EPA/600/R-20/079 | April 2020
www.epa.gov/homeland-security-research

United States
Environmental Protection
Agency

Development and Optimization of a Rapid Viability Polymerase Chain Reaction (RV-PCR) Protocol for Detection of *Yersinia pestis* in Water Samples

Office of Research and Development
Homeland Security Research Program

EPA/600/R-20/079
April 2020

FINAL REPORT

Development and Optimization of a Rapid Viability Polymerase Chain Reaction (RV-PCR) Protocol for Detection of *Yersinia pestis* in Water Samples

by

Sanjiv R. Shah, Ph.D.
U.S. Environmental Protection Agency
Washington, DC 20460

and

Staci Kane, Ph.D.
Teneile Alfaro
Lawrence Livermore National Laboratory
U.S. Department of Energy
Livermore, CA 94551

EPA IA DW-89-92328201-0
Homeland Security Research Program

Disclaimer

U.S. Environmental Protection Agency

The United States Environmental Protection Agency (U.S. EPA) through its Office of Research and Development funded and managed the research described here (EPA IA DW-89-92328201-0). This report has been reviewed and approved for public release in accordance with the policies of the U.S. EPA. Note that approval does not signify that the contents necessarily reflect the views of the Agency. Mention of trade names, products, or services does not convey EPA approval, endorsement, or recommendation.

Lawrence Livermore National Laboratory

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 Nickel.Kathy@epa.gov or McCall.Amelia@epa.gov for assistance.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

Ab ..... antibody
ABI ..... Applied BioSystems, Inc.
ATD ..... Arizona Test Dust
Ave ..... average
B. anthracis ..... *Bacillus anthracis*
BHI ..... brain heart infusion
BHQ ..... Black Hole Quencher
bp ..... base pair
BSC ..... biosafety cabinet
BSL-3 ..... BioSafety Level-3
°C ..... degrees Centigrade
CDC ..... Centers for Disease Control and Prevention
CESER ..... Center for Environmental Solutions and Emergency Response
CFU ..... colony-forming units
CRP ..... Critical Reagents Program
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_{12}) or T_{12} C_T ..... C_T value after 12 hours incubation
C_T (T_{24}) or T_{24} C_T ..... C_T value after 24 hours incubation
C_T (T_{40}) or T_{40} C_T ..... C_T value after 40 hours incubation
ΔC_T ..... delta cycle threshold
DD ..... distilled, deionized
DE ..... diatomaceous earth
DNA ..... deoxyribonucleic acid
DOE ..... Department of Energy
dNTP ..... deoxynucleotide triphosphate
dsDNA ..... double-stranded DNA
ERLN ..... Environmental Response Laboratory Network
EPA ..... Environmental Protection Agency
F. tularensis ..... *Francisella tularensis*
FAM 6-carboxyfluorescein
fg/µL ..... femtogram per microliter
FDA ..... Food and Drug Administration
FERN ..... Food Emergency Response Network
g ..... gram
hr ..... hour(s)
HSMMD ..... Homeland Security and Materials Management Division
HSRP ..... Homeland Security Research Program
IMS ..... immunomagnetic separation
ISO ..... International Organization for Standardization
IPA ..... isopropanol
kb ..... kilobase
LLNL ..... Lawrence Livermore National Laboratory

LOD ..... limit of detection
LRN ..... Laboratory Response Network
µg ..... microgram
µg/L ..... micrograms per liter
µg/mL ..... micrograms per milliliter
µm ..... micrometer
Mb ..... mega base pairs
MF ..... modified filtration
mg ..... milligram
MG ..... Miracle Gro®
min ..... minute
µL ..... microliter
mL ..... milliliter
mg/L ..... milligrams per liter
mm ..... millimeter
mM ..... millimolar
NA ..... not applicable
NDT ..... non-detect
ng/µL ..... nanograms per microliter
NIST ..... National Institute of Standards and Technology
nm ..... nanometer
NTC ..... No-Template Control
OD_{600} ..... optical density at 600 nm
ORD ..... Office of Research and Development
OW ..... Office of Water
PBS ..... phosphate buffered saline
PCR ..... polymerase chain reaction
pg ..... picogram
PMP ..... paramagnetic particles
ppm ..... parts per million
PI ..... Principal Investigator
QA ..... quality assurance
QC ..... quality control
RCF ..... relative centrifugal force
RNA ..... ribonucleic acid
ROX 6-carboxyl-X-rhodamine
rpm ..... revolutions per minute
RV ..... rapid viability
RV-PCR ..... rapid viability–polymerase chain reaction
SAP ..... superabsorbent polymers
SD ..... standard deviation
sec ..... second
T_0 ..... time 0, prior to incubation
T_2 ..... after 2 hr of incubation
T_9 ..... after 9 hr of incubation
T_{12} ..... after 12 hr of incubation

T_{24} .....after 24 hr of incubation
T_{40} .....after 40 hr of incubation
T_{48} .....after 48 hr of incubation
T_f .....time final, after incubation
TBA..... Tryptose Blood Agar base
TNTC .....too numerous to count
UNG .....uracil-N-glycosilase
UV.....ultraviolet
WLA ..... Water Laboratory Alliance
Y. pestis ..... *Yersinia pestis*
YPEB ..... *Y. pestis* Enrichment Broth
1X.....1-fold concentrated (no concentration)
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
AmpliTaq Gold®	Life Technologies	Carlsbad, CA
Autovials™	GE Healthcare	Noblesville, IN
Bacto™	Difco Laboratories	Franklin Lakes, NJ
Black Hole Quencher®	Biosearch Technologies	Petaluma, CA
Difco™	Becton Dickinson	
Dynamag™	Life Technologies	Carlsbad, CA
Epicentre®	Epicentre Biotechnologies Inc.	Madison, WI
Invitrogen®	Life Technologies	Carlsbad, CA
Life Technologies™	Life Technologies	Carlsbad, CA
LIVE/DEAD® BacLight™	Life Technologies	Carlsbad, CA
MagneSil® Blood Genomic	Promega	Madison, WI
MasterPure®	Epicentre Biotechnologies	Madison, WI
Millipore®, Milli-Q™	Millipore Corp.	Billerica, MA
MicroFunnel™	Pall Corp.	Ann Arbor, MI
Miracle Gro®	The Scotts Company	Maryville, OH
Pathatrix®	Life Technologies	Carlsbad, CA
PicoGreen®	Life Technologies	Carlsbad, CA
Quant-iT™	Life Technologies	Carlsbad, CA
Qubit®	Life Technologies	Carlsbad, CA
TaqMan®	Life Technologies	Carlsbad, CA
Whatman®	GE Healthcare, Life Sciences	Pittsburgh, PA

Acknowledgments

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory (LLNL) under Contract DE-AC52-07NA27344. Funding for this research was provided by the Homeland Security Research Program of the U.S. Environmental Protection Agency's (EPA's) Office of Research and Development (ORD).

Research Team

Staci Kane and Teneile Alfaro [Lawrence Livermore National Laboratory (LLNL)]

EPA Technical Lead

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

Technical Reviewers

Gene Rice (U.S. EPA-ORD- CESER-HSMMD,)

Worth Calfee (U.S. EPA-ORD- CESER-HSMMD)

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

Jafrul Hasan (U.S. EPA Office of Chemical Safety Pollution Prevention – Office of Pesticide Programs Microbiology Laboratory)

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-ORD- CESER-HSMMD)

Technical Editor

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

Executive Summary

Due to the historical usage of *Yersinia pestis* as a biological weapon and the occurrence of natural plague outbreaks, there is a need for rapid and sensitive analytical methods for detection of viable *Y. pestis*. These incidents can result in contamination of water infrastructure. Vegetative bacterial pathogens such as *Yersinia pestis*, the bacteria that cause plague, may remain viable and infectious for some time in certain environments including water. The EPA Office of Water (OW) is responsible for protecting and managing water resources. In this research effort, the Rapid Viability Polymerase Chain Reaction (RV-PCR) method was developed for use by the Water Laboratory Alliance (WLA), a network of laboratories for water sample analysis established by the EPA's OW.

The RV-PCR method, which combines high throughput sample processing, short-incubation broth culture, and sensitive, specific real-time PCR analysis before and after sample incubation, 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 agent. In partnership between the scientists at EPA's Center for Environmental Solutions and Emergency Response within the Office of Research and Development (ORD) and the scientists 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 *Y. pestis* detection. This method may serve as a model for detection of other vegetative bacterial pathogens of concern.

In this research effort, the RV-PCR method was developed and evaluated for *Y. pestis* cells with the following key features: 24-hr incubation (shortened from preliminary 48-hr incubation); sample incubation in 48-well plates for high throughput culture and sample handling; sensitive, 10-cell level (10-99 cells) limit of detection (LOD); and robust sample processing steps that accommodate complex sample backgrounds. By optimizing the cell processing procedure and incorporating a deoxyribonucleic acid (DNA) extraction/purification procedure (based on Promega Magnesil® reagents), a shorter incubation period was demonstrated with maintenance of the 10-cell level LOD. These reagents showed good DNA yield and quality and are compatible with automated processing, although other existing manual or automated platforms could be used as well. Furthermore, improvements in the culturing step enabled reproducible growth even at low inoculum levels (< 10 cells per mL). Additional recommendations were made based on challenge testing with potential soluble and insoluble chemical interferences, and live, non-target or dead target biological interferences, addressing a range of potential "real world" complex sample types. These included extending the incubation time to 36 hr to further reduce the method false negative rate for samples with high dead target cell backgrounds ($\geq 10^4$ cells/mL). This effort also included a preliminary investigation that showed RV-PCR was compatible with front-end methods to concentrate cells from larger volume water samples such as immunomagnetic separation (IMS) using magnetic beads coated with *Y. pestis*-specific antibodies and a modified filtration approach using superabsorbent polymers to maintain moisture in filtration devices. However, both of these approaches had their inherent limitations, which could challenge their use during the real-world sample analysis. While outside the scope of this effort, RV-PCR is expected to be compatible with ultrafiltration for cell concentration since a 10-cell level LOD was observed even in complex samples containing chemical and biological interferences.

The RV-PCR method for detection of viable *Y. pestis* from water samples will help enhance the WLA's capability for rapid, reliable, and high throughput sample analysis in case of a natural outbreak, laboratory accident, or a criminal or terrorist incident of plague.

1.0 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. EPA is also designated as the Sector-Specific Agency for water and wastewater systems. Decision makers will need timely and reliable water sample analysis results during biological agent response and recovery efforts. *Y. pestis* is one of these agents which could be introduced into water infrastructure due to a natural outbreak, laboratory accident, or intentional contamination. It is known that vegetative bacterial pathogens such as *Yersinia pestis* (*Y. pestis*, the causative agent of plague) may remain viable and infectious for some time in certain environments including water. Due to the historical usage of *Y. pestis* as a biological weapon and the occurrence of natural plague outbreaks, there is a need for rapid and sensitive analytical methods for detection of viable *Y. pestis*. Within EPA, the Office of Water (OW) is responsible for protecting and managing water resources. The OW 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). The WLA needs rapid and reliable sample analysis methods to assess the presence of live *Y. pestis*.

To help meet this need, the Rapid Viability Polymerase Chain Reaction (RV-PCR) method was developed and optimized for rapid detection of viable *Y. pestis* in water samples. This method can serve as a template for the detection of other vegetative bacterial pathogens, where modifications can be made for differences in growth requirements and characteristics.

The current culture-based methods used for *Y. pestis* detection are labor-intensive and low throughput such that only 30–40 samples can be processed per day per laboratory, with confirmed results obtained days later. More rapid viability methods are needed as part of the EPA's capabilities to ensure public safety and to help mitigate impacts of facility and infrastructure closures following a biological agent release. It is well understood that rapid detection methods such as real-time polymerase chain reaction (PCR) cannot distinguish between live (potentially infectious) and dead pathogens; however, features of real-time PCR were leveraged for development of RV-PCR. The RV-PCR method combines high throughput sample processing, short-incubation broth culture, and 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, referred to as the change in cycle threshold (C_T) or ΔC_T, between the initial (before sample incubation) C_T at time 0 (C_T T_0) and the final C_T after incubation (C_T T_f). Example PCR response curves are shown in Figure 1 along with the criteria for positive detection, namely $\Delta C_T \geq 6$. 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.

