10^3	**3**	1	35.0	33.2	**2.3**
			2	36.0	32.5	
			3	34.6	32.9	
			Avg. (SD)	**35.2 (0.7)**	**32.9 (0.3)**	
	1.2 (0.3) × 10^3	**Overall Avg. (SD)**		**35.8 (0.9)**	**32.6 (0.3)**	**3.2 (1.0)**

*C_T value from Promega DNA extraction minus the C_T value from Roche DNA extraction.
Acronyms: Avg. Average; SD, standard deviation; CFU, colony-forming units; C_T, cycle threshold.

Table 16. Comparison of Promega and Roche DNA Extraction-Purification Procedures for Detection of *F. tularensis* Schu S4 Cells by PCR After 36-Hour Incubation

Avg. Initial CFU/mL (from inoculum)	Avg. T_{36} CFU/mL (from plating)	Sample Replicate	PCR Replicate	Promega C_T T_{36}	Roche C_T T_{36}	C_T T_{30} Difference*
41	**6.0 (0.9) × 10^4**	**1**	1	28.7	26.8	**1.7**
			2	28.6	26.8	
			3	28.6	27.0	
			Avg. (SD)	**28.6 (0.06)**	**26.9 (0.1)**	
	5.5 (0.8) × 10^4	**2**	1	28.2	26.4	**1.8**
			2	28.4	26.5	
			3	28.4	26.6	
			Avg. (SD)	**28.3 (0.1)**	**26.5 (0.09)**	
	2.9 (1.4) × 10^4	**3**	1	28.8	26.5	**2.2**
			2	28.8	26.6	
			3	28.7	26.7	
			Avg. (SD)	**28.8 (0.08)**	**26.6 (0.07)**	
	4.8 (1.7) × 10^4	**Overall Avg. (SD)**		**28.6 (0.2)**	**26.7 (0.2)**	**1.9 (0.3)**
4.1	**8.2 (0.1) × 10^3**	**1**	1	32.5	31.1	**1.2**
			2	32.1	31.0	
			3	32.5	31.1	
			Avg. (SD)	**32.3 (0.2)**	**31.1 (0.08)**	
	4.7 (0.7) × 10^3	**2**	1	32.3	30.2	**2.1**
			2	32.0	30.1	
			3	32.2	30.1	
			Avg. (SD)	**32.2 (0.1)**	**30.1 (0.06)**	
	4.3 (0.8) × 10^3	**3**	1	31.7	30.1	**1.6**
			2	32.1	30.5	
			3	32.3	30.7	
			Avg. (SD)	**32.0 (0.3)**	**30.4 (0.3)**	
	5.7 (1.9) × 10^3	**Overall Avg. (SD)**		**32.2 (0.3)**	**30.5 (0.5)**	**1.7 (0.5)**

*C_T value from Promega DNA extraction minus the C_T value from Roche DNA extraction.
Acronyms: Avg. Average; SD, standard deviation; CFU, colony-forming units; C_T, cycle threshold.

Table 17. Comparison of Promega and Roche DNA Extraction-Purification Procedures for Detection of *F. tularensis* Schu S4 Cells by PCR After 30-Hour Incubation – Replicate Experiment

Avg. Initial CFU/mL (from inoculum)	Avg. T_{30} CFU/mL (from plating)	Sample Replicate	PCR Replicate	Promega C_T T_{30}	Roche C_T T_{30}	C_T T_{30} Difference*
57	**2.2 (0.2) × 10^4**	**1**	1	28.9	28.2	**0.7**
			2	28.9	28.3	
			3	28.9	28.1	
			Avg. (SD)	**28.9 (0.04)**	**28.2 (0.1)**	
	2.5 (0.4) × 10^4	**2**	1	28.9	27.9	**1.0**
			2	28.8	27.9	
			3	28.8	27.8	
			Avg. (SD)	**28.8 (0.05)**	**27.9 (0.04)**	
	2.3 (0.3) × 10^4	**3**	1	28.6	27.3	**1.3**
			2	28.7	27.3	
			3	28.6	27.3	
			Avg. (SD)	**28.6 (0.05)**	**27.3 (0.01)**	
	2.4 (0.3) × 10^4	**Overall Avg. (SD)**		**28.8 (0.1)**	**27.8 (0.4)**	**1.0 (0.3)**
5.7	**4.3 (0.6) × 10^3**	**1**	1	31.5	30.5	**1.3**
			2	31.9	--**	
			3	31.6	30.2	
			Avg. (SD)	**31.7 (0.2)**	**30.4 (0.2)**	
	3.5 (0.4) × 10^3	**2**	1	32.5	30.7	**1.7**
			2	32.5	30.9	
			3	32.6	30.6	
			Avg. (SD)	**32.5 (0.1)**	**30.8 (0.1)**	
	2.8 (0.2) × 10^3	**3**	1	32.7	30.8	**1.7**
			2	32.7	30.9	
			3	32.5	30.9	
			Avg. (SD)	**32.6 (0.1)**	**30.9 (0.05)**	
	3.6 (0.7) × 10^3	**Overall Avg. (SD)**		**32.3 (0.5)**	**30.7 (0.3)**	**1.6 (0.2)**

*C_T value from Promega DNA extraction minus the C_T value from Roche DNA extraction.
**Only two of three PCR replicates provided valid data.
Acronyms: Avg. Average; SD, standard deviation; CFU, colony-forming units; C_T, cycle threshold.

Table 18. Comparison of Promega and Roche DNA Extraction-Purification Procedures for Detection of *F. tularensis* Schu S4 Cells by PCR After 36-Hour Incubation – Replicate Experiment

Avg. Initial CFU/mL (from inoculum)	Avg. T_{36} CFU/mL (from plating)	Sample Replicate	PCR Replicate	Promega C_T T_{36}	Roche C_T T_{36}	C_T T_{36} Difference*
57	**2.4 (0.5) × 10^4**	**1**	1	28.2	27.2	**1.0**
			2	28.1	27.0	
			3	28.2	27.2	
			Avg. (SD)	**28.1**	**27.1**	
	2.5 (0.8) × 10^4	**2**	1	28.0	27.0	**1.1**
			2	28.2	27.0	
			3	28.1	26.9	
			Avg. (SD)	**28.1**	**27.0**	
	3.2 (1.4) × 10^4	**3**	1	27.7	26.3	**1.5**
			2	27.8	26.3	
			3	27.7	26.4	
			Avg. (SD)	**27.8**	**26.3**	
	2.7 (0.9) × 10^4	**Overall Avg. (SD)**		**28.0 (0.2)**	**26.8 (0.4)**	**1.2 (0.3)**
5.7	**4.7 (3.0) × 10^3**	**1**	1	30.5	29.6	**1.0**
			2	30.4	29.5	
			3	30.5	29.6	
			Avg. (SD)	**30.5**	**29.5**	
	5.4 (1.5) × 10^3	**2**	1	30.2	28.6	**1.5**
			2	30.1	28.8	
			3	30.2	28.6	
			Avg. (SD)	**30.2**	**28.7**	
	5.5 (1.6) × 10^3	**3**	1	29.0	29.0	**0.0**
			2	29.3	29.1	
			3	29.1	29.2	
			Avg. (SD)	**29.1**	**29.1**	
	5.2 (1.9) × 10^3	**Overall Avg. (SD)**		**30.0 (0.6)**	**29.1 (0.4)**	**0.8 (0.8)**

*C_T value from Promega DNA extraction minus the C_T value from Roche DNA extraction.
Acronyms: Avg. Average; SD, standard deviation; CFU, colony-forming units; C_T, cycle threshold.

4.3 Task 3. Evaluate and optimize an RV-PCR protocol for *F. tularensis* Schu S4 detection in water samples with relevant challenges

4.3.1 Objectives

In this task, RV-PCR method performance with regard to method inhibition, analytical sensitivity of detection, and time-to-results, was evaluated with a limited range of conditions under which samples are likely to be obtained, including samples with both soluble and insoluble contaminants. Iron sulfate and humic acid levels were selected that were representative (although

at the high end) of levels expected from actual water samples (NRC, 1979; WHO, 1996). In addition, water samples containing native AZ Test Dust representing both chemical (metals, oxides) and biological (live, non-target cells) interferences were also evaluated. The characterization data are included in Materials and Methods Section 2.4.

4.3.2 Evaluation of chemical and biological challenges for RV-PCR analysis of F. tularensis Schu S4 in water samples

In previous experiments, PBS buffer was used as a surrogate for water. Challenges included debris containing non-target microorganisms represented by native (non-autoclaved) ATD, as characterized by Rose et al. (2011) used at 4 mg/mL or 12 mg/3 mL sample (with ~5×10^3 CFU/mg ATD according to Rose et al.). Control treatments used PBS buffer without debris. In addition, ferrous sulfate (10 μg Fe^{2+}/mL) and humic acid (50 μg/mL) were tested together since iron minerals and organic matter such as humic acid are often present in environmental samples including water samples, and the iron minerals and organic matter are known PCR inhibitors (Schrader et al., 2012; Sidstedt et al., 2015). The *F. tularensis* Schu S4 cell suspensions were prepared as described for previous experiments (for Tasks 1 and 2) and held at 4°C for 24 hours to mimic sampling and storage at 4°C during shipment prior to analysis. The 30-h incubation period was used based on the previous experiments to obtain measurable C_T values even if growth and/or PCR inhibition were observed in the presence of the soluble or insoluble challenge materials.

The results for three different starting *F. tularensis* Schu S4 cell levels (CFU/mL) for the first experiment are shown in **Table 19**, **Table 20**, and **Table 21** for average cell densities of ~1,200, 120, and 12 CFU/3-mL sample, respectively. The results are shown as T_0 C_T, T_{30} C_T, and ΔC_T. As mentioned in Sections 2.11 and 2.12, if two or three of three PCR replicates were NDT, then a C_T value of 45 (i.e., the number of PCR cycles run) was assigned as the average T_0 C_T or T_{30} C_T (as appropriate) to calculate ΔC_T; whereas, if two or three of three replicates showed positive C_T values, the average of two or three replicate C_T values was used to calculate ΔC_T. Results for a replicate experiment with slightly higher cell densities of ~2,500, 250, and 25 CFU/mL are shown in **Table 22**, **Table 23**, and **Table 24**, respectively. Individual PCR values are shown as well as the average and standard deviation for the replicate PCR analyses. In addition, 10-fold dilutions of DNA extracts from the ATD treatments were included to check for PCR inhibition (referred to as ATD 1:10 in the treatment column.

The T_{30} C_T data showed that PBS buffer with Fe and humics had no apparent inhibition with lower T_{30} C_T values compared to clean controls (~2–7 C_T decrease depending on starting cell number) for both replicate experiments. For the first experiment with the 10^3-cell level (~1,200 CFU/sample), the Fe/humics treatment showed ΔC_T values that were more than double the

Table 19. RV-PCR Results for *F. tularensis* Schu S4 Cells (~1,200 CFU/3-mL Sample) in the Presence of Fe/Humics or ATD

Treatment	Sample Replicate	PCR Replicate	T_0 C_T	T_{30} C_T	ΔC_T (T_0–T_{30})
Control	1	1	34.5	25.1	9.3
		2	33.9	25.1	
		3	34.4	25.0	
		Avg. (SD)	**34.3 (0.3)**	**25.0 (0.04)**	
	2	1	33.7	26.6	7.4
		2	33.8	26.6	
		3	34.5	26.6	
		Avg. (SD)	**34.0 (0.5)**	**26.6 (0.03)**	
	3	1	34.1	25.7	8.3
		2	33.6	25.7	
		3	34.2	25.7	
		Avg. (SD)	**34.0 (0.3)**	**25.7 (0.02)**	
Fe/Humics	1	1	NDT	22.3	22.7
		2	NDT	22.3	
		3	NDT	22.3	
		Avg. (SD)	**NDT**	**22.3 (0.04)**	
	2	1	42.1	22.7	18.7
		2	42.0	22.7	
		3	40.0	22.7	
		Avg. (SD)	**41.4 (1.2)**	**22.7 (0.02)**	
	3	1	37.3	21.6	14.5
		2	35.7	21.6	
		3	35.4	21.6	
		Avg. (SD)	**36.1 (1.0)**	**21.6 (0.01)**	
ATD	1	1	NDT	33.2	5.5
		2	39.7	33.6	
		3	38.3	33.8	
		Avg. (SD)	**39.0 (1.0)**	**33.5 (0.3)**	
	2	1	38.2	33.1	5.3
		2	38.8	NDT	
		3	NDT	33.4	
		Avg. (SD)	**38.5 (0.4)**	**33.2 (0.2)**	
	3	1	37.0	33.5	3.7
		2	37.5	34.1	
		3	NDT	33.1	
		Avg. (SD)	**37.2 (0.4)**	**33.5 (0.5)**	
ATD 1:10*	1	1	38.7	36.6	2.1
		2	NDT	37.4	
		3	39.0	36.0	
		Avg. (SD)	**38.8 (0.3)**	**36.7 (0.7)**	
	2	1	NDT	37.3	8.1
		2	NDT	36.6	
		3	NDT	36.6	
		Avg. (SD)	**NDT**	**36.9 (0.4)**	
	3	1	NDT	36.7	8.3
		2	NDT	36.9	
		3	NDT	36.6	
		Avg. (SD)	**NDT**	**36.7 (0.2)**	

***** The same ATD concentration was used with the DNA extract analyzed after 10-fold dilution with PCR H_2O.
Acronyms: Avg. Average; SD, standard deviation; CFU, colony-forming units; C_T, cycle threshold.

Table 20. RV-PCR Results for *F. tularensis* Schu S4 Cells (~120 CFU/3-mL Sample) in the Presence of Fe/Humics or ATD

Treatment	Sample Replicate	PCR Replicate	T_0 C_T	T_{30} C_T	ΔC_T (T_0–T_{30})
Control	1	1	38.9	28.6	9.3
		2	37.0	28.6	
		3	37.7	28.5	
		Avg. (SD)	**37.9 (1.0)**	**28.6 (0.02)**	
	2	1	37.0	28.2	8.9
		2	NDT	28.3	
		3	37.1	28.2	
		Avg. (SD)	**37.1 (0.07)**	**28.2 (0.04)**	
	3	1	36.7	28.2	9.0
		2	36.6	28.0	
		3	37.7	28.3	
		Avg. (SD)	**37.0 (0.6)**	**28.2 (0.1)**	
Fe/Humics	1	1	NDT	26.8	16.2
		2	42.9	26.4	
		3	42.5	26.4	
		Avg. (SD)	**42.7 (0.3)**	**26.5 (0.2)**	
	2	1	NDT	27.0	18.3
		2	NDT	26.8	
		3	NDT	26.2	
		Avg. (SD)	**NDT**	**26.7 (0.4)**	
	3	1	38.3	23.6	15.4
		2	40.3	23.7	
		3	38.4	23.7	
		Avg. (SD)	**39.0 (1.1)**	**23.7 (0.06)**	
ATD	1	1	NDT	38.7	7.2
		2	NDT	36.2	
		3	NDT	38.5	
		Avg. (SD)	**NDT**	**37.8 (1.4)**	
	2	1	NDT	37.8	8.2
		2	NDT	35.7	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**36.8 (1.5)**	
	3	1	NDT	36.7	8.2
		2	NDT	37.0	
		3	NDT	36.8	
		Avg. (SD)	**NDT**	**36.8 (0.1)**	
ATD 1:10*	1	1	NDT	39.5	5.3
		2	NDT	39.8	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**39.7 (0.2)**	
	2	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	
	3	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	

***** The same ATD concentration was used with the DNA extract analyzed after 10-fold dilution with PCR H_2O.
Acronyms: Avg. Average; SD, standard deviation; CFU, colony-forming units; C_T, cycle threshold.