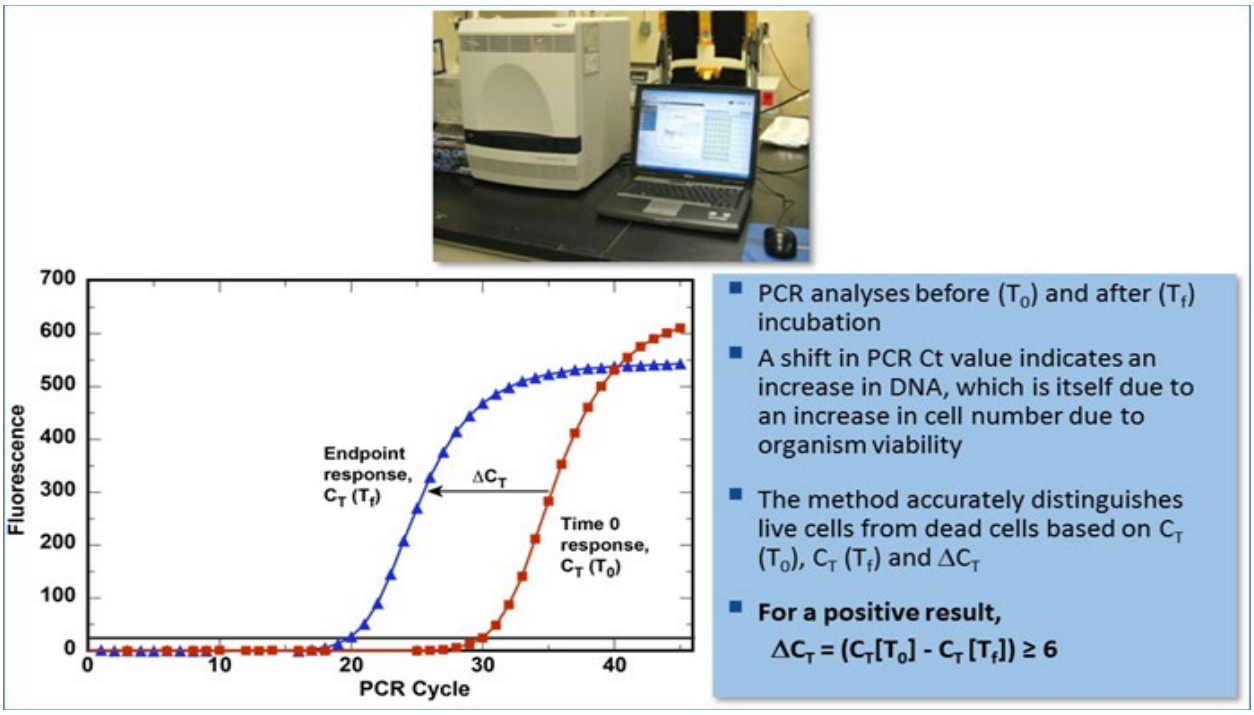

Figure 1. Example real-time PCR response curves showing parameters (C_T [T_0], C_T [T_f], and ΔC_T) used in determining presence or absence of viable spores in the original sample. A significant shift in PCR response curve indicates an increase in DNA and thus, cell number. A curve is shown for the Time 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) in order to calculate a ΔC_T value.

The RV-PCR method for *Y. pestis* detection not only generates rapid results but also can provide a higher throughput capability as compared to the traditional culture-based methods, and hence, increases the laboratory capacity for sample analysis. In place of multiple sample dilutions, several growth media agar plates, and enrichment culture per sample used by the culture method, the RV-PCR method uses a single well per sample on a 48-well plate (Figure 2).

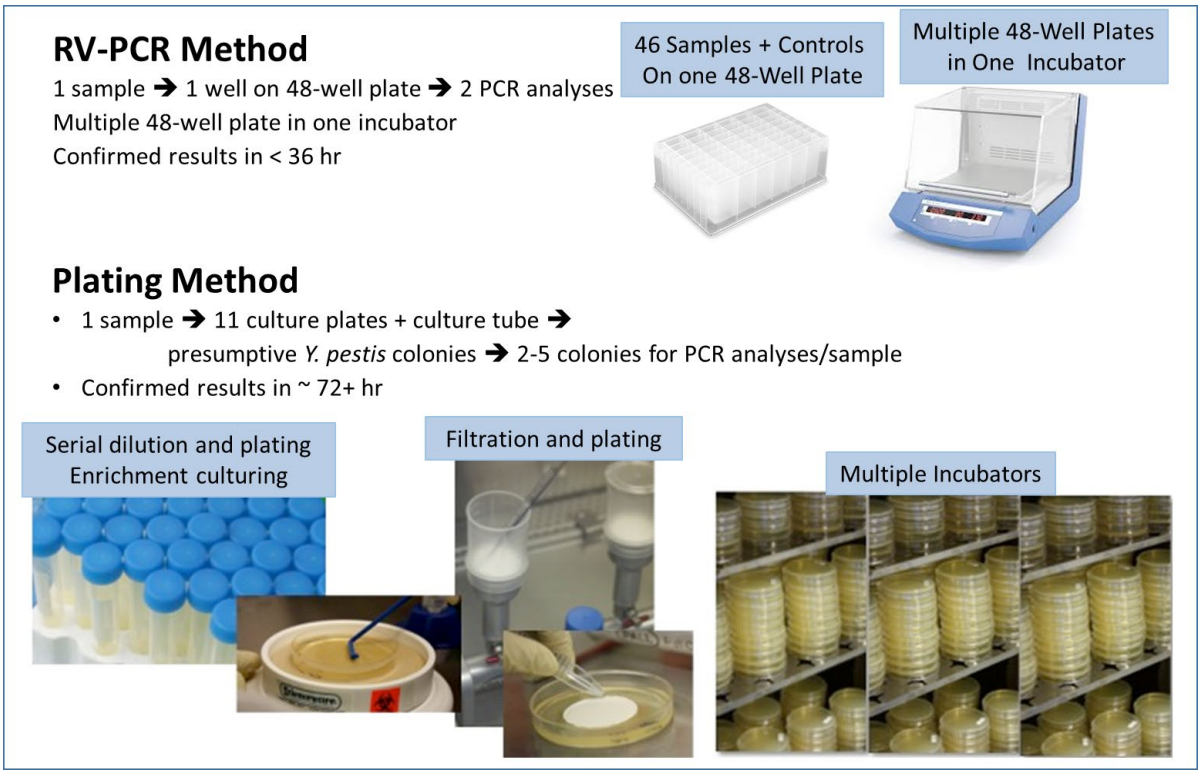

Figure 2. Comparison of the RV-PCR method vs. the traditional culture method for *Y. pestis*.

This effort significantly expanded a previous effort where LLNL and EPA scientists developed preliminary RV-PCR protocols for *Y. pestis* and *Francisella tularensis* (*F. tularensis*). The project work led to protocols for *Y. pestis* and *F. tularensis* cells from wet wipes and buffered water samples (US EPA Internal Report, 2010). 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 current effort described here.

This project focused on the 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 dead *Y. pestis* cells and microorganisms present in non-autoclaved ATD including *Bacillus* spp. and other non-target bacteria as well as fungal species (Rose et al., 2011). The virulent *Y. pestis* CO92 reference strain was used for protocol development. In addition, the attenuated *Y. pestis* CO92 pgm^- strain was used in specific cases as identified in the report; in particular, this strain was used in evaluation of the immunomagnetic separation (IMS) and modified filtration (MF) approaches as well as generation of a dead cell background for RV-PCR method evaluation. In this effort, DNA 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 linear range (~8 logs), and 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.

The RV-PCR protocol steps and some of the equipment for *Y. pestis* are shown in Figure 3. The manual RV-PCR protocol (without cell concentration) developed in this study could be readily automated for higher throughput since the same materials and procedures would be used. In addition, automated platforms for DNA extraction and concentration are often available in laboratories including those in the Centers for Disease Control and Prevention Laboratory Response Network (CDC LRN).

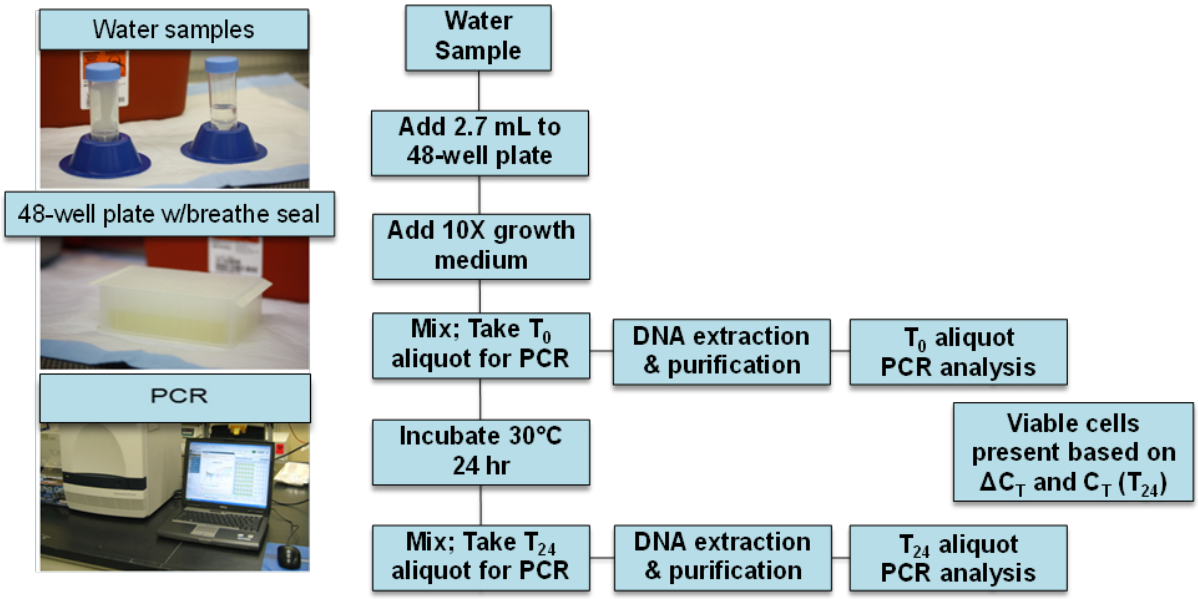

Figure 3. Flow chart for RV-PCR analysis of *Y. pestis* cells from water samples. Using a 48-well plate, up to 3.5 milliliter (mL) total volume can be used per 5-mL well, such as 2.7 mL water sample with 0.3 mL 10X broth (as shown) or up to 3.15 mL water sample with 0.35 mL 10X broth.

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

Task 1. Incorporate DNA extraction/purification procedure into *Y. pestis* RV-PCR protocol

Task 2. Further develop and optimize the sample processing procedure for *Y. pestis* cell recovery and cell growth

Task 3. Further develop and optimize the RV-PCR protocol for *Y. pestis*

Tasks 1 and 2 included development and optimization of procedures for *Y. pestis* 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: (i) humic acid and ferrous sulfate as potential chemical interferents; (ii) live (native) ATD as a source of potential growth-competing microorganisms (non-target cells/spores) and metal oxides; and (iii) dead target cells as background for either post-decontamination or natural degradation scenarios.

With the RV-PCR method developed in this effort, *Y. pestis* Enrichment Broth (YPEB) diluted from 10X to 1X concentration with the water sample was shown to be optimal for *Y. pestis* growth in the 48-well format. The 10-cell level (10-99 cells) limit of detection (LOD) was observed after 24 hr incubation even in the presence of soluble chemicals, insoluble particulates, and non-target cells and spores. Based on the results, a longer incubation period of 36 hr was recommended for samples containing high concentrations of dead *Y. pestis* cells (>10^4/mL). The RV-PCR method enabled higher throughput and shorter time to results (~36 hr for 48 samples and controls with a 24-hr incubation period) compared with traditional culture methods.

2.0 Materials and Methods

2.1 Bacterial Strains and Growth Conditions

In this effort, the pathogenic *Y. pestis* CO92 strain and the attenuated *Y. pestis* CO92 pgm$^-$ strain were used. The pgm$^-$ strain lacks the 102-kilobase (kb) *pgm* locus, which contains a pigmentation section and a high pathogenicity island with virulence genes (Buchrieser et al., 1999). The two strains are from the LLNL strain collection and were verified by performing real-time PCR analysis on genomic DNA using primers and probes specific to the *Y. pestis* chromosome and plasmids. Although the pgm$^-$ strain has a deleted region, none of the assays used in this effort targeted this region so all three assays also detected this strain.

The *Y. pestis* strains were initially grown in Brain Heart Infusion (BHI) broth (prepared from dehydrated powder; Becton Dickinson, Cat. No. 237500) at 28 degrees Centigrade (°C) and on BHI agar plates (with Bacto™ Agar) at 28°C; however, during this study, it was determined that improved cell growth occurred with plates prepared from Tryptose Blood Agar base without blood (TBA; Becton Dickinson, Cat. No. 223220) and *Y. pestis* Enrichment Broth (YPEB) (Doran et al., 2013) rather than BHI broth. Therefore, TBA and YPEB were used for the majority of the experiments (Please see the SOP).

In addition, 30°C was used for some subsequent experiments where noted. Frozen stocks (-80°C) were prepared with 10% glycerol and used to start cultures on TBA plates for experiments. Two different types of solid agar plates were used for the study. Initially BHI plates were used; however, since inconsistent results were observed within and between experiments, a recommendation was made to use TBA (without blood). TBA plates led to high cell counts relative to BHI plates as well as more consistent results (less variability between replicate plates).

2.2 *Y. pestis* Cell Suspension Preparation

For each experiment, cells were propagated starting with agar plates (BHI or TBA) that were streaked from -80°C glycerol stocks. Plates were incubated at 28°C for 2 to 4 days prior to selecting 2–3 colonies with similar morphology for inoculating 5-mL liquid cultures in 50-mL conical tubes.

For initial experiments with BHI broth, successive overnight (18–26 hr) 5-mL cultures were used in each case starting with an optical density at 600 nanometer (nm) (OD_{600}) of ~0.1 (adjusted by dilution with BHI), which corresponds to approximately 6–7 × 10^6 CFU/mL. After three overnight cultures, cells were harvested by centrifugation (3,100 relative centrifugal force [RCF] at 4°C for 15 min) and suspended in BHI directly for experiments testing 1X BHI and suspended in PBS for experiments in which one part 10X (10-fold concentrated) BHI was added to nine parts cell suspension in PBS (referred to as 1X BHI prepared from 10X BHI).

Since YPEB provided more consistent growth, the majority of experiments used a 5-mL YPEB overnight culture with incubation at 28–30°C. The cells were prepared as described for culturing in BHI broth except 1X YPEB was used directly or 10X YPEB was added to cells in PBS to bring the final concentration to 1X YPEB. Cells were then diluted in 1X YPEB or PBS to OD_{600} ~0.1, and ten-fold serial dilutions were performed in 1X YPEB or PBS buffer to achieve the desired starting cell density (CFU/mL) in three mL final volume per well of a 48-well plate.

2.3 Water Sample Types 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 *Y. pestis* cell suspensions prepared in PBS. Materials were added to this buffer including i) iron sulfate and humic acid (Sigma-Aldrich, Cat. No. 53680-10G) to represent chemical interferences, ii) ATD (Section 2.4) to represent chemical, biological (live, non-target microorganisms), and physical challenges, and iii) dead *Y. pestis* cells to assess the background effect for post-decontamination applications.

2.4 Addition of Dust Background

ATD (ISO 12103-1, A3 Medium Test Dust; Powder Technology, Arden Hills, MN) was used to evaluate biological and chemical inhibition effects on *Y. pestis* 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. Microbiological analysis showed that the dust had ~5 x 10^4 CFU/10 mg background microbes including fungi and bacterial spores (Rose et al., 2011). Dust was non-sterilized, 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 (heptahydrate; Sigma-Aldrich, Cat. No. 215422) and humic acid (Sigma-Aldrich Cat. No. 53680-10G) were added to samples to test for chemical interferences effecting *Y. pestis* 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 microgram (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 Preparation of Dead *Y. pestis* Cell Suspensions

Different killing methods were evaluated to generate intact, dead cells that did not lose their DNA upon disinfection. Initially, UV irradiation and desiccation were proposed; however, from previous studies it was known that these methods were not very reproducible and for desiccation, long time periods (e.g., 2–4 weeks) were required to produce complete and nearly complete disinfection of *Y. pestis* cells (Staci Kane, personal communication). Therefore, in this effort, autoclaving, antibiotic exposure, and isopropanol (IPA) exposure were investigated. It was determined that autoclaving led to DNA degradation, so it was not used for generating a dead cell background. For antibiotic treatment, doxycycline was selected since there have been no reports of resistance to this antibiotic. In order to avoid generating a resistant pathogenic strain, the pgm$^-$ strain was used. Doxycycline was used at 160 µg/mL for 24 and 48 hr; this concentration is more than 100 times that reported for the minimum inhibitory concentration (Hernandez et al., 2003). While the antibiotic treatment showed that cell proliferation measured by spectrometry (OD_{600}) was halted after treatment, significant viable cells still remained when the suspension was harvested, washed in PBS, and plated. Therefore, IPA was investigated since it is often used to generate dead cells as negative controls for cell staining kits for viability analysis using microscopy or flow cytometry (e.g., LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit, Life Technologies, Inc.).

For preparation of IPA-killed cells, an overnight culture of *Y. pestis* CO92 pgm$^-$ was prepared. From this culture, a 100 mL culture was started after dilution of cells to OD_{600} ~0.01 in YPEB. The culture was incubated at 30°C (with shaking at 180 revolutions per min [rpm]) until OD_{600} ~0.3–0.4 was achieved. Cells were then split into four 20-mL aliquots in 50 mL conical tubes and harvested by centrifugation (3,100 RCF at 4°C for 15 min). Pellets were then suspended in 6 mL of PBS. For the cells treated with IPA, 14 mL of 99+% IPA were added to the 6-mL suspension to yield a final IPA concentration of ~70%. For the control treatment, 14 mL PBS were added. The cell suspensions were mixed gently and incubated for 1 and 2 hr at room temperature. Suspensions were gently mixed every 30 min. After incubation, the suspensions were centrifuged at 4,000 rpm and 4°C for 15 min. The supernatant was removed, and the pellets washed in 20 mL PBS once more followed by centrifugation, removal of supernatant and final suspension in PBS to 20 mL. During washes and preparation of final cell suspension, the fractions were retained for subsequent PCR analysis as outlined below.

The IPA-killed cell suspensions were divided into 1-mL aliquots for storage at 4°C until use, with one aliquot used for each experiment. The calculated concentration based on the controls processed in parallel was ~4.1×10^7 per mL. The IPA-killed aliquots were used over a 40-day period and in each case the cells were tested by PCR prior to use to ensure that DNA was not leaking from the cells. Testing included heat lysis of both the original IPA-killed suspension and the cell pellet after centrifugation of 0.5 mL and removal of 300 µL supernatant (as for RV-PCR sample analysis). Heat lysis was conducted at 95°C for 5 min followed by placement on ice for 2 min, centrifugation (20,800 RCF at 4°C for 5 min), and removal of liquid for PCR analysis (leaving cell debris pellet in tube). An aliquot from the 300 µL supernatant was also analyzed by PCR to determine if significant DNA was lost during this step, or the DNA largely remained with the cell pellet. The

original cell suspension prior to IPA treatment as well as the control treatments (treated with PBS instead of IPA) were serially diluted and plated onto TBA plates for quantitation. For experiments with different concentrations of dead cells, the stock suspension was diluted with PBS to achieve the desired dead cell level based on plate counts from control processed in parallel with IPA-killed cells.

2.7 PCR Evaluation of Dead *Y. pestis* Cell Suspensions

PCR analysis was used to determine whether dead cells remained intact, and DNA remained inside the cells. During cell suspension preparation, washed cells and supernatants were analyzed. For the initial supernatant following IPA treatment and centrifugation, 10- and 50-fold dilutions were analyzed to attempt to dilute out the IPA and obtain PCR data. It was important to evaluate how much DNA may have been lost during each processing step.

In addition, prior to using stored IPA-killed cells for an experiment, the suspension was centrifuged at 20,800 RCF at 4°C for 5 min. The supernatant was used to test for DNA loss by PCR analysis with the YC2 assay. In addition, the remaining cell pellet was lysed by heat and analyzed by PCR. The original IPA-killed cell suspension was also subjected to heat lysis and PCR analysis. Results from the supernatant, heat-lysed cell pellet, and heat lysed IPA-killed cell suspension were compared to determine if DNA would be lost during RV-PCR sample processing and analysis (which includes an additional centrifugation step). The YC2 assay was used following the conditions outlined in Section 2.10.

2.8 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. In contrast to the RV-PCR protocol for *B. anthracis* spores, 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 was not vacuum filtered but rather was prepared using 10X-concentrated broth, in the proper ratio, in order 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 3.15 mL water sample and 0.35 mL 10X broth could be used. In this study 2.7 mL sample was added to 0.3 mL 10X broth.

After mixing by pipettor, a 0.5 mL aliquot was removed from the total 3.0 mL in each sample well before incubation (T_0 aliquot), transferred to 2 mL Eppendorf tubes, and centrifuged at 20,800 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 DNA extraction and PCR analysis following the protocol detailed below. During method development, in some cases (as specified in the report), 0.25 mL aliquots were removed and processed as described above except that only 50 µL were removed, leaving 0.2 mL pellets. The 48-well plate was sealed with a sterile AeraSeal™ breathable adhesive seal (Excel Scientific, Cat. No. BS-25) and incubated for different time periods from 12 to 40 hr at 28–30°C with shaking at 180 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., 3, 8-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, Cat. No. MD1360). This kit enables DNA recovery from multiple complex samples simultaneously using a magnetic bead-based cleanup method. The method was modified from that developed for *B. anthracis* cells (US EPA, 2012), as described below. 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 magnetic rack and 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 allowed to cool 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).

2.9 *Y. pestis* CO92 DNA Standards for Real-Time PCR

Y. pestis CO92 DNA standards were generated from harvested cells from overnight incubation of 5 mL YPEB cultures inoculated from 2-3 individual colonies from TBA plates. A MasterPure™ Complete DNA and RNA (ribonucleic acid) Purification Kit (Epicentre® Biotechnologies Inc. 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 a large number of 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, Cat. No. Q32854) with a Qubit™ fluorometer (Cat. No. Q33216). Standard concentrations prepared in PCR-grade water ranged from 1 nanogram (ng)/µL to 1 femtogram (fg)/µL. Each PCR plate contained seven 10-fold dilutions, ranging from 5 ng per 25-µL PCR to 5 fg 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 developed by Dr. Sanjiv Shah (while at Edgewood Chemical and Biological Center of the Department of Defense) and LLNL using computational tools for primer and TaqMan® probe design were used to design *Y. pestis* real-time PCR assays (personal communication). In addition, *in silico* analysis and rigorous wet-chemistry screening approaches were used to further down-select candidate signatures, by screening against an extensive panel of environmental extracts, bacteria, eukaryotes, near-neighbors, and target strain DNAs. Furthermore, the down-selected assays were tested against 12 target DNA templates in order to yield sensitive assays targeting the chromosome (YC2 assay) and the pMT1 plasmid (YpMT1 assay). In addition, an assay for pPCP1 (EPA-YpP1, referred to as YpP1 for this study) developed by Dr. Shah met the stringent screening requirements and showed excellent sensitivity in the previous effort. Therefore, these three assays were used in this effort to optimize the RV-PCR protocol for detection of viable *Y. pestis* from water samples.

An Applied Biosystems® 7500 Fast Real-Time PCR System was used to perform Real-time PCR. Each well of a 96-well PCR plate contained five µL sample aliquots added to 20 µL of PCR mix. While three assays were used as mentioned, the majority of analysis used the YC2 assay. This enabled more accurate analysis of DNA recovery efficiency and comparison with culture data since the copy number for the chromosome marker could be more accurately assumed to be one per cell. This assumption was confirmed by analysis of the *Y. pestis* CO92 genome sequence.

The YC2 assay targets a hypothetical protein with similarity to the *Bordetella pertussis* BapA protein and the *Escherichia coli* YchA protein. These are autotransporter proteins of a type V secretion system and these systems have been linked to virulence in Gram-negative bacteria (Derbise et al., 2010). The YpP1 assay, also referred to as Yp-EPA1 was developed by EPA (Sanjiv Shah, personal communication) and targets the plasminogen activator/coagulase (*pla*) gene, which plays a role in virulence. Finally, the YpMT1 assay targets the caf1R gene, a positive regulator of the F1 operon (encoding the F1 capsule antigen involved in virulence).

The PCR mix contained TaqMan® 2X Universal PCR Master Mix (Life Technologies, Cat. No. 4304437), which includes AmpliTaq Gold® DNA polymerase, deoxynucleotide triphosphates (dNTPs), a 6-Carboxyl-X-Rhodamine (ROX) passive reference dye (for signal normalization), and AmpErase® UNG (uracil-N-glycosilase) which prevents 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. The assay primer and probe sequences are listed in Table 1. PCR-grade water was used to make the mix volume up to 20 µL per reaction and 5 µL of sample were 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 for 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 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 *Y. pestis* RV-PCR Analysis

Assay	Forward Primer	Reverse Primer	Probe	Amplicon Length (bp)
YC2	CAACGACTAGCCAG GCGAC	CATTGTTCGCACG AAACGTAA	TTTTATAACGAT GCCTACAACGGC TCTGCAA	78
YpP1	TGGGTTCGGGCACA TGATA	CCAGCGTTAATT ACGGTACCATAA	CTTACTTTCCGT GAGAAGACATC CGGCTC	101
YpMT1	GGTAACAGATTCGT GGTTGAAGG	CCCCACGGCAGT ATAGGATG	TCCCTTCTACCC AACAAACCTTTA AAGGACCA	99

* Sequences are listed in 5' to 3' orientation. bp = base pair.

2.11 Interpretation of RV-PCR Results

As a starting point for RV-PCR detection of viable *Y. pestis* cells, the criteria developed for *B. anthracis* were employed; specifically, for positive detection, the 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) are required. For initial optimization, most of the work was conducted with 24 hr incubation, such that $T_f = T_{24}$. For cases where no PCR response was obtained (non-detect results), the C_T values were set to 45 (since 45 PCR cycles were used), in order to calculate ΔC_T. A $\Delta C_T \geq 6$ represented an increase in DNA concentration of approximately 2-log, as a result 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 in DNA concentration), and a corresponding lower end point (C_T of ≤ 36) could be used. For individual replicates within an experiment, the RV-PCR result was considered positive when at least 2 of 3 replicates met the algorithm requirement.