Table 21. RV-PCR Results for *F. tularensis* Schu S4 Cells (~12 CFU/3-mL Sample) in the Presence of Fe/Humics or ATD

Treatment	Sample Replicate	PCR Replicate	T_0 C_T	T_{30} C_T	ΔC_T (T_0–T_{30})
Control	1	1	NDT	35.3	10.3
		2	NDT	33.9	
		3	NDT	34.8	
		Avg. (SD)	**NDT**	**34.7 (0.7)**	
	2	1	NDT	34.5	10.9
		2	NDT	34.3	
		3	NDT	33.5	
		Avg. (SD)	**NDT**	**34.1 (0.6)**	
	3	1	NDT	32.4	12.7
		2	NDT	32.3	
		3	NDT	32.4	
		Avg. (SD)	**NDT**	**32.3 (0.07)**	
Fe/Humics	1	1	NDT	27.3	17.8
		2	NDT	27.2	
		3	NDT	27.1	
		Avg. (SD)	**NDT**	**27.2 (0.07)**	
	2	1	NDT	29.1	15.9
		2	NDT	29.2	
		3	NDT	29.0	
		Avg. (SD)	**NDT**	**29.1 (0.08)**	
	3	1	NDT	28.5	16.5
		2	NDT	28.5	
		3	NDT	28.6	
		Avg. (SD)	**NDT**	**28.5 (0.07)**	
ATD	1	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	
	2	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	
	3	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	
ATD 1:10*	1	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	
	2	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	
	3	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	

***** The same ATD concentration was used with the DNA extract analyzed after 10-fold dilution with PCR H_2O.
Acronyms: Avg. Average; SD, standard deviation; CFU, colony-forming units; C_T, cycle threshold.

values for the clean controls since the T_0 C_T values were higher than the values for the clean controls. This treatment also showed more variability in average T_0 C_T values between sample replicates potentially due to PCR inhibition and slight differences in sample replicate composition (e.g., pipetting variability), whereas T_{30} C_T values were similar between sample replicates (21.6 – 22.7). For the other cell levels in the first experiment and for all cell levels in the second experiment, the T_0 C_T values were comparable between Fe/Humics and clean control treatments, and higher ΔC_T values were attributed to lower T_{30} C_T values.

It is possible that cells were limited for Fe or nutrients present with the humic acid formulation, suggesting that improvements to the growth medium could enhance *F. tularensis* growth. However, in the presence of competing microbes, the addition of Fe and humics may not specifically enhance *F. tularensis* growth, and other microorganisms could out-compete *F. tularensis* given its slower growth character. Growth inhibition was observed in the presence of test dust (including background microbes) resulting in no detection at the 10^1 CFU/mL level (~12–25 cells) for both replicate experiments (**Table 21** and **Table 24**).

In general, PCR inhibition did not appear to contribute to higher C_T values since the difference between T_{30} C_T values for undiluted and 1:10 diluted ATD DNA extracts showed roughly a three C_T difference, as expected. In some cases (10^2-cell level for both experiments), the 10-fold diluted DNA extracts showed non-detect T_0 C_T results while values were measured for undiluted extracts; these results are expected for data near the PCR assay detection limit. The 10-fold diluted extracts showed higher ΔC_T values in the 10^3-cell level for both experiments (**Table 19** and **Table 22**), since the T_0 C_T values were NDT and set to 45 (the number of PCR cycles run) to calculate ΔC_T. These data also suggest that a 30-h to 36-h incubation period should be used rather than 24-h to improve detection of viable *F. tularensis* cells in complex samples containing background microbes. The overall results for effect of the Fe/Humics and ATD on the RV-PCR method are summarized in **Table 25**.

Table 22. RV-PCR Results for *F. tularensis* Schu S4 Cells (~2,500 CFU/3-mL Sample) in the Presence of Fe/Humics or ATD – Replicate Experiment

Treatment	Sample Replicate	PCR Replicate	T_0 C_T	T_{30} C_T	ΔC_T (T_0–T_{30})
Control	1	1	34.7	25.3	9.3
		2	34.0	25.1	
		3	34.7	25.2	
		Avg. (SD)	**34.4 (0.4)**	**25.2 (0.07)**	
	2	1	34.2	26.2	7.9
		2	33.8	26.3	
		3	34.3	26.2	
		Avg. (SD)	**34.1 (0.3)**	**26.2 (0.04)**	
	3	1	33.6	26.2	7.2
		2	33.4	26.1	
		3	33.0	26.1	
		Avg. (SD)	**33.4 (0.3)**	**26.1 (0.04)**	
Fe/Humics	1	1	33.7	22.0	11.5
		2	33.4	21.9	
		3	33.1	21.9	
		Avg. (SD)	**33.4 (0.3)**	**21.9 (0.03)**	
	2	1	33.8	20.5	13.3
		2	34.0	20.6	
		3	33.7	20.6	
		Avg. (SD)	**33.8 (0.1)**	**20.5 (0.02)**	
	3	1	37.5	19.9	17.2
		2	37.8	19.9	
		3	36.3	20.0	
		Avg. (SD)	**37.2 (0.8)**	**20.0 (0.03)**	
ATD	1	1	37.6	38.3	-0.1
		2	39.6	39.3	
		3	NDT	38.4	
		Avg. (SD)	**38.6 (1.4)**	**38.7 (0.5)**	
	2	1	38.0	37.5	2.4
		2	38.8	35.1	
		3	37.8	34.8	
		Avg. (SD)	**38.2 (0.6)**	**35.8 (1.5)**	
	3	1	NDT	34.6	10.7
		2	NDT	33.5	
		3	NDT	34.7	
		Avg. (SD)	**NDT**	**34.3 (0.7)**	
ATD 1:10*	1	1	NDT	35.8	9.2
		2	NDT	36.3	
		3	NDT	35.1	
		Avg. (SD)	**NDT**	**35.8 (0.6)**	
	2	1	NDT	35.9	9.2
		2	NDT	35.5	
		3	NDT	36.1	
		Avg. (SD)	**NDT**	**35.8 (0.3)**	
	3	1	NDT	37.8	8.3
		2	NDT	36.1	
		3	NDT	36.1	
		Avg. (SD)	**NDT**	**36.7 (1.0)**	

***** The same ATD concentration was used with the DNA extract analyzed after 10-fold dilution with PCR H_2O.
Acronyms: Avg. Average; SD, standard deviation; CFU, colony-forming units; C_T, cycle threshold.

Table 23. RV-PCR Results for *F. tularensis* Schu S4 Cells (~250 CFU/3-mL Sample) in the Presence of Fe/Humics or ATD – Replicate Experiment

Treatment	Sample Replicate	PCR Replicate	T_0 C_T	T_{30} C_T	ΔC_T (T_0–T_{30})
Control	1	1	NDT	29.9	7.8
		2	38.3	29.7	
		3	36.8	29.8	
		Avg. (SD)	**37.6 (1.1)**	**29.8 (0.1)**	
	2	1	38.8	28.9	8.1
		2	36.3	29.2	
		3	36.3	28.9	
		Avg. (SD)	**37.1 (1.4)**	**29.0 (0.1)**	
	3	1	36.1	29.6	6.8
		2	37.0	29.5	
		3	35.7	29.2	
		Avg. (SD)	**36.3 (0.7)**	**29.4 (0.2)**	
Fe/Humics	1	1	37.2	24.9	12.5
		2	37.8	24.9	
		3	37.2	24.8	
		Avg. (SD)	**37.4 (0.3)**	**24.9 (0.05)**	
	2	1	38.7	25.5	12.6
		2	38.4	25.5	
		3	37.1	25.5	
		Avg. (SD)	**38.1 (0.9)**	**25.5 (0.01)**	
	3	1	38.0	24.9	13.9
		2	NDT	24.8	
		3	39.4	24.8	
		Avg. (SD)	**38.7 (1.0)**	**24.8 (0.08)**	
ATD	1	1	NDT	38.4	7.5
		2	NDT	37.3	
		3	NDT	36.7	
		Avg. (SD)	**NDT**	**37.5 (0.9)**	
	2	1	NDT	40.6	5.2
		2	NDT	40.3	
		3	NDT	38.5	
		Avg. (SD)	**NDT**	**39.8 (1.1)**	
	3	1	NDT	37.6	7.0
		2	NDT	39.2	
		3	NDT	37.2	
		Avg. (SD)	**NDT**	**38.0 (1.1)**	
ATD 1:10*	1	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	
	2	1	NDT	NDT	6.4
		2	NDT	38.0	
		3	NDT	39.2	
		Avg. (SD)	**NDT**	**38.6 (0.8)**	
	3	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	

***** The same ATD concentration was used with the DNA extract analyzed after 10-fold dilution with PCR H_2O.
Acronyms: Avg. Average; SD, standard deviation; CFU, colony-forming units; C_T, cycle threshold.