The RV-PCR method LOD was equivalent to the *Y. pestis* cell level where 100% of the spiked samples had C_T (T_f) of ≤ 39 with a $\Delta C_T \geq 6$. This was essentially an analytical LOD of the RV-PCR method and did not take into account 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 T_f C_T. Data tables show both individual PCR replicates as well as averages and standard deviations calculated in Microsoft Excel®. If a single PCR replicate was positive and the other two replicates were non-detect, the sample was considered negative or non-detect (NDT) and the sample C_T was set to 45, in order to calculate ΔC_T. Single replicate positive high C_T values (e.g., 39–44) were likely due to cross contamination.

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 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.

2.13 Estimation of DNA Copy Numbers and Cell Numbers from Real-Time PCR Results

Comparison was made between actual CFU/mL measured by plate counts and corrected for dilution and estimated cell numbers from Real-time PCR analysis using the YC2 chromosomal assay. The following equation was used to calculate fg/target (assuming one target per genome):

$$\frac{bp}{target\ (genome)} \times \left(\frac{mol\ bp}{6.023 \times 10^{23}}\right) \times \left(\frac{650\ g}{mol\ bp}\right) \times \left(\frac{10^{15}\ fg}{g}\right) = \frac{fg}{target}$$

As an example for *Y. pestis*,

$$\left(\frac{4.83 \times 10^6}{target}\right) \times \left(\frac{mol\ bp}{6.023 \times 10^{23}}\right) \times \left(\frac{650\ g}{mol\ bp}\right) \times \left(\frac{10^{15}\ fg}{g}\right) = \frac{5.21\ fg}{target}$$

Assumptions included that there was one genome copy per cell and that the number of base pairs (bp) per genome copy was 4.83×10^6 (by adding the bp from one copy of the chromosome [4.65 mega base pairs, Mb] and one copy each of the three plasmids, pCD1 [70.3 kb], pPCP1 [9.6 kb], and pMT1[96.2 kb]).

In order to estimate the CFU/mL from the PCR C_T value the following equation was used:

$$C_T = -m(\log fg\ DNA) + b$$

where m is the slope and b is the y-intercept from the standard curve obtained when plotting C_T vs. log fg DNA for genomic DNA concentrations ranging from 5 ng to 5 fg (10-fold dilutions). The log fg DNA (per 5 µL) was then converted to fg DNA per mL by multiplying with the appropriate dilution factor. In order to convert fg DNA per mL to targets (or genome equivalents) per mL, the value was multiplied by $\frac{5.21\ fg}{target}$. It was assumed that genome equivalents were equal to cell equivalents (i.e., one genome copy per cell). The CFU per plate was also converted to CFU per mL, based on the amount of the sample plated and the dilution plated. To obtain the log difference, the targets per mL and CFU/mL were converted to log values and subtracted from each

other. For the data shown, the log difference = log targets from PCR/mL – log CFU from plating/mL.

2.14 Immunomagnetic Separation of *Y. pestis* Cells

Since large volume water samples may need to be processed for detection of *Y. pestis* cells, immunomagnetic separation (IMS) was investigated as a front-end concentration method upstream of RV-PCR analysis. IMS has been used successfully to concentrate *Y. pestis* cells from complex food rinsates (Himathongkham et al., 2007; Amoako et al., 2012; Darcy Hanes, Food and Drug Administration [FDA], personal communication) with both an Applied Biosystems™ Pathatrix® Auto concentrator (Life Technologies) and an *i*CropTheBug system (Filtaflex, Inc., Almonte, Ontario, Canada). In addition, IMS approaches have been used to capture bacterial spores from soil (Laura Rose, CDC, personal communication). Figure 4 shows the Pathatrix instrument, which uses a disposable syringe system for mixing the sample with the antibody-coated magnetic beads. In this effort, a Pathatrix system was used to concentrate the initial suspension (up to 60 mL although 33 mL was used in this case) to 0.1 mL solution containing cells bound to magnetic beads conjugated with a *Y. pestis*-specific antibody (330-fold concentration). For this effort, polyclonal antibodies specific to *Y. pestis* were obtained from the Critical Reagents Program (CRP) through BEI Resources, Inc., Manassas, VA (Cat. No. AB-G-YERS). The protocol for bead conjugation and Pathatrix operation from Life Technologies was followed.

It should be noted that the captured cells could be processed directly by culture, immunoassay, or real-time PCR (following DNA extraction), or alternatively the cells with beads could be used for RV-PCR analysis, as was done in this case. Although not tested in this effort, it may be possible for the beads to be reused for subsequent rounds of IMS for the same sample by transferring the 0.5 mL bead solution to additional 60-mL aliquots and repeating the capture process. Although there would be some bead loss for each round of IMS, using the same beads for multiple aliquots of the same sample could lead to greater cell concentration factor.

For the IMS experiment, cells were grown overnight in YPEB at 28°C with shaking at 180 rpm. The OD_{600} was measured and the cells were washed with 1X PBS. The cell suspension was adjusted to approximately 10, 100, and 1000 cells per mL (based on dilutions of suspensions at OD_{600} ~0.1). Triplicate three mL suspensions for each cell level were used for IMS. To each 3-mL sample, 30 mL 1X PBS and 50 µL antibody-coated beads were added, and the sample was processed by the Pathatrix instrument (Figure 4). A negative control without *Y. pestis* cells was also included. A flow chart for sample processing using IMS is shown in Figure 5. The Pathatrix instrument processes five samples concurrently within 15 min, excluding sample handling, which can add 10 min per set of five samples. Therefore, the overall time for RV-PCR analysis would increase from approximately 36 to 40 hr for 48 samples by including IMS.

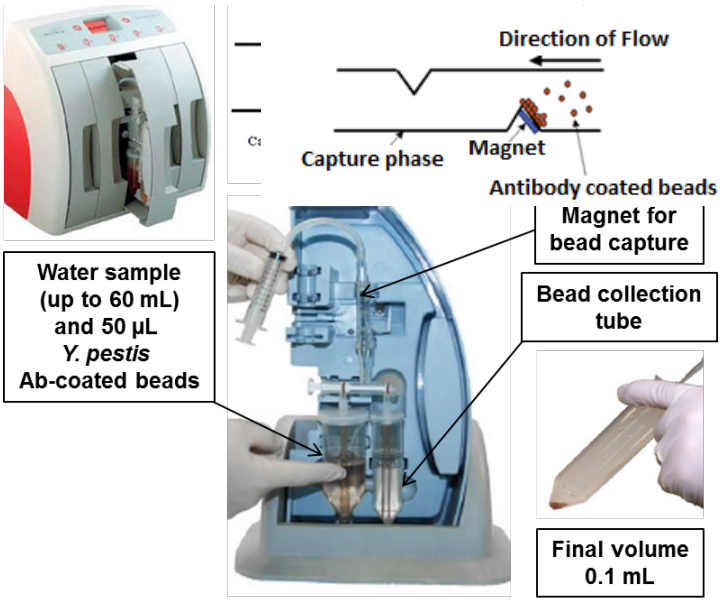

Figure 4. Pathatrix Immunomagnetic Separation system (Life Technologies, Inc.). The system has the ability to concentrate cells up to 600-fold (60 mL down to 0.1 mL). Five samples can be processed in 15 min during which the sample is passed across the magnet with antibody (Ab)-coated beads about 400 times.

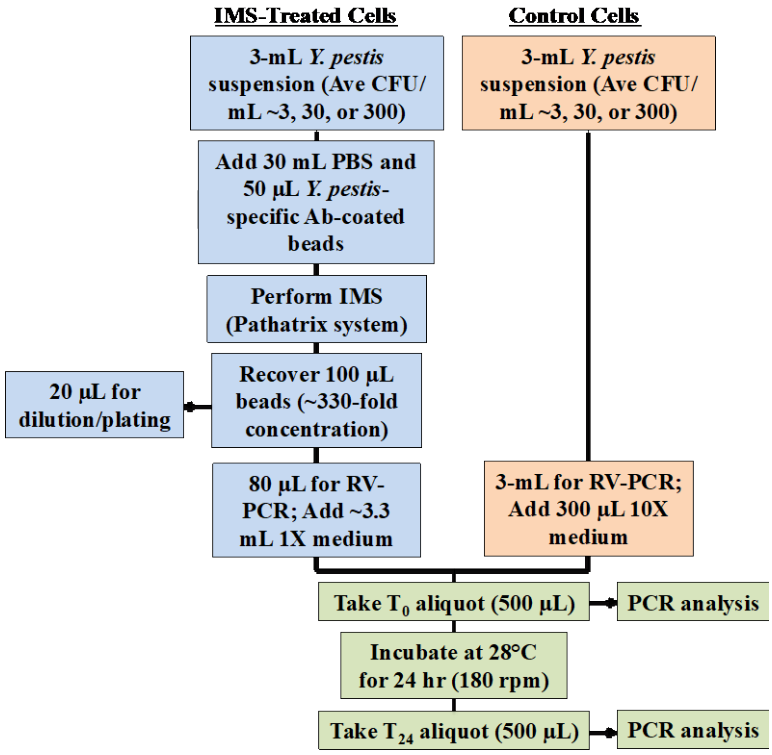

Figure 5. Flow chart for IMS-treated and control *Y. pestis* cell suspensions.

Traditional culture analysis was used to determine recovery percentage relative to the inoculum level from a portion of the recovered beads (20 µL of total 100 µL bead suspension), including both direct plating (without dilution) of the resulting bead suspension and/or filter-funnel plating (of the solution remaining after bead capture). The remaining bead solution (80 µL) was added to a 48-well plate with 3.3 mL 1X YPEB. After mixing by pipettor, a T_0 aliquot (500 µL) was removed and processed for DNA extraction as described in Section 2.8. Control cell suspensions (3 mL for each cell level) were added to the 48-well plate and 300 µL 10X YPEB was added, mixed by pipetting up and down, and a T_0 aliquot was removed for DNA extraction and PCR analysis. The plate was incubated at 28°C for 24 hr with shaking at 180 rpm after which a 500 µL aliquot was removed for DNA extraction and PCR analysis.

2.15 Modified Filtration for Concentration of *Y. pestis* Cells

Modified filtration (MF) methods were investigated to prevent desiccation of cells on standard membranes during vacuum filtration. In this task, different compounds were added to filter devices (Whatman® Filtration Autovials™, GE Healthcare, Cat. No. AV125NPUPSU) to retain moisture on the filter membrane to maintain cell viability during filtration. Autovials were qualified by comparing their performance to that of the previously used Whatman® Autocups™ (Cat. No. 1602 - 0475), since the latter was discontinued; similar RV-PCR LOD results were obtained for the two different devices and the protocol was modified slightly to accommodate the smaller volume of the Autovials (12.5 mL compared with 20 mL for the Autocups). In addition, vacuum manifolds modified to accommodate the larger diameter Autovials were used.

Materials used to test MF included diatomaceous earth (DE) and different types of superabsorbent polymers (SAPs). Due to its high silica content, DE has been used for nutrient and moisture retention. SAPs have been used for similar and related applications including biosolids dewatering, fuel filtering to remove water, diaper manufacturing, and as soil additives for moisture retention. These materials are typically polymers of polyacrylic acid or co-polymers of poly(isobutylene) and poly (maleic acid), which have different properties (absorption characteristics) dependent on polymer chemistry and cross-linking. SAPs used in the testing included H-200, H-300, H-400, and H-500 from JRM Chemical, Inc. (Cleveland, OH) with smaller numbers representing smaller particle sizes) and 100% polyacrylamide "Water Storing Crystals" (Miracle-Gro™ abbreviated MG; Scotts Miracle-Gro, Marysville, OH). DE was food-grade material packaged as "Kleen-N-Fresh", which was obtained from Garden Fresh® (Pleasant Hill, CA). 0.1 to 0.2 gram (g) amounts of SAP and/or DE materials were used per Autovial. The MF approach for cell concentration used in this study is shown in **Figure 6**.