Table 24. RV-PCR Results for *F. tularensis* Schu S4 Cells (~25 CFU/3-mL Sample) in the Presence of Fe/Humics or ATD – Replicate Experiment

Treatment	Sample Replicate	PCR Replicate	T_0 C_T	T_{30} C_T	ΔC_T (T_0–T_{30})
Control	1	1	NDT	34.3	10.6
		2	NDT	34.7	
		3	NDT	34.0	
		Avg. (SD)	NDT	**34.4 (0.4)**	
	2	1	NDT	33.6	11.4
		2	NDT	33.6	
		3	NDT	33.6	
		Avg. (SD)	**NDT**	**33.6 (0.02)**	
	3	1	NDT	32.6	12.3
		2	NDT	32.9	
		3	NDT	32.6	
		Avg. (SD)	**NDT**	**32.7 (0.2)**	
Fe/Humics	1	1	NDT	28.5	16.4
		2	NDT	28.6	
		3	NDT	28.6	
		Avg. (SD)	**NDT**	**28.6 (0.08)**	
	2	1	NDT	28.3	16.8
		2	NDT	28.2	
		3	NDT	28.1	
		Avg. (SD)	**NDT**	**28.2 (0.07)**	
	3	1	NDT	28.6	16.5
		2	NDT	28.4	
		3	NDT	28.4	
		Avg. (SD)	**NDT**	**28.5 (0.08)**	
ATD	1	1	NDT	44.4	1.2
		2	NDT	43.3	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**43.8 (0.8)**	
	2	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	
	3	1	NDT	41.4	4.9
		2	NDT	NDT	
		3	NDT	38.8	
		Avg. (SD)	**NDT**	**40.1 (1.8)**	
ATD 1:10*	1	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	
	2	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	
	3	1	NDT	NDT	0.0
		2	NDT	NDT	
		3	NDT	NDT	
		Avg. (SD)	**NDT**	**NDT**	

***** The same ATD concentration was used with the DNA extract analyzed after 10-fold dilution with PCR H_2O.
Acronyms: Avg. Average; SD, standard deviation; CFU, colony-forming units; C_T, cycle threshold.

Table 25. Summary of RV-PCR Results Based on Condition and Starting Cell Level (Tables 21–24)

Condition	Approximate Starting CFU per 3-mL Sample	ΔC_T Range	Positive RV-PCR Results
No Challenge	2500	7.2–9.3	3 of 3
	1200	7.4–9.3	3 of 3
	250	6.8–8.1	3 of 3
	120	8.9–9.3	3 of 3
	25	10.6–12.3	3 of 3
	12	10.3–12.7	3 of 3
Fe/Humics	2500	11.5–17.2	3 of 3
	1200	14.5–22.7	3 of 3
	250	12.5–13.9	3 of 3
	120	15.4–18.3	3 of 3
	25	16.4–16.8	3 of 3
	12	15.9–17.8	3 of 3
ATD (undiluted or 10-fold diluted)*	2500	9.2–10.7	3 of 3
	1200	5.5–8.3	2 of 3
	250	6.4–7.5	3 of 3
	120	7.2–8.2	3 of 3
	25	0–4.9	0 of 3
	12	0 - 0	0 of 3

* The ΔC_T range includes the highest ΔC_T value per ATD sample replicate, either from undiluted or 10-fold diluted DNA extracts.
Highlighted cells represent conditions and starting cell numbers where three of three sample replicates had positive RV-PCR results.
Acronyms: ATD, Arizona Test Dust; C_T, cycle threshold.

5.0 Summary and Conclusions

In summary, an optimized RV-PCR method was developed for detection of viable *F. tularensis* cells from water samples, which along with the *Y. pestis* RV-PCR method (US EPA, 2016), could serve as a model method for the detection of vegetative bacterial pathogens. The RV-PCR method was optimized by improving procedures for high-throughput culturing (in 48-well plates) and DNA extraction/purification using an automated platform. The optimized method was evaluated with regard to sensitivity and performance with complex water samples, including both biological and chemical challenges.

Specifically, previous work on *F. tularensis* growth optimization (Morris et al., 2017) using a BHI-Vitox-Fildes-Histidine (BVFH) medium was leveraged for development of 6X-concentrated growth medium for RV-PCR analysis. The 6X BVFH medium diluted to 1X supported *F. tularensis* cell growth comparable to the growth observed for the 1X BVFH medium; values were within ~72–75% of the cell growth that for 1X BVFH medium ($p = 0.01 – 0.18$, two-tailed, pairwise t-test). However, 10X BVFH diluted to 1X did not support adequate growth due to precipitation of medium components. Concentration of the medium enabled use of a larger volume water sample (3-mL plus 0.6 mL 6X BVFH medium per 5-mL well) and demonstrated reproducible growth even at low inoculum levels (< 10 cells per mL).

Prior to RV-PCR method evaluation, real-time PCR assay optimization showed that addition of 1.25 U Platinum™ Taq Polymerase to the reaction resulted in better sensitivity with average C_T values reduced with the enzyme addition by an average 9.8 and 8.9 C_T units for F4 and F5 assays, respectively. Therefore, the additional enzyme was included along with the AmpliTaq Gold® DNA polymerase from the MasterMix.

Testing was also performed to determine whether storing the cells at 4°C for 24 h impacted RV-PCR analysis (and the corresponding cell growth), thus mimicking conditions if samples were shipped overnight at 4°C for processing the next day. Using a 24-h incubation period for RV-PCR analysis of clean samples, two replicate experiments with starting cell levels ranging from 45 to 7,000 CFU/mL showed that holding cells for 24 h prior to analysis led to slightly lower increases in log CFU/mL; however, results were significant only for a couple of cases ($p < 0.01$),) and corresponding ΔC_T values were not significantly different between treatments. Overall, the data suggested that the assay sensitivity for viable *F. tularensis* cells using 24-h incubation for RV-PCR analysis was in the range of 135 to 210 cells per 3-mL for clean water samples.

Challenge testing with potential soluble (iron, humic acid) and insoluble chemical interferences (metal oxides in ATD) and live, non-target biological interferences (in ATD) addressed a range of potential "real world" complex sample types. Based on the testing with ATD (12 mg/3-mL sample), it is possible that extending the incubation time to 36 h might reduce the risk of false negative results for samples with low target cell numbers (10^1 CFU level or 10-99 cells per 3-mL sample) and high non-target microbial backgrounds ($\geq 10^4$–10^5 CFU/3-mL sample); however, 30-h incubation was sufficient for water samples with potential chemical inhibitors including iron (up to 10 µg/mL) and humic acid (up to 50 µg/mL). For these samples, the T_{30} C_T values were approximately 3.2 – 7.5 units lower than the clean controls (PBS). Growth of *F. tularensis*

cells may have been enhanced by iron and/or nutrients associated with the humic acid formulation. These data suggest that the growth medium could be further optimized, although this could also enhance growth of competing microbes. Conversely, in the presence of ATD, *F. tularensis* growth appeared to be inhibited for low cell levels (10^1 CFU/3-mL sample) leading to non-detect results, which could not be attributed to PCR inhibition because both undiluted and 10-fold diluted DNA extracts were non-detect. Additional testing is needed to assess RV-PCR method performance with real-world water samples as well as with a dead *F. tularensis* cell background for natural decay or post-decontamination scenarios.

Together, these findings showed that the RV-PCR method could be applied to *F. tularensis* in water samples containing potential inhibitors, demonstrating good sensitivity and method performance with complex sample matrices. While traditional culture methods are considered the gold standard for viability analysis, this effort demonstrated that RV-PCR could detect 10^1 to 10^2-cell level per 3-mL sample in < 36 h (sample processing and analysis time) compared to more than 72 h required for confirmed results from plate culture analysis. In addition, the RV-PCR method would produce significantly less waste and have a smaller laboratory space footprint for analysis. To illustrate these characteristics, RV-PCR uses a single 48-well plate for 48 samples (and controls) for growth compared with the culture method that uses 11 Chocolate Agar plates, dilution tubes, and an enrichment culture tube per sample, with additional plates to re-streak from the enrichment culture for target colony isolation.

In addition, other manual or automated DNA extraction platforms could be integrated into the RV-PCR method. An advantage with the Promega MagneSil® procedure used in this effort was that it required less centrifugation compared to other manual protocols (i.e., Qiagen QIAamp®). Centrifugation is more time-consuming under BSL-3 conditions, requiring transfer of the rotor to the BSC for sample processing. In addition, the Roche automated protocol could enable use of existing MagNA Pure® platforms in LRN laboratories, thus providing a more rapid analysis with more reproducible RV-PCR results. In this study, DNA extracts from the Roche MagNA Pure® Compact showed an average 1.8 ± 0.9 lower C_T values than the manual Promega MagneSil® DNA extracts. The real-time PCR assays used in this effort consistently demonstrated < 30 genome equivalent sensitivity; however, other assays in use for *F. tularensis* detection could readily be integrated into the RV-PCR method.

An SOP was developed (Appendix A) providing specific details for conducting sample processing and analysis as part of a tularemia incident response. This SOP can interface with a front-end sampling processing/concentration procedure such as ultrafiltration (e.g., Francy et al., 2009) and secondary filtration, like the procedure used for *Y. pestis* (US EPA, 2016), although this requires further testing for relevant filtrate samples. The RV-PCR method for detection of viable *F. tularensis* from water samples will help enhance the WLA capability for rapid, reliable, and high-throughput sample analysis to effectively respond to an intentional, accidental, or natural outbreak incident resulting in water infrastructure contamination. Additionally, subsequent to following sample-type-specific processing and concentration steps, this RV-PCR method can be used not just for water samples, but also for other sample types during a response to a wide-area tularemia incident.

6.0 References

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Appendix A. Standard Operating Procedure – Manual Protocol for Rapid Viability Polymerase Chain Reaction (RV-PCR) for Analysis of *Francisella tularensis* in Water Samples

Rapid Viability Polymerase Chain Reaction (RV-PCR)

This protocol describes processing and analysis of 40 mL water samples, first by stabilizing the samples with addition of 4.5 mL of 10X PBS to bring the final PBS concentration to approximately 1X concentration. For RV-PCR analysis, the standard incubation period is 30 h (to allow *Francisella tularensis* cell propagation prior to DNA extraction/purification and analysis).

Acronyms

BHI.....Brain Heart Infusion
BSC .....biosafety cabinet
BSL..... Biosafety Level
BVFH.....BHI/Vitox/Fildes/Histidine
BHI..... brain heart infusion
CA..... Chocolate Agar
CFU..... colony forming units
C_T ..... cycle threshold
DD ..... deionized, distilled
DNA..... deoxyribonucleic acid
EDTA..... ethylenediaminetetraacetic acid
hr .....hour
PAPR .....powered air purifying respirators
PBS .....phosphate buffered saline
PES .....polyethersulfone
PMPs .....paramagnetic particles
PPE .....personal protection equipment
RCF ..... relative centrifugal force
RV-PCR.....rapid viability-polymerase chain reaction
TE.....Tris EDTA
T_0.....time 0, prior to incubation
T_{30} ..... after 30 hours of incubation
TNTC.....too numerous to count

Trademarked Products

Trademark	Holder	Location
AeraSeal™	Excel Scientific, Inc.	Victorville, CA
Bacto™	BD Biosciences	Franklin Lakes, NJ
Biopur® Safe-Lock®	Eppendorf	Hamburg, Germany
Clorox®	Clorox Company	Oakland, CA
Costar®	Corning	Corning, NY
Dispatch®	Clorox Company	Oakland, CA
Dynamag™	Life Technologies	Carlsbad, CA
Lazy-L™	Millipore Sigma	Burlington, MA

Trademark	Holder	Location
MagneSil®	Promega	Madison, WI
MasterPure®	Epicentre Biotechnologies	Madison, WI
MagNA Pure®	Roche Diagnostics	Indianapolis, IN
MaxQ™	Thermo Scientific	Waltham, MA
Millipore®, Milli-Q™	Millipore Corp.	Billerica, MA
Oxoid®	Oxoid Limited	Cheshire, England
Remel™	Remel	Lenexa, KS
TaqMan®	Life Technologies	Carlsbad, CA
Tyvek® suit	DuPont	Wilmington, DE
Ziploc®	Johnson and Johnson	New Brunswick, NJ

Laboratory set-up

- Don PPE (personal protection equipment) on for Biosafety Level 3 (BSL-3): Tyvek® suit, powered air purifying respirators (PAPR), booties, double gloves.
- Prepare fresh bleach solution (1 volume bleach [Ultra Clorox® Germicidal bleach; 6.15% sodium hypochlorite] + 9 volumes water). Date and label with initials.
- Clean/bleach biosafety cabinet (BSC) and bench surfaces allowing 30-minute (min) contact time. Rinse with DD water or 70% isopropanol.
- All sample manipulations are performed in the BSC.

General Laboratory Supplies

- Gloves (e.g., latex, vinyl, or nitrile)
- Bleach wipes (Dispatch® Cat. No. 69150 or equivalent)
- Ultra Clorox® Germicidal bleach (VWR Cat. No. 76245-190 or equivalent)
- Acetic Acid, Glacial (VWR Cat. No. CA71006-436 or equivalent)
- Ziploc® bags (large ~20" (inches) × 28", medium ~12" × 16", small ~7" × 8")
- Sharps waste container
- Absorbent pad
- Medium and large biohazard bag(s) and rubber band(s)
- Squeeze bottle with 70% isopropyl alcohol
- Squeeze bottle with deionized, distilled (DD) water
- Autoclave tape
- Autoclave bags
- Aluminum foil or Kraft paper
- Large photo-tray or similar tray for transport of racks
- Laboratory marker
- Timer
- Disposable aerosol filter pipet tips: 1000 µL, 200 µL, 10 µL (Rainin Cat. No. SR-L1000F, SR-L200F, GP-10F or equivalent)
- 1.5 mL Eppendorf Snap-Cap Microcentrifuge Biopur® Safe-Lock® tubes (Fisher Scientific Cat. No. 05-402-24B or equivalent)
- 50 mL conical tubes (VWR Cat. No. 21008-951 or equivalent)
- 15 mL conical tubes (VWR Cat. No. 21008-918 or equivalent)
- 250 mL and 1 L filter systems, polyethersulfone (PES), 0.2 µm (Fisher Scientific Cat. No. 09-741-04, 09-741-03 or equivalent)
- Tubes, sterile 2 mL DNase, RNase-free, gasketed, screw caps (National Scientific Cat. No. BC20NA-PS or equivalent)
- Glass Petri dishes, 100 × 15 mm
- Disposable Serological Pipettes 5 mL serological pipettes 5mL (VWR Cat. No. 89130-896 or equivalent)
- Disposable Serological Pipettes 10 mL serological pipettes 10mL (VWR Cat. No. 89130-898 or equivalent)
- Disposable Serological Pipettes 25 mL serological pipettes 25mL (VWR Cat. No. 89130-900 or equivalent)

- Disposable Serological Pipettes 50 mL serological pipettes 50mL (VWR Cat. No. 89130-902 or equivalent)
- 500-mL bottles (Sigma-Aldrich Cat. No. 1395-500HTC or equivalent)
- 1-L bottles (Sigma-Aldrich Cat. No. 1395-1LHTC or equivalent)