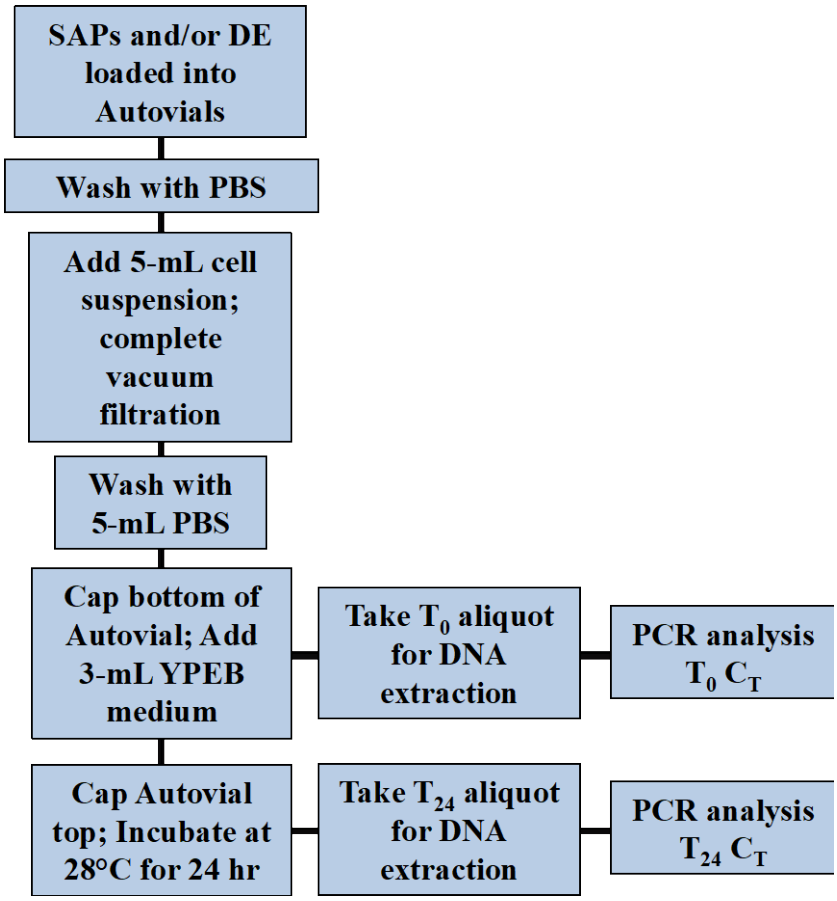

Figure 6. Flow chart for sample processing using the modified filtration approach followed by RV-PCR analysis for *Y. pestis* cells. Vacuum filtration of the Autovials was stopped as soon as filtration was complete in a given vial to enable direct comparison between treatments for biocompatibility and growth without assessing which material retained more moisture. The vials with and without materials added were washed with 10 mL PBS before addition of cells.

3.0 Quality Assurance and Quality Control

3.1 Laboratory Inspections

Monthly laboratory inspections were conducted by the project principal investigator (PI) to comply with DOE and Centers for Disease Control and Prevention (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 biosafety cabinet (BSC), robotic enclosure, and autoclave
- Reviewing waste handling procedures
- Taking inventory of select agents (in addition, 25% inventory conducted quarterly)

- Reviewing personnel training

3.2 Calibration

The Applied BioSystems™ Inc. (ABI) 7500 Fast PCR instrument was calibrated and underwent preventative 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 PI.

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 phosphate-buffered saline, PBS) to test for cross-contamination were conducted for each experiment.

3.5 Real-time PCR Analysis

During the experiment, *Y. pestis* CO92 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 *Y. pestis* CO92 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 average colony-forming units (CFU; for the spread plate method), with corresponding standard deviation (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

This research effort was to develop a qualitative, RV-PCR method of *Y. pestis*. 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 of the acceptable range (i.e., if a negative control showed 1 of 3 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 1 C_T value of the average). For individual replicates within an experiment, the RV-PCR result was considered positive when at least 2 of 3 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.

4.0 Results and Discussion

The following section presents results for the three project tasks and also provides some discussion of results. In addition, particular details relevant to the given experiment are included such as cell concentrations tested, broth used, and PCR assay employed. This allows the relevant information to be in close proximity to 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: Incorporate DNA Extraction and Purification Steps into RV-PCR Protocol for *Y. pestis* and Evaluate Protocol Parameters (Incubation Period, LOD)

4.1.1 Objectives

As stated, the objective of the first task was to shorten the *Y. pestis* RV-PCR method incubation period by incorporating a DNA extraction/purification procedure, which also enabled concentration of the resulting DNA. During the initial method development for *Y. pestis* (US EPA Internal Report, 2010), crude DNA extracts were obtained from samples by heat lysis with no subsequent DNA concentration or cleanup performed. In this task, a DNA extraction/purification procedure was incorporated into the protocol, which used chemical lysis to break open cells and release their DNA. Specifically, the objective was to maintain the 10-cell level detection limit (10–99 cells per sample) while significantly shortening the incubation from 48 hr; in this case a 24-hr incubation period was targeted since a shorter incubation would lead to a shorter time for results and increased sample throughput. However, in addition to chemical lysis used by the DNA extraction/purification protocol, heat lysis followed by DNA concentration/purification was evaluated as a potentially more streamlined approach. Since *Y. pestis* is a Gram-negative vegetative cell and more easily lysed than Gram-positive cells, it was thought that the processing time could be shortened, and heat lysis could more quickly release DNA than chemical lysis steps.

4.1.2 Overall Approach for Evaluating DNA Extraction and Purification Protocols for Y. pestis Cells

The RV-PCR protocol for *B. anthracis* used a MagneSil® Blood Genomic, Max Yield System kit (Promega Corp., Madison, WI) consisted of several buffers for (i) cell lysis and recovery of DNA onto magnetic beads, (ii) washes to purify the DNA, and (iii) elution of the purified DNA. The reagents were shown to lyse the Gram-positive *B. anthracis* vegetative cells and not the spores. In this effort, these reagents were evaluated for *Y. pestis* cell lysis and DNA concentration/purification. Simpler protocols with fewer reagents and steps were tested for ability to generate DNA with sufficient quantity and quality for subsequent analysis. In particular, one of the lysis wash steps and one of the alcohol wash steps were omitted.

In this task, *Y. pestis* CO92 cells were used for determining yield of DNA (based on PCR response) from different extraction/purification procedures. Cell concentrations ranged from 10^1 to 10^6 per sample and were determined for each experiment by serial dilution and plating. Real-time PCR analysis used *Y. pestis*-specific assays including YC2 (chromosome), YpMT1 (pMT1), and YpP1 (pPCP1).

4.1.3 Evaluation of Y. pestis-Specific Real-Time PCR Assays

As mentioned, three real-time PCR assays were down-selected in the previous effort (US EPA Internal Report, 2010). In the current effort, the sensitivity of the assays was confirmed using 10-fold dilutions of prepared genomic DNA stocks from *Y. pestis* CO92. Genomic DNA was prepared and quantified as described in the Materials and Methods Section 2.9. PCR C_T data are shown below for 7-log standards for two plasmid assays, YpP1 (pPCP1 plasmid) and YpMT1 (pMT1 plasmid), and the chromosomal assay, YC2. The data showed good assay performance even down to the 5-fg level (Table 2). The greater sensitivity of the YpP1 assay compared to the other assays could be due in part to there being multiple pPCP1 plasmids per cell due to its small size (~10 kb).

Table 2. Real-time PCR Results for *Y. pestis* CO92 Genomic DNA Dilutions With Assays for pPCP1 and pMT1 Plasmids and the Chromosomal Gene Targets

***Y. pestis* DNA (pg)**	**Average* C_T (SD) by Assay**		
	YpP1 (pPCP1)	**YC2 (chromosome)**	**YpMT1 (pMT1)**
5000	16.9 (0.3)	17.8 (0.2)	18.8 (0.6)
500	20.9 (0.4)	21.2 (0.2)	22.2 (0.1)
50	24.8 (0.3)	24.7 (0.2)	25.6 (0.3)
5	29.4 (0.6)	28.4 (0.2)	29.6 (0.2)
0.5	33.7 (0.6)	32.0 (0.2)	33.2 (0.5)
0.05	38.2 (0.6)	35.9 (0.4)	37.2 (0.4)
0.005	41.6 (0.4)	38.0 (1.2)	40.3 (0.4)

* Average and standard deviation (SD) based on four replicates.

4.1.4 Comparison of Cell Number Estimated from PCR Results and Viable Cell Counts – Evaluation of Modified Chemical Lysis (Promega MagneSil) Protocol

In this effort, viable cell counts were compared with cell number estimates based on real-time PCR results in order to estimate the efficiency of cell lysis and DNA concentration. The project scope did not include an extensive optimization of the DNA extraction protocol but rather included a smaller effort to assess DNA yield and quality (assessed together as PCR performance) when using fewer wash steps. In this case, one fewer lysis wash step and one fewer alcohol wash step were used compared to the protocol for DNA extraction of *B. anthracis* cells (US EPA, 2012).

DNA target concentrations were estimated from the resulting C_T, using the standard curve and assuming one target copy per cell and correcting for dilution (as described in Section 2.13). The one copy per cell estimate was likely valid since the chromosomal assay YC2 was used. It was also assumed that only live cells contributed DNA and that the dead cell population was negligible. These assumptions seemed valid since 24 hr (T_{24}) was not an excessively long incubation period which would include entry into stationary phase for *Y. pestis*; this was supported by the measured CFU/mL at T_{24} that ranged from about 4×10^5 to 3×10^7. The log difference between cell numbers estimated from PCR analysis and those from culture varied from -0.2 to 0.7 with positive values showing higher CFU based on PCR estimates and negative values showing higher CFU for culture (Table 3). The average log difference for samples starting with 10, 100, or 1000 cells and using 24 hr incubation was 0.4 ± 0.3 showing that results from PCR gave significantly higher estimates than CFU values measured from plating. Many factors can affect the estimated CFU/mL for both plating and PCR analysis (e.g., pipetting variability, variation in target copy number per cell) and PCR does not generate absolute cell counts, but in general the results suggested that good DNA yields were obtained using the modified DNA extraction protocol for *Y. pestis* cells.

Table 3. Comparison of Estimated CFU/mL from Real-Time PCR Analysis* (YC2 Assay) With CFU/mL from Culture Analysis (With 1X YPEB Prepared From 10X)

Inoculum Log CFU/mL from Culture	Sample Replicate	Average Log CFU/mL from Culture at T_{24}	Estimated Average Log CFU/mL from PCR at T_{24}**	Log Difference (PCR - Culture)
1.2	**1**	5.9	6.6	0.6
	2	5.6	6.2	0.6
	3	5.8	6.2	0.4
	Ave (SD)	**5.8 (0.2)**	**6.3 (0.2)**	**0.5 (0.1)**
2.2	**1**	6.8	6.8	0.0
	2	6.9	7.5	0.6
	3	6.8	7.5	0.7
	Ave (SD)	**6.8 (0.1)**	**7.3 (0.4)**	**0.5 (0.4)**
3.2	**1**	7.5	8.0	0.5
	2	7.4	8.0	0.6
	3	7.5	7.3	-0.2
	Ave (SD)	**7.5 (0.1)**	**7.8 (0.4)**	**0.3 (0.4)**
	Overall Ave (SD)			**0.4 (0.3)**

* DNA extracts were obtained from T_{24} aliquots for RV-PCR analysis.
** Log CFU/mL was estimated assuming one target copy per cell, a genome size of 4.83 Mb (5.21 fg/target), using the PCR C_T value at T_{24} corrected for dilution. A T_{24} aliquot of 500 µL was used. Each sample replicate was analyzed in triplicate by culture and PCR analyses.
CFU, colony forming units; SD, standard deviation.

4.1.5 Comparison of PCR Results with Universal Reagents/Standard Cycling and Fast Reagents/Fast Cycling Conditions

In addition to the data shown in Table 2 generated with Universal Master Mix and Standard cycling (Universal/Standard) conditions, the same DNA standard concentrations were run with Fast Master Mix and Fast cycling (Fast/Fast) conditions. The PCR cycling conditions for both Fast and Standard Modes are shown in Table 4.

Table 4. Thermal Cycling Parameters for the Different Real-Time PCR Configurations

Cycling Type (7500 Fast System)	Thermal-Cycling Profile					Overall PCR Run Time (hh:mm)**
	Parameter	UNG Incubation†	Polymerase Activation‡	PCR (45 cycles)		
		Hold	Hold	Denature	Anneal/ Extend*	
	Temp. (°C)**	50	95	95	60	
Standard Mode (Universal Master Mix)	Time (mm:ss)	02:00	10:00	00:05	00:20	00:55
Fast Mode (Fast Master Mix)	Time (mm:ss)	02:00	00:20	00:03	00:20	00:45

† Required for optimal UNG activity.
‡ Required to activate the DNA polymerase.
* Based on a single-channel (FAM) measured; if all four channels were measured the Anneal/Extend period would need to be extended to 01:00 for Standard Mode and 00:30 for Fast Mode.
** hh = hour; mm = minutes, and ss = seconds (in double digit format).

Aside from different Master Mix used for the different PCR conditions, the same concentration of primers and probe and the same concentration of DNA standards were used. The average C_T differences across 7-log *Y. pestis* genomic DNA concentrations for the two PCR conditions were 4.5 ± 1.3 and 1.7 ± 0.2 for YpP1 and YpMT1, respectively (Table 5); the YC2 assay did not show differences (Table 6).