Supplies for Rapid Viability-Polymerase Chain Reaction (RV-PCR) Analysis

- Disposable nylon forceps (VWR Cat. No. 12576-933 or equivalent)
- 50 mL conical tubes (VWR Cat. No. 21008-951 or equivalent)
- Disposable serological pipets: 50 mL, 25 mL, 10 mL, 5 mL
- Single 50 mL conical tube holder (Bel-Art Cat. No. F187950001 or equivalent)
- Screw cap tubes, 2 mL (VWR Cat. No. 89004-298 or equivalent)
- 96-well microcentrifuge tube rack(s) for 2 mL tubes (8 × 12 layout) (Bel-Art, Cat. No. F188450031 or equivalent)
- 2 mL Eppendorf tubes (Fisher Scientific Cat. No. 05-402-24C or equivalent)
- 48-well plates for rapid viability-polymerase chain reaction (RV-PCR) sample incubation (E&K Scientific Cat. No. EK-2044 or equivalent)
- 0.2 micron Ultrafree-MC filter units (Millipore Cat. No. UFC30GV0S) for filtration following DNA extraction

Supplies for Real-time PCR Analysis

- 96 well PCR plates (ABI Cat. No. 4346906 or equivalent)
- 96 well plate holders, Costar®, black (VWR Cat. No. 29442-922 or equivalent)
- Edge seals for 96 well PCR plates (Adhesive Plate Sealers, Edge Bio Cat. No. 48461 or equivalent)
- 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
- Optical seals (ABI Cat. No. 4311971 or equivalent)
- PCR-grade water, sterile (Teknova Cat. No. W3350 or equivalent)

Supplies for Culture

- Petri dishes, sterile, disposable, 100 × 15 mm
- Inoculating loops and needles, sterile, disposable
- Disposable cell spreaders (such as L-shaped, Fisher Scientific Cat. No. 03-392-150 or equivalent)
- Racks for 50 mL centrifuge tubes
- 50 mL conical tubes (VWR Cat. No. 21008-951 or equivalent)
- Pipet tips with aerosol filter for 1000 µL and 100 µL (Rainin Cat. No. SR-L1000F and GP-100F or equivalent)
- Biotransport carrier (Nalgene, Thermo Scientific Cat. No. 15-251-2 or equivalent)
- Sterile, breathable adhesive seals (AeraSeal™ Film, Thomas Scientific Cat. No. 6980A25 or equivalent).
- Chocolate Agar plates (Hardy Diagnostics Cat. No. E14 or equivalent)

Equipment

- Biosafety cabinet (BSC) – Class II or Class III
- PCR preparation hood (optional)
- Shaker incubator for RV-PCR (Thermo Scientific, MaxQ™ 4000 Cat No. SHKE4000 or equivalent) and Universal 18" × 18" shaker platform (Thermo Scientific, MaxQ™ Cat. No. 30110 or equivalent)
- Balance, analytical, with Class S reference weights, capable of weighing 20 g ± 0.001 g
- ABI 7500 Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA)
- Refrigerated centrifuge with PCR plate adapter and corresponding safety cups (Eppendorf Cat. No. 5804R, 5810R or equivalent) or PCR plate spinner (placed in BSC) (VWR, Cat. No. 89184-608 or equivalent)
- Refrigerated microcentrifuge for Eppendorf tubes with aerosol-tight rotor (Eppendorf, Cat. No. 5415R or equivalent)
- Platform vortexer for RV-PCR (VWR Cat. No. 58816-115 or equivalent) with Velcro® straps
- Single-tube vortexer
- Heating block for RV-PCR (VWR Cat. No. 12621-096 or equivalent) and 2 mL tube blocks (VWR Cat. No. 12985-048 or equivalent) or water bath set at 95°C
- Single-channel micropipettors (1000 µL, 200 µL,100 µL, 20 µL, 10 µL)
- Serological pipet aid
- Dynamag™ magnetic racks for RV-PCR (Invitrogen Cat. No. 123-21D or equivalent)
- Incubator(s), microbiological type, maintained at 35-37 °C
- Autoclave or steam sterilizer, capable of achieving 121°C (15 psi) for 30 minutes
- Cold block for 2 mL tubes (Eppendorf Cat. No. 3880 001.018 or equivalent)
- pH meter (VWR Cat. No. 89231-664 or equivalent)

Reagents

- PCR-grade water, sterile (Teknova Cat. No. W3350 or equivalent)
- MilliQ® H_2O or equivalent
- Phosphate buffered saline (PBS) buffer (Teknova Cat. No. P0261 or equivalent)
- 10X PBS buffer (Teknova Cat. No. P0195 or equivalent)
- TE buffer (1X Tris-HCl-EDTA [Ethylenediaminetetraacetic acid]) buffer, pH 8.0, Fisher Scientific, Cat. No. BP2473-500 or equivalent)
- 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)
  - 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)
- 100% Ethanol (200-proof) for preparation of 70% ethanol by dilution with PCR-grade water
- MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Cat. No. 03730 964001)
- MagNA Pure LC Total Nucleic Acid Isolation Kit-Additional Lysis/Binding Buffer (Roche Cat. No. 03246779001)
- TaqMan® Universal PCR Master Mix (Life Technologies, Cat. No. 4304437)
- TaqMan® Platinum® Taq DNA Polymerase (Life Technologies, Cat. No. 10966-034)
- Primers and probe for *F. tularensis* Type A specific strains F4_pdpD targeting the pathogenicity determinant protein D (obtained from a commercial supplier, Biosearch Technologies or similar)
  - Forward Primer (F4_pdpD_F) –
    5'- TTG CTC CAG TAG CTG CAA GAT T -3'
  - Reverse Primer (F4_pdpD_R) –
    5'- CCA AGT GCT TGG TGG TGG TA -3'
  - Probe (F4_pdpD_Pr) –
    5'-6FAM- TGC TGC CGA GAT GTT TTC ATT ATT AAC TGA TGC –BHQ1-3'
- Primers and probe for *F. tularensis* Type A specific strains F5_pdpD targeting the pathogenicity determinant protein D (obtained from a commercial supplier, Biosearch Technologies or similar)
  - Forward Primer (F5_pdpD_F) –
    5'-GAG ACA TCA ATT AAA AGA AGC AAT ACC TT-3'
  - Reverse Primer (F5_pdpD_R) –
    5'-CCA AGA GTA CTA TTT CCG GTT GGT-3'
  - Probe (F5_pdpD_Pr) –
    5'-6FAM-AAA ATT CTG CTC AGC AGG ATT TTG ATT TGG TT-BHQ1-3'
- Bacto™ Brain Heart Infusion Broth Base (BD Biosciences Cat No. 237500)
- L-histidine (Sigma-Aldrich Cat. No. H8000-5G)
- Oxoid® Vitox Supplement (Thermo Fisher Scientific Cat. No. SR0090A)
- Remel™ Fildes Enrichment supplement (Thermo Fisher Scientific Cat. No. R45037)

6X 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)

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1. Weigh 22.2 g Bacto™ Brain Heart Infusion Broth Base powder into 500 mL flask or bottle.
2. Weigh 0.6 g L-histidine into the same 500 mL flask or bottle.
3. Add 28 mL MilliQ® H_2O or equivalent.
4. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
5. Allow broth to cool down to room temperature under a BSC before storing at 4°C.

6. Check pH and adjust by addition of NaOH pellets if pH is under 6, such that the pH is 7.2 ± 0.2.
7. 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).
8. 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).
9. 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.

1X Phosphate-Buffered Saline (1X PBS)

Note: 1X PBS may be prepared from 10X PBS Buffer or used as 1X (pH 7.4; Teknova Cat. No. P0261)

1. Add 100 mL 10X PBS buffer (Teknova Cat. No. P0195) to a 1-L flask or bottle.
2. Add 900 mL MilliQ® H_2O or equivalent.
3. Sterilize 15 min at 15 psi and 121°C or through 0.2 micron 1-L disposable filtration unit.
4. Store in refrigerator.

10% Bleach-pH amended (prepared daily)

- Prepare bleach solution by adding 1-part bleach (Ultra Clorox® Germicidal bleach), 1-part acetic acid and 8 parts reagent-grade water as described below.
- 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 should be used to measure pH as opposed to pH strips or kit. When mixed, place a lid on the mixture to reduce chlorine escape and reduce worker exposure.

<u>Sample Processing and Plating for Water Samples</u>

Note: Gloves should be used and changed between samples and as indicated below.

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 phosphate-buffered saline (PBS) to 40 mL water sample (final ~1X PBS concentration).
   a. Using a 50-mL serological pipet, transfer 40 mL of water sample to a 50-mL screw capped centrifuge tube. If the sample volume is greater than 40 mL, process the sample by adjusting the PBS volume (final concentration ~1X PBS) and the centrifugation step (Step 1c., in multiple tubes, if necessary), to have a final suspension volume of 3 mL (Step 1e).
   b. Repeat steps above for each sample.

c. Make sure tubes are balanced and place 50-mL tubes into sealing centrifuge buckets and decontaminate centrifuge buckets before removing them from the BSC.
d. Centrifuge tubes at 3,500 × g, with the brake off, for 15 minutes in a swinging bucket rotor.

Note: A higher × g (up to 4,500 x g) is preferred as long as the speed is within the tube specifications.

e. 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.
f. Vortex mix the remaining 3 mL and the pellet.
g. Remove suspension (or combined suspension) from one tube with a sterile 5 mL pipet (recording the volume) and transfer to a corresponding sample well of a 48-well plate.

2. Add concentrated growth medium (6X BVFH) and process for RV-PCR analysis.
   a. 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 medium well.
   b. For each well, transfer 500 µL from each sample well of the 48-well plate and transfer to an appropriately labeled screw cap tube. This is a T_0 aliquot for each sample. Repeat for each sample.
   c. Store aliquots on ice or in cold block (4ºC).
3. Seal and incubate 48-well plate
   a. Seal 48-well plate with sterile, breathable seal.
   b. Place in Zip-lok®Ziploc bag and seal bag.
   c. Incubate 48-well plate at 37ºC on a shaker incubator at 180 rpm for 30 hour (h).
4. Process T_0 aliquot for DNA Extraction-Purification
   a. Centrifuge tubes at 14,000 rpm (20,800 RCF) for 10 min at 4ºC.
   b. Remove 300 µL supernatant and dispose to waste. Store pellets on ice or in cold block (4ºC). Alternatively, the pellet may be stored at -20ºC until ready to process for DNA extraction (see **DNA Extraction/Purification Procedure** section).
5. At T_{30} (after 30-h incubation), transfer 500 µL from each sample well to an appropriately labeled 2-mL screw cap tube. Ensure that the T_{30} aliquot for each sample is taken from the same well from which the T_0 aliquot for the corresponding sample was taken. This is a T_{30} aliquot for each sample.

   Note: For post-decontamination, field samples (with potentially high concentrations of dead F. tularensis cells), the incubation period may be extended to 36 h.

   a. Centrifuge tubes at 14,000 rpm (20,800 RCF) for 10 min at 4ºC.
   b. Remove 300 µL supernatant and dispose to waste. Store pellets on ice or in cold block (4ºC). Alternatively, the pellet may be stored at -20ºC until ready to process for DNA extraction.

6. Process the pellets from T_0 and T_{30} aliquots by the **DNA Extraction/Purification Procedure** below**.**

RV-PCR Analysis: DNA Extraction/Purification Procedure (Promega MagneSil® Blood kit)

Note: T_0 and T_{30} extractions can be completed separately.

1. Thaw T_0 and T_{30} aliquots if they were stored at -20°C.
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.
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 as necessary between samples. Incubate the T_0 and T_{30} lysate tubes at room temperature for 5 minutes.
4. Vortex the paramagnetic particles (PMPs) on high (~1800 rpm) for 30–60 seconds, or until they are uniformly resuspended. Resuspend PMPs by briefly vortexing (3–5 seconds) as necessary.
5. Uncap one tube at a time and add 600 µL of PMPs to each T_0 and T_{30} lysate (containing 1 mL sample), hereafter referred to as "T_0 and T_{30} tubes". Mix by briefly vortexing (use a new tip for each sample and discard used tips in a sharps container).
6. Repeat for all T_0 and T_{30} tubes.
7. Vortex each T_0 and T_{30} tube for 5−10 seconds (high), incubate at room temperature for 5 minutes, briefly vortex, and then place on the magnetic stand with hinged-side of the tube facing toward the magnet. After all the tubes are in the stand, invert tubes 180 degrees (upside-down) turning away from you, then right side-up, then upside down toward you, then right side-up (caps up) position. This step allows all PMPs to contact the magnet. Check to see if any beads are in the caps and if so, repeat the tube inversion cycle again. Let the tubes sit for 5−10 seconds before opening. Maintain the tube layout when transferring tubes between the magnetic stand and the 96-well tube rack. Alternatively, tubes may be vortexed while in removable rack that interfaces with magnetic stand.
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.
9. Uncap each T_0 and T_{30} 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 tips in a sharps container. Cap and vortex on low setting for 5−10 seconds, and transfer to 96-well tube rack.

10. After adding lysis buffer to all the T_0 and T_{30} 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 above.
11. Remove all the liquid as described above, except that a glove change between samples is not required. Use a new tip for each T_0 and T_{30} tube (discard used tips in a sharps container). Recap the tube.
12. Repeat liquid transfer for all tubes.
13. 1st Salt Wash: Uncap each T_0 and T_{30} tube, one at a time, and add 360 µL of Salt Wash solution (VWR Cat. No. PAMD1401 or equivalent). Use a new tip for each T_0 and T_{30} tube and discard used tips in a sharps container. Cap and transfer to 96-well tube rack.
14. After adding the Salt Wash solution to all of the T_0 and T_{30} 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 above.
15. Remove liquid as described above. Use a new tip for each T_0 and T_{30} tube and discard used tips in an appropriate waste container. Recap the tube. Repeat for all T_0 and T_{30} tubes.
16. 2nd Salt Wash: Repeat Salt Wash for all T_0 and T_{30} tubes.
17. 1st Alcohol Wash: Uncap each T_0 and T_{30} tube, one at a time, and add 500 µL of alcohol wash solution (VWR Cat. No. PAMD1411 or equivalent). Use a new tip for each sample and discard used tips in a sharps container. Cap and transfer to 96-well tube rack.
18. After adding alcohol wash solution to all the T_0 and T_{30} tubes, vortex each tube for 5−10 seconds (low speed) and place on the magnetic stand. After all the T_0 and T_{30} tubes are in the stand, follow the tube inversion cycle, as described above.
19. Remove liquid as described above. Use a new tip for each T_0 and T_{30} tube and discard used tips in a sharps container. Recap the tube.
20. 2nd Alcohol Wash: Repeat Alcohol Wash for all T_0 and T_{30} tubes.
21. 3rd Alcohol Wash: Repeat Alcohol Wash for all T_0 and T_{30} tubes except use 70% ethanol wash solution. After the liquid is removed, recap the tube and transfer to the 96-well tube rack.
22. Open all T_0 and T_{30} tubes and air dry for approximately 2 minutes.
23. Heat the open T_0 and T_{30} tubes in the heat block at 80°C ± 2°C until the PMPs are dry (~20 minutes). Allow all the alcohol solution to evaporate since alcohol may interfere with analysis.
24. DNA elution: While they are in the heating block, add 200 µL of elution buffer (VWR Cat. No. PAMD1421 or equivalent) to each T_0 and T_{30} tube, and close tube.
25. Vortex for 10 sec (seconds) and let the tubes sit in the heating block for 80 seconds.
26. Briefly vortex the tubes (5−10 sec) taking care to prevent the liquid from entering the tube cap and let the tube sit in the heating block for one minute.
27. Repeat vortexing/heating cycle four more times.
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 5 minutes.