Table 5. Real-time PCR Results for the Plasmid Assays YpP1 (pPCP1) and YpMT1 (pMT1) Using Fast/Fast and Universal/Standard Conditions With *Y. pestis* DNA Standards

Y. pestis DNA (pg)	YpP1 Assay Ave C_T (SD)		C_T Difference	YpMT1 Assay Ave C_T (SD)		C_T Difference
	Fast/Fast	Universal/ Standard		Fast/Fast	Universal/ Standard	
5000	14.2 (0.1)	16.9 (0.3)	2.7	17.2 (0.1)	18.8 (0.6)	1.6
500	17.7 (0.2)	20.9 (0.4)	3.2	20.8 (0.1)	22.2 (0.1)	1.4
50	21.2 (0.3)	24.8 (0.3)	3.6	24.1 (0.1)	25.6 (0.3)	1.5
5	24.4 (0.1)	29.4 (0.6)	5.0	27.7 (0.1)	29.6 (0.2)	1.9
0.5	28.4 (0.1)	33.7 (0.6)	5.3	31.7 (0.3)	33.2 (0.5)	1.5
0.05	31.8 (0.1)	38.2 (0.6)	6.4	35.4 (0.1)	37.2 (0.4)	1.8
0.005	36.3 (0.8)	41.6 (0.4)	5.3	38.2 (0.3)	40.3 (0.4)	2.1
		Ave (SD)	**4.5 (1.3)**		**Ave (SD)**	**1.7 (0.2)**

* Average (Ave) and standard deviation (SD) based on 3–4 replicates. pg = picogram.

The larger differences especially for the YpP1 assay which targets a ~10-kb plasmid could be due to plasmid supercoiling and the possibility that the Fast reagents and Fast cycling program amplified supercoiled DNA more efficiently.

Table 6. Real-time PCR Results for the YC2 (Chromosomal) Assay Using Fast/Fast and Universal/Standard Conditions With *Y. pestis* DNA Standards

Y. pestis DNA (pg)	YC2 Assay Ave C_T (SD)		C_T Difference
	Fast/Fast	Universal/ Standard	
5000	18.2 (0.1)	17.8 (0.2)	-0.4
500	21.6 (0.1)	21.2 (0.2)	-0.4
50	24.9 (0.1)	24.7 (0.2)	-0.2
5	28.5 (0.1)	28.4 (0.2)	-0.1
0.5	32.3 (0.2)	32.0 (0.2)	-0.3
0.05	36.2 (0.8)	35.9 (0.4)	-0.3
0.005	38.3 (0.1)	38.0 (1.2)	-0.3
		Ave (SD)	**-0.3 (0.1)**

* Average (Ave) and standard deviation (SD) based on 3–4 replicates. pg = picogram

Since results were similar between the two modes/conditions for the YC2 assay, the Universal/Standard condition was used for the subsequent experiments with the YC2 assay; however, Fast reagents and Fast cycling profiles could be considered for improved sensitivity using the plasmid assays.

4.1.6 Evaluation of Heat Lysis Vs. Chemical Lysis for Y. pestis Cells

Since heat lysis could simplify the DNA extraction/purification procedure in the RV-PCR protocol (based currently on chemical lysis with Promega reagents), both lysis methods were tested in parallel with *Y. pestis* CO92 cells. After the initial lysis step, the purification steps were the same between both procedures (Figure 7). Lysis buffer was added to heat lysates only to ensure proper chemistry for DNA binding and the samples were not incubated in this buffer. The goal was to determine if the modified protocol provided the same DNA yield and quality (as determined by real-time PCR analysis) as the original protocol. Cells were from overnight cultures which were diluted to OD_{600} ~0.1. One-mL aliquots were processed to recover DNA using the standard DNA extraction/purification protocol, after initially concentrating the cell suspension to 200 µL by centrifugation (as described in Materials and Methods Section 2.8). All three assays were used for PCR analysis, YC2, YpP1 and YpMT1.

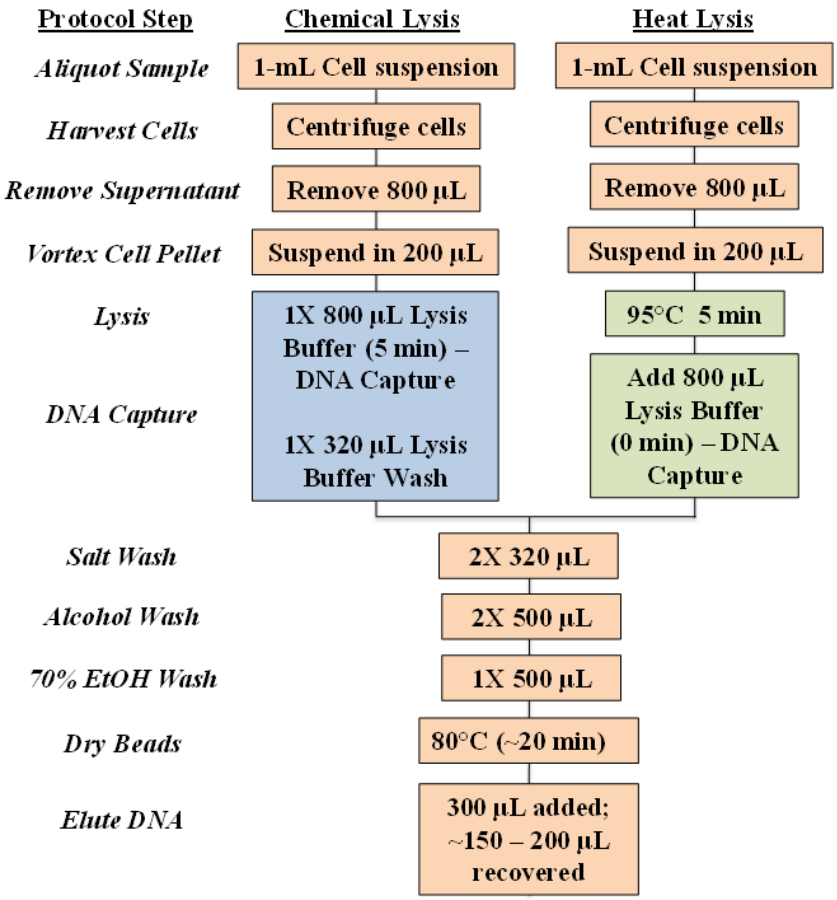

Figure 7. Outline of Protocol Steps for Chemical Lysis (Promega MagneSil kit) and Heat Lysis Procedures for DNA Extraction and Purification. For the heat lysis procedure, 800 µL Lysis Buffer was added after heat treatment to create proper conditions for released DNA to bind to MagneSil magnetic beads. The samples were put immediately on a magnetic rack and the liquid was removed to limit chemical lysis activity.

Results showed the overall process times were similar for the different lysis treatments; approximately 3 hr for 24 samples processed manually. This is in part due to the fact that the same steps were used after lysis. RV-PCR results from triplicate samples and triplicate PCR analyses per sample replicate showed that DNA extracts from chemical lysis treatment produced significantly lower average C_T values (3–4 units lower) for the first replicate experiment, ranging from 16.6–19.8 compared to 20.5–23.0 for heat lysis for all replicates and assays (Table 7) with p-values ranging from 2.2×10^{-5} to 7.5×10^{-7} (paired, two-tailed T-test). The data suggested that chemical lysis was more effective for reproducibly generating good quality, amplifiable genomic DNA. Diluted extracts (10-fold) showed the same trends although the ranges overlapped with C_T values of 20.6–23.2 and 22.6–24.3, for chemical and heat lysis treatments, respectively. The data suggested some level of PCR inhibition for heat lysates that was not observed for extracts from chemical lysis since the average C_T differences between undiluted and 10-fold diluted extracts for the three assays ranged from 1.3-2.6 for heat lysates and 3.4-4.0 for chemical lysates (i.e., C_T differences > 3 for 10-fold dilutions suggest a lack of PCR inhibition). Ten-fold dilution of extracts from heat lysates appeared to relieve the PCR inhibition at least in part, as shown by more similar C_T values for diluted extracts from the different lysis methods.

A replicate experiment was conducted using the same conditions as described above. PCR results in terms of averages and standard deviations from three biological replicates and triplicate PCR analyses are shown in Table 8. Unlike the first replicate experiment, there were much smaller differences (0.1–0.8) between heat lysis and chemical lysis treatments with average C_T values for undiluted extracts ranging from 16.4–19.4 and 16.3–18.8 across assays, respectively. As mentioned, the experiments were conducted in the same manner although the estimated cell number differed slightly; OD_{600} values were 0.11 and 0.18 for the first and second experiments, respectively, which corresponds to about 1.6-fold difference in estimated cell numbers. In general, the C_T values for the first replicate experiment were higher since fewer cells were used for this experiment. Although there were differences in results, the data suggested that chemical lysis provided more consistent, lower C_T values (more DNA recovery and/or lack of PCR inhibition), therefore this extraction method was used in the RV-PCR protocol for *Y. pestis* analysis.

Table 7. Real-time PCR Results for DNA Extracted from *Y. pestis* Cells by Heat or Chemical Lysis (followed by Promega Kit Purification) and Analyzed by Chromosomal (YC2) and Plasmid Assays (YpP1 and YpMT1) – First Replicate Experiment

DNA Extraction Method	Sample Replicate-PCR Replicate	C_T* by *Y. pestis* Assay					
		YpP1		YC2		YpMT1	
		Undiluted	10-Fold Dilution	Undiluted	10-Fold Dilution	Undiluted	10-Fold Dilution
Heat Lysis	1-1	22.2	23.3	21.8	23.8	23.5	25.3
	1-2	21.7	23.4	21.3	23.9	23.9	25.3
	1-3	21.4	24.0	21.1	24.1	24.1	25.7
	Ave (SD)	**21.8 (0.4)**	**23.6 (0.4)**	**21.4 (0.4)**	**23.9 (0.1)**	**23.9 (0.3)**	**25.4 (0.2)**
	2-1	20.6	21.7	20.2	22.5	22.6	23.7
	2-2	20.3	22.2	19.8	22.3	22.3	23.8
	2-3	19.8	22.4	19.8	22.9	22.6	23.9
	Ave (SD)	**20.2 (0.4)**	**22.1 (0.3)**	**19.9 (0.2)**	**22.6 (0.3)**	**22.5 (0.2)**	**23.8 (0.1)**
	3-1	20.3	21.7	20.5	22.6	22.5	23.5
	3-2	19.8	22.1	19.9	22.8	22.1	23.8
	3-3	20.3	22.4	20.2	22.7	22.8	23.9
	Ave (SD)	**20.2 (0.3)**	**22.1 (0.3)**	**20.2 (0.3)**	**22.7 (0.1)**	**22.5 (0.4)**	**23.7 (0.2)**
	Overall Ave (SD)	**20.7 (0.8)**	**22.6 (0.8)**	**20.5 (0.7)**	**23.1 (0.7)**	**23.0 (0.7)**	**24.3 (0.8)**
Promega Kit (Chemical Lysis)	1-1	17.0	20.5	18.5	22.1	20.3	23.4
	1-2	16.7	20.6	18.6	22.2	20.1	23.5
	1-3	16.8	21.1	18.7	22.6	19.9	23.7
	Ave (SD)	**16.8 (0.1)**	**20.7 (0.3)**	**18.6 (0.1)**	**22.3 (0.3)**	**20.1 (0.2)**	**23.5 (0.2)**
	2-1	16.7	20.4	18.7	21.9	20.2	22.9
	2-2	16.5	20.6	18.4	21.9	19.6	23.1
	2-3	16.5	20.7	18.2	22.1	19.8	23.7
	Ave (SD)	**16.6 (0.1)**	**20.6 (0.2)**	**18.4 (0.2)**	**22.0 (0.1)**	**19.9 (0.3)**	**23.2 (0.4)**
	3-1	16.4	20.2	18.2	21.7	19.5	22.9
	3-2	16.6	20.5	18.2	21.7	19.2	22.8
	3-3	16.5	20.7	18.2	22.0	19.4	23.3
	Ave (SD)	**16.5 (0.1)**	**20.5 (0.2)**	**18.2 (0.0)**	**21.8 (0.2)**	**19.4 (0.1)**	**23.0 (0.2)**
	Overall Ave (SD)	**16.6 (0.2)**	**20.6 (0.2)**	**18.4 (0.2)**	**22.0 (0.3)**	**19.8 (0.4)**	**23.2 (0.3)**

* C_T = cycle threshold; SD = standard deviation. Average (Ave) and SD values are based on triplicate samples.