29. Briefly vortex each tube (5−10 seconds) on low speed. Place tube in 96-well tube rack.
30. Briefly vortex each tube and place on the magnetic stand for at least 30 seconds. Bring the cold block to the BSC.
31. Collect liquid from each T_0 or T_{30} tube with a micropipettor (~80–90 µL) and transfer to a clean, labeled, 1.5 mL tube on a cold block (check tube labels to ensure the correct order). Use a new tip for each tube and discard tips in a sharps container. Visually verify absence of PMP carryover during final transfer. If magnetic bead carryover occurred, place 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).
32. Centrifuge tubes at 14,000 rpm at 4°C for 5 min 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).
33. Store T_0 and T_{30} DNA extract tubes "referred to as T_0 and T_{30} 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, store DNA extracts at -20ºC.

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

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).
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).
3. Add 300 µL phosphate-buffered saline (PBS; 1X PBS, Teknova, Cat. No. P0261) to each PC Tube.
4. Vortex tubes for 10 sec on single tube vortexer at 1800-2000 rpm (VWR, IKA Model MV1 or equivalent).
5. Incubate tubes for 30 min at room temperature (in BSC). Every 5 min invert tubes 5 times to mix.

DNA Purification Protocol

Note: Run samples in batches of 8 on the instrument.

1. 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.
2. 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.
3. 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).

MagNA Pure® Compact Protocol Using the MagNA Pure Compact Nucleic Acid Isolation Kit I

Isolate nucleic acids according to the protocol below:

4. Turn on the instrument; ensure that the Tube Rack is seated correctly in the instrument.
5. Remove the Elution Tube Rack from the instrument.
6. Click the Run button on the Main Menu Screen to access Sample Ordering Screen 1.
7. Follow the software-guided workflow.
8. Remove a prefilled Reagent Cartridge from the blister pack. Handle each Reagent Cartridge as follows:
    a. Always wear gloves when handling the cartridge.
    b. Hold the cartridge only at the barcode imprinted area and the opposite side.
    c. Avoid touching the sealing foil covering the cartridge wells.
    d. Avoid touching the two single open wells and do not use them as handles.
    e. 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.
9. 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.
10. Scan the cartridge barcode using the barcode scanner supplied with the instrument.
11. With the two isolated wells pointing away from you, insert all the wells on the Reagent Cartridge into the holes in the Cartridge Rack.
12. Use the guide slots on the rack to help position the cartridge.
13. Repeat the steps above for the desired numbers of samples (1 to 8).
14. Proceed to Sample Ordering Screen 2.
15. Select the appropriate purification protocol from the Protocol menu (DNA_Blood_external_lysis).
16. Select the elution volume (100 µL).
17. Optional: Select the Internal Control Volume (0 µL).
18. Insert the appropriate number of Tip Trays (one per sample) into the assigned position in the instrument Tip Rack.
19. 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.
20. 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.
21. Proceed to Sample Ordering Screen 3.
22. Scan the sample barcode from the primary sample tube or enter the sample ID.

23. 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.
24. Scan the bar codes of the Elution Tubes.
25. Place the Elution Tubes into the Elution Tube Rack. Make sure the brim of the tubes seats solidly on the rack.
26. On the Confirmation Screen, check the information display.
27. If the information is correct, confirm it by touching the "Confirm Data" button, close the front cover, and start the run.
28. After the purification run has ended, the Result Screen appears showing the result of the isolation process for each channel.
29. The result will be PASS if the isolation run was completed without any warning or error.
30. 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.
31. 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.
32. 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.
33. Optional: Start the automated liquid waste discard.
34. Always empty the MagNA Pure® Compact Waste Tank after every purification run.
35. 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).
36. Store DNA extract tubes "referred to as T_0 or T_{30} 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.

Cleanup Procedure

- Dispose of all biological materials (double bagged) in autoclave bags and sealed.
- Autoclave all waste materials.
- Decontaminate counters and all equipment with bleach (1 volume water and 9 volumes commercial bleach) made fresh daily. Follow this with rinsing by 70% isopropanol and/or by rinsing with deionized water.

Real-time PCR Analysis

1. Prepare PCR Mix according to the table below (PCR Mix for All Selected *F. tularensis* Assays).
2. Set up 96 well PCR plate with PCR mix according to plate layout in PCR-preparation hood, seal, and transfer to BSC.
3. Analyze T_0 and T_{30} DNA extracts on same PCR plate.

4. If samples were frozen, transfer them to BSC and let them thaw to room temperature.
5. Perform 1:10 dilution of samples. Alternatively, only run samples undiluted (5 µL plus 20 µL PCR Master Mix).
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.
7. Mix sample up and down 5 times and transfer 10 µL to plate wells, following the plate layout.
8. Mix diluted samples up and down 10 times and transfer 5 µL from plate well or tube to the PCR plate (with PCR Mix). Seal PCR plate with clear Edge Seal.
9. Centrifuge sealed PCR plate for 1 min at 2000 rpm.
10. Open safety cup in BSC, place plate on photo-tray, change gloves, transfer PCR plate to ABI thermocycler.
11. Run PCR cycle (see below).
12. After cycle completion, discard sealed PCR plate to waste. Autoclave. PCR plates with amplified product are never to be opened in the laboratory.
13. Follow laboratory cleanup procedure.

PCR Thermal Cycling Conditions

STEPS	**UNG Incubation**	**AmpliTaq Gold Activation**	**PCR, 45 cycles**	
	HOLD	**HOLD**	**Denaturation**	**Annealing/Extension**
Temperature	50°C	95°C	95°C	60°C
Time	2 min	10 min	5 sec	20 sec

Fast Ramp: 3.5°C/sec up and 3.5°C/sec down.

PCR Mix for F4 and F5 *F. tularensis* Real-time PCR Assays

Reagent	**Volume (µL)**	**Final Concentration**
TaqMan® 2X Universal Master Mix	12.5	1X
Platinum® *Taq* 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	

RV-PCR Data Interpretation

Calculate an average C_T from the replicate reactions for T_0 and T_{30} DNA extracts of each sample. Subtract the average C_T of the T_{30} DNA extract from the average C_T of the T_0 DNA extract. If there is no C_T value for the T_0 or T_{30} DNA extract (i.e., non-detect), use 45 (total number of PCR cycles used) as the C_T value. The significant change (decrease) in the average C_T value from T_0 to T_{30} (ΔC_T) indicates a positive result suggesting the presence of viable *F. tularensis* cells in the

sample. A ΔC_T criterion of ≥ 6 (an approximate two log difference in DNA concentration) and a corresponding T_{30} 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) shall be used (i.e., 36 h for post-decontamination, field samples with high concentrations of dead *F. tularensis* cells). However, $(\Delta C_T) \geq 6$ algorithm should still be used for a positive result. A minimum of two out of three T_0 PCR replicates must result in C_T values ≤ 44 (in a 45-cycle PCR) to calculate the average T_0 C_T. A minimum of two out of three T_{30} 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 because 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 because 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 are considered valid or if the PCR analyses must be repeated.

Traditional culturing of diluted cell suspensions on Chocolate Agar (or other appropriate media)

1. Inoculate Chocolate Agar plates with 100 µL of each sample (each dilution is plated in triplicate).
2. Using one Lazy-L™ cell spreader (Millipore Sigma, Burlington, MA) per suspension, spread sample to obtain a uniform liquid layer on plate.
   Note: do not spread liquid to plate edge.
3. After all liquid is absorbed, invert plates.
4. Incubate Chocolate Agar plates at 37°C for 3 days.
5. Place sealed sample tubes in a secondary container (re-sealable bag); store tubes at 4°C.
6. After three days, confirm growth for *F. tularensis*. Confirm that a subset of the colonies is characterized as *F. tularensis* based on real-time PCR analysis using the F4 or F5 *F. tularensis*-specific chromosomal assay.

7. Count colony-forming units (CFU) and record on supplied data sheet:
   - If colony counts are ≤ 250 – record the actual number
   - If colony counts are greater than 250, record as TNTC (too numerous to count)

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