Table 8. Real-time PCR Results for DNA Extracted from *Y. pestis* Cells by Heat or Chemical Lysis (followed by Promega Kit Purification) and Analyzed by Chromosomal (YC2) and Plasmid Assays (YpP1 and YpMT1) – Second Replicate Experiment

DNA Extraction Method	Sample Replicate - PCR Replicate	C_T* by *Y. pestis* Assay					
		YpP1		YC2		YpMT1	
		Undiluted	10-Fold Dilution	Undiluted	10-Fold Dilution	Undiluted	10-Fold Dilution
Heat Lysis	1-1	18.3	19.9	17.4	18.1	20.9	21.3
	1-2	17.9	19.9	17.3	17.9	20.2	20.9
	1-3	17.6	19.7	17.0	18.4	19.6	21.2
	Ave (SD)	**17.9 (0.4)**	**19.8 (0.1)**	**17.2 (0.2)**	**18.2 (0.3)**	**20.3 (0.7)**	**21.1 (0.2)**
	2-1	17.2	20.0	17.0	18.4	19.8	20.9
	2-2	17.6	20.1	16.4	18.5	19.2	20.8
	2-3	17.5	20.1	15.6	18.7	18.6	21.3
	Ave (SD)	**17.4 (0.2)**	**20.1 (0.1)**	**16.3 (0.7)**	**18.5 (0.1)**	**19.2 (0.6)**	**21.0 (0.3)**
	3-1	18.0	19.8	15.9	18.2	19.3	20.7
	3-2	17.5	19.7	15.7	18.3	18.9	20.6
	3-3	16.8	19.9	15.4	18.3	17.9	20.8
	Ave (SD)	**17.4 (0.6)**	**19.8 (0.1)**	**15.7 (0.2)**	**18.3 (0.1)**	**18.7 (0.7)**	**20.7 (0.1)**
	Overall Ave (SD)	**17.6 (0.5)**	**19.9 (0.2)**	**16.4 (0.8)**	**18.3 (0.2)**	**19.4 (0.9)**	**20.9 (0.2)**
Promega Kit (Chemical Lysis)	1-1	17.3	19.6	16.4	17.9	19.2	20.1
	1-2	16.6	19.7	15.7	18.0	18.0	20.3
	1-3	16.5	19.6	15.4	18.1	17.7	20.1
	Ave (SD)	**16.8 (0.4)**	**19.6 (0.1)**	**15.8 (0.5)**	**18.0 (0.1)**	**18.3 (0.8)**	**20.2 (0.1)**
	2-1	17.0	19.8	17.1	18.7	20.3	20.6
	2-2	16.9	19.7	16.3	18.2	18.4	20.7
	2-3	16.7	19.7	16.3	18.6	18.3	20.8
	Ave (SD)	**16.9 (0.2)**	**19.7 (0.0)**	**16.6 (0.4)**	**18.5 (0.3)**	**19.0 (1.1)**	**20.7 (0.1)**
	3-1	17.3	20.1	16.9	18.7	19.2	21.0
	3-2	17.0	20.1	16.5	18.7	20.0	20.9
	3-3	16.8	20.2	15.7	18.7	18.1	21.1
	Ave (SD)	**17.1 (0.3)**	**20.1 (0.0)**	**16.4 (0.6)**	**18.7 (0.0)**	**19.1 (1.0)**	**21.0 (0.1)**
	Overall Ave (SD)	**16.9 (0.2)**	**19.8 (0.2)**	**16.3 (0.6)**	**18.4 (0.3)**	**18.8 (0.9)**	**20.6 (0.4)**

* C_T = cycle threshold; SD = standard deviation. Average (Ave) and SD values are based on triplicate samples.

With the RV-PCR method using chemical lysis, the overall sample processing and analysis time for 48 samples and controls (3 mL volume) would be ~36 hr including: 2–3 hr for sample receipt and set up in 48-well plates and taking the T_0 aliquots; 24 hr for incubation (during this time, T_0 aliquots are processed for DNA extraction); 3–4 hr for taking the T_{24} aliquots and processing the first set of 24 samples/controls for DNA extraction; ~3 hr for DNA extraction of the second set of 24 samples/controls; and ~3 hr for PCR setup and analysis (T_0 and T_{24} DNA extracts for all 48 samples/controls). In addition, multi-channel pipettors or automated platforms may be used with RV-PCR to further enhance throughput and shorten the time to results.

4.2 TASK 2: Further development and optimization of sample processing protocols for *Y. pestis* cell recovery and growth

4.2.1 Objectives

The objectives of this task were to optimize the recovery efficiency of *Y. pestis* cells from water samples and enhance subsequent growth kinetics in the RV-PCR format. Culture conditions in 48-well plates were modified to enhance growth kinetics with the goal of shortening the method incubation period from 48 hr to 24 hr. This represented a focused effort in which different liquid growth media were evaluated such as YPEB (Doran et al., 2013) recommended by Dr. Darcy Hanes (FDA) (personal communication), and BHI broth as used in a previous effort (US EPA Internal Report, 2010) and by Gilbert et al. (2014). Aliquots were removed over time with care to not significantly deplete the culture volume and affect the growth rate; in this regard separate 48-well plates were set up for different incubation periods so that only one aliquot each were removed for the T_0 and T_f time points. Serial dilution and plating was also conducted at the same end points to assess cell growth. The overall goal was to leverage the results from optimization of cell recovery and growth conditions (from this task) and optimized methods for recovery of purified DNA from cells from Task 1, such that an improved RV-PCR protocol could be evaluated in Task 3.

4.2.2 Approaches Used for Y. pestis CO92 Growth Optimization

Initial culture was performed using BHI agar plates; however, poor growth was observed such that TBA (base without blood; Becton Dickinson Difco™, Cat. No. 223220) was substituted. TBA was more consistent often showing >2-fold higher plate counts from the same cell suspension and sometimes providing data where no counts were obtained for BHI plates. Results reported below are based on TBA plate counts corrected for dilution. For propagation of broth cultures in the 48-well plate format, initial experiments tested BHI broth at 1X concentration, followed by testing of broth prepared with nine parts PBS (for cell suspensions) or distilled deionized water (for only broth reconstitution) and one part 10X BHI broth. Subsequent experiments tested YPEB in both the 1X and reconstituted formats as for BHI broth.

4.2.3 Evaluation of Y. pestis Growth in 1X BHI Broth in 48-Well Plate Format

Growth experiments were initiated with -20°C glycerol stock of *Y. pestis* CO92 and used 5 mL overnight cultures as described in the Materials and Methods section. Three successive overnight cultures in BHI broth were prepared by diluting to an OD_{600} ~0.1 using a portable UV spectrophotometer) and propagated overnight (18–26 hr) at 28°C with orbital shaking at 180 rpm. Initially, successive overnight cultures were propagated based on previous reports of improved growth in BHI broth using this approach; however, later experiments used only one overnight culture as described. After overnight incubation, the OD_{600} values were ~1.8 to > 2 (i.e., the maximum reading on the UV spectrophotometer). To set-up the 48-well plate, the culture was again diluted to OD_{600} value of ~ 0.1 which corresponded to ~ 1×10^7 CFU/mL (actual counts from plating were ~6–7 × 10^6 CFU/mL). Subsequent 10-fold dilutions were performed in BHI broth to produce cell suspensions down to ~ 1×10^1 CFU/mL; 1×10^2 – 1×10^4 cell concentrations were also included. Each well contained three mL of culture at the appropriate dilution and triplicates

were included per cell level. The plate also contained negative controls with broth although without cells. Plates were covered with adhesive AeraSeal (Molecular Devices, Sunnyvale, CA) and incubated at 28°C (with shaking at 180 rpm) for 24 and 40 hr. Separate plates were prepared in the same manner and used for each time point.

The growth curves from a 48-well plate experiment (based on TBA plate counts) are shown in Table 9. Results showed about 1-log increase over 24 hr for 6×10^1 and 6×10^2, and a > 3-log increase for 6×10^3. The inoculum with ~6 CFU/mL did not increase over time.

Table 9. Growth of *Y. pestis* Cells in 48-Well Plates (3 mL 1X BHI)* for ~6×10^1 – 6×10^3 CFU/mL Starting *Y. pestis* Cell Concentrations

Time Point	Average (SD) Measured CFU/mL for Different Starting Cell Concentrations (CFU/mL)**			
	6	**60**	**600**	**6000**
0	5.9 (1.6) × 10^0	5.9 (1.6) × 10^1	5.9 (1.6) × 10^2	5.9 (1.6) × 10^3
24	3.0 (1.7) × 10^1	7.7 (8.3) × 10^2	1.8 (0.4) × 10^3	4.1 (0.7) × 10^7
40	1.5 (0.7) × 10^1	3.8 (3.0) × 10^5	1.8 (0.1) × 10^7	1.4 (0.1) × 10^8
Log Increase ($T_0 - T_{24}$)	0.7	1.1	0.5	3.8
Log Increase ($T_0 - T_{40}$)	0.4	3.8	4.6	4.4

* Cells were prepared by three sequential overnight cultures, harvested by centrifugation, suspended in 1X BHI, transferred to 48-well plates (3 mL), and incubated at 28°C (180 rpm) for 24 or 40 hr.
** Results are averages and standard deviations (SD) from triplicate samples with one replicate plate count per sample. Data represent the average and SD from inoculum reference plating, corrected for dilution.

At each time point, a 1-mL aliquot from each sample (as well as two negative controls without cells) was extracted for DNA using the Promega Magnesil reagents. The protocol for *Y. pestis* cells was based on that for *B. anthracis* (US EPA, 2012) and included the following: (1) two lysis buffer steps; (2) two salt wash buffer steps; (3) two alcohol wash buffer steps; (4) one 70% ethanol wash buffer step; (5) bead drying; and (6) DNA elution. PCR was performed on undiluted DNA extracts using the YC2 assay with 45 amplification cycles. The resulting C_T data were used to estimate *Y. pestis* cells based on *Y. pestis* genomic DNA standard curves assuming one target DNA copy per cell and assuming the size of a *Y. pestis* genome is 4.83 Mb (see Materials and Methods Section 2.13).

The data showed that good DNA yields were obtained from the Promega kit for the 48-well cultures with estimated DNA copies greater on average than those measured from culture analysis. For all assays, the difference between estimated cell numbers from PCR analysis and measured cell numbers from culture analysis was 0.4 ± 0.6 log higher for all assays and for both time points (0.5 ± 0.5 for the YC2 chromosomal assay) (see Section 2.13 for calculations). The estimates have inherent error due to assumptions for converting C_T data to cell number.

4.2.4 Evaluation of Y. pestis Growth in 1X BHI Broth Prepared From 10X BHI

A similar experiment was conducted (with multiple overnight cultures) where in this case *Y. pestis* cells were harvested by centrifugation and washed with 1X phosphate-buffered saline (PBS) prior to setting up the cell suspensions in a 48-well plate. Cell suspensions were diluted to different starting concentrations as described previously and prepared with 10X BHI broth to yield 1X BHI. This was done to match the conditions that would be used for RV-PCR analysis of water samples, which would be mixed with 10X BHI to yield 1X concentration. The overnight cultures used BHI prepared from 10X BHI as well.

Results are shown in Table 10 for plates incubated at 28°C (with shaking at 180 rpm) for 24 and 40 hours, again with separate plates used for each time point. The data showed better growth after 24 hr in this case for ~8-800 CFU/mL, and comparable growth for ~8,000 CFU/mL. Unlike the earlier experiment, significant growth was observed for the 6 CFU/mL level. The growth after 40 hr was similar for all but the ~6 CFU/mL level, which as mentioned did not show growth over time in the previous experiment. It should be noted that plate counts for initial cell concentrations ~6 and 60 CFU/mL at 40 hr were actually above the counted CFU since the plate dilution was missed and plates were too numerous to count (TNTC); the data points are shown at greater than the plate count limit corrected for dilution. Overall, the data suggested that use of reconstituted broth to generate 1X BHI would work well for the protocol with actual water samples.

Table 10. Growth of *Y. pestis* Cells in 48-Well Plates (3 mL 1X BHI Prepared Using 10X BHI)*

Time Point	Average (SD) Measured CFU/mL for Different Starting Cell Concentrations (CFU/mL)**			
	8.4	**84**	**840**	**8400**
0	$8.4\ (0.6) \times 10^0$	$8.4\ (0.6) \times 10^1$	$8.4\ (0.6) \times 10^2$	$8.4\ (0.6) \times 10^3$
24	$4.4\ (3.1) \times 10^2$	4.2×10^4 †	3.9×10^5 †	$6.2\ (0.8) \times 10^6$
40	$> 3 \times 10^4$ ***	$> 3 \times 10^5$ ***	$1.7\ (0.2) \times 10^7$	$4.1\ (1.1) \times 10^7$
Log Increase ($T_0 - T_{24}$)	1.7	2.7	2.7	2.9
Log Increase ($T_0 - T_{40}$)	> 3.6	> 3.6	4.3	3.7

* Cells were prepared by three sequential overnight cultures, harvested by centrifugation, suspended in 1X PBS, mixed with 10X BHI in 48-well plates to yield 1X BHI (3 mL), and incubated at 28°C (180 rpm) for 24 or 40 hr.
** Data points show the average and standard deviation (SD) from triplicate analyses for inoculum reference plating (0 time point) and after 24 and 40 hr incubation, corrected for dilution.
*** Values for 6 and 60 CFU/mL at T_{40} were greater than the values shown due to incorrect dilutions plated.
† Data are from single replicates.

As for the experiment using 1X BHI (Section 4.2.3), a 1-mL aliquot was taken at T_{24} or T_{40} from each sample and negative control for DNA extraction and purification; one aliquot was taken per each well since two separate 48-well plates were used for each incubation period, T_{24} or T_{40}. Likewise, good DNA yields were obtained such that the estimated CFU/mL from PCR data was on average within 2-fold of the expected value based on plate counts (with 0.0 ± 0.2 log representing the difference between PCR and culture analysis for both time points across all assays, and a 0.1 ± 0.2 log difference for the YC2 assay) (see Section 2.13 for calculations). As mentioned,

the estimates for cell number from PCR data have inherent error due to assumptions for converting C_T data to cell number and variability in pipetting and standard curve generation.

4.2.5 Evaluation of Y. pestis Growth in 1X YPEB Compared to 1X BHI Broth Prepared From 10X BHI Broth

An experiment was conducted comparing *Y. pestis* growth on 1X BHI (prepared using 10X BHI) and 1X YPEB. In this case, a single overnight culture of *Y. pestis* cells in either 1X BHI or 1X YPEB was generated, harvested by centrifugation (15 min at 4,000 rpm at 4°C), washed in PBS buffer, and diluted to ~10 to ~10^4 CFU/mL in 1X BHI broth (prepared using 10X BHI broth) or 1X YPEB in two separate 48-well plates. The actual average CFU/mL from plate counts at T_0 were ~3.5 to ~3.5 × 10^3 for BHI and ~2.8 to ~2.8 × 10^3 for YPEB. The total culture volume per well was 3 mL. The 48-well plates were incubated at 28°C (with shaking at 180 rpm).

Results showed poor growth for BHI-grown cells (data not shown), demonstrating inconsistency with previous experiments possibly due to use of a single overnight culture rather than three overnight cultures. However, results from YPEB-grown cells (from a single overnight culture) showed significant increases in cell density representing a 3.6 to 4-log increase over the 24-hr period (Table 11). Based on inconsistent growth on BHI, *Y. pestis* CO92 cells were only propagated on YPEB for the remainder of the experiments.

Table 11. Growth of *Y. pestis* Cells in 48-Well Plates (3 mL 1X YPEB)*

Time Point	Average (SD) Measured CFU/mL for Different Starting Cell Concentrations (CFU/mL)**			
	3	**30**	**300**	**3000**
0	2.8 (0.1) × 10^0	2.8 (0.1) × 10^1	2.8 (0.1) × 10^2	2.8 (0.1) × 10^3
24	2.4 (1.2) × 10^4	1.7 (0.6) × 10^5	1.5 (0.1) × 10^6	8.5 (2.0) × 10^6
Log Increase ($T_0 - T_{24}$)	4.0	3.9	3.8	3.6

* Cells were prepared by one overnight culture, harvested by centrifugation, suspended in 1X YPEB, added to 48-well plates (3 mL), and incubated at 28°C (180 rpm) for 24 hr.
** Data points show the average and standard deviation (SD) from triplicate analyses for inoculum reference plating (0 time point) and after 24 hr incubation, corrected for dilution.

4.2.6 Growth of Y. pestis in 48-Well Plates and RV-PCR Analysis With Different T_0 and T_f Aliquot Volumes

An experiment was conducted with 1X YPEB (prepared from 10X YPEB similar to that described for preparation of 1X BHI broth from 10X BHI broth) to evaluate the impact of different aliquot volumes on *Y. pestis* growth and ΔC_T values at two different incubation periods, 12 and 24 hr. *Y. pestis* cells were prepared as described above and used to inoculate YPEB. *Y. pestis* cells from a single overnight culture were harvested, washed in PBS buffer, and diluted to ~10 to 10^3 CFU/mL (the actual CFU/mL were ~14–1.4 × 10^3 from plating). The experiment used different 48-well plates for each incubation period. Different aliquot volumes including 250 µL and 500 µL were tested, with the same volume used for T_0 and T_{12} or T_0 and T_{24} (the total culture volume per well was 3 mL). In addition, colony counts were obtained from these time points by serial dilution and plating onto TBA plates and used to assess growth with 1X YPEB prepared with 10X YPEB in the 48-well format. Aliquots were centrifuged and the supernatant was removed to leave 200 µL

for DNA extraction for both aliquot volumes to allow direct comparison of results. The Promega MagneSil kit was used as described in the Materials and Methods Section 2.8 and resulting extracts were analyzed undiluted using the YC2 assay with 45 amplification cycles.

Culture analysis showed ~4.3–4.8 log increase over a 24-hr period and ~2-log increase during 12-hr incubation. This experiment showed that even with removal of either 250 or 500 µL at T_0 (including cells present in this aliquot), good growth conditions were observed. Results from PCR analysis of the extracts are shown in Table 12 in terms of ΔC_T for the two different incubation periods. For each starting cell level, the 500 µL extract gave higher average ΔC_T values. For T_{12}, ΔC_T ranged from 5.9–7.3 for the 250 µL aliquots and 6.3–9.6 for T_{12} for the 500 µL aliquots. For T_{24}, ΔC_T ranged from 13.2–17.7 for the 250 µL aliquots and 15.3–19.8 for the 500 µL aliquots. While it was expected that the 500 µL aliquot volume would contain DNA from twice as many cells as that for the 250 µL aliquot, it also resulted in twice as many cells being removed at T_0 such that they could not contribute to cell propagation. Statistical analyses of culture and PCR results for samples processed using 250 or 500 µL volumes for both T_0 and T_{24} did not show significant differences for the different volumes (p-values ranged from 0.1 to 0.9); however, since average ΔC_T values for 500 µL aliquots were greater than those for 250 µL aliquots, 500 µL aliquots were used for subsequent RV-PCR experiments.

Table 12. Effect of Time Point Aliquot Volume (250 and 500 µL) on ΔC_T for RV-PCR Analysis: *Y. pestis* Cells in YPEB (Prepared Using 10X YPEB)*

Starting CFU/mL	Aliquot Volume	ΔC_T** (YC2 Assay)			
		T_0 - T_{12}		T_0 - T_{24}	
		Ave	SD	Ave	SD
10^3	250 µL	6.9	0.3	16.0	0.4
		6.0	0.3	12.8	0.2
		5.2	0.4	10.7	0.1
	Ave	**6.1**	**0.8**	**13.2**	**2.3**
	500 µL	6.2	0.2	14.8	0.1
		7.0	0.1	15.0	0.3
		5.8	0.3	16.0	0.2
	Ave	**6.3**	**0.6**	**15.3**	**0.6**
10^2	250 µL	6.9	0.1	16.0	0.8
		3.4	0.5	16.1	0.8
		7.3	0.7	15.9	0.9
	Ave	**5.9**	**1.9**	**16.0**	**0.7**
	500 µL	7.1	0.4	17.3	0.6
		7.6	1.4	15.8	0.5
		5.9	0.8	17.3	0.7
	Ave	**6.9**	**1.1**	**16.8**	**0.9**
10^1	250 µL	6.5	0.4	17.2	0.1
		6.8	0.7	18.6	0.1
		8.6	0.2	17.1	0.1
	Ave	**7.3**	**1.1**	**17.7**	**0.7**
	500 µL	10.5	0.0	20.3	0.1
		9.4	0.2	20.2	0.1
		8.8	0.3	19.0	0.1
	Ave	**9.6**	**0.8**	**19.8**	**0.6**

* Cells were prepared from one overnight culture, harvested by centrifugation, suspended in 1X PBS, reconstituted with 10X YPEB in 48-well plates to yield 1X YPEB (3 mL), and incubated at 28°C (180 rpm) for 24 hr.
** Average and standard deviation (SD) were based on triplicate samples.

Results also showed that good DNA yields were obtained corresponding to an average of 0.2 ± 0.3 log higher CFU/mL estimated from PCR results relative to those measured by plate counts (corrected for dilution) from the same sample time point (see Section 2.13 for calculations). Greater estimated CFU/mL from PCR analysis compared to culture analysis likely resulted from assumptions in copy number calculation and variations in pipetting, PCR and culture efficiency. It should also be reiterated that PCR data cannot be used to determine absolute cell counts. Regardless, the data showed DNA extraction and purification procedures using the Promega reagents were quite effective. Based on these results, 1X YPEB (prepared from 10X) was used for subsequent *Y. pestis* cultures and the 500 µL aliquot volume was selected for RV-PCR analysis.

4.2.7 Modified Filtration for Concentration of Y. pestis Cells from Larger Volume Water Samples

With regard to concentration and recovery of *Y. pestis* cells, the methods developed for *B. anthracis* spores needed to be modified for vegetative cells to obtain improved limits of detection. Unlike spores, vegetative cells are susceptible to killing by desiccation during filtration. The feasibility of a modified filtration (MF) approach was evaluated for *Y. pestis* cells from complex samples to allow filtration and retain moisture for collection of viable *Y. pestis* cells. Compatibility with RV-PCR analysis was also investigated in a proof-of-principle MF test using SAPs with different particle sizes and chemical compositions and DE in current filtration devices, like those used for collection and concentration of *B. anthracis* spores. The SAP materials were inherently inexpensive since many processes use them in large quantities. Filtration properties (i.e., speed), moisture retention, and biocompatibility (i.e., cell viability maintenance and cell outgrowth) were evaluated, with the initial down-selection based on filtration properties. Different SAP types and amounts were tested with Whatman™ Autovials since the previously used Autocups were discontinued by the vendor (GE Healthcare). Autovials use similar membrane materials (polyethylene sulfone compared with nylon) and the same pore size, 0.45 micron; however, their volume capacity is 12.5 mL, which is less than that for the Autocups, 20 mL, such that lower volumes must be filtered at a time. Different top and bottom caps were also identified for the new filter vials in order to perform sample incubation following cell collection and broth addition. A flow chart in Materials and Methods Section 2.15 shows the MF sample processing method used upstream of RV-PCR analysis.

Prior to this study, it was not known whether the diatomite material could retain sufficient moisture to maintain cell viability or whether the material was sufficiently biocompatible to allow cell propagation. It should be noted that excessive vacuum durations were not used to fully test desiccation of the SAPs or DE materials in this study; the assessment was simply a proof-of-principle study to see how the materials functioned in the filter devices (Autovials) and whether they were also biocompatible with *Y. pestis* cells. In this regard, individual filter samples were under vacuum until the liquid had completely filtered and then the vacuum was released, thereby removing the variable of drying (or desiccation) time.

For the proof-of-principle study, DE, aqueous-based SAPs H-400, and H-500 (JRM Chemical, Inc.), and "Water Storing Crystals" (Miracle-Gro, MG; another type of SAP) were used. Initial studies focused on physical properties of wetting and filtration using different amounts of SAPs and/or DE. Based on the initial findings, an RV-PCR experiment was initiated to evaluate different amounts of DE, MG, and SAPs alone or in combination with other materials, as follows:

1) 0.1 g DE
2) 0.2 g DE
3) 0.1 g DE plus 0.1 g MG
4) 0.1 g MG
5) 0.1 g H-300 plus 0.1 g H-400
6) Control without SAPs, MG, or DE

Y. pestis cells were diluted from an overnight culture to 4×10^4 CFU in 5 mL PBS (determined from reference plating onto TBA plates) and added to pre-washed SAPs and/or DE materials in Autovials. After filtering the cell suspension, the vials were rinsed with 10 mL PBS. In each case, vacuum filtration was stopped as soon as filtration was complete in a given Autovial. This allowed direct comparison between treatments without assessing which material retained more moisture for given vacuum conditions. The only treatment that did not slow the filtration speed relative to the control (Autovial alone) was 0.1 g MG; other treatments added 1 min to > 5 min per filtration step. The treatment with 0.1 g H-300 plus 0.1 g H-400 gelled and took longer to filter completely. In addition, debris-containing samples are expected to have longer filtration times, but were not tested in this effort.

After the wash step, the vial bottom was capped and 5 mL YPEB were added. Due to the experiment timing, a T_0 aliquot could not be taken; however, T_2 samples were taken after 2-hr incubation for both culturing and RV-PCR analysis. The Autovial samples were incubated at 30°C with shaking at 180 rpm, and after 24 hrs another 1 mL aliquot was removed (T_{24} aliquot). Aliquots from both time-points were extracted for DNA using the Promega MagneSil reagents, and undiluted and 10-fold diluted extracts were analyzed using the YC2 assay in triplicate with Universal reagents and Standard cycling conditions (45 amplification cycles).

Since results showed that C_T values for 10-fold diluted DNA extracts were lower than those from undiluted extracts (suggesting PCR inhibition), only results for 10-fold diluted extracts are shown in Table 13. The 0.1 g MG treatment compared most favorably to the control treatment in terms of average T_{24} C_T values (15.8 and 14.9, respectively). The MG treatment also showed good filtration behavior, allowing filtration similar to the case without MG material (i.e., control). Limited plate count analysis (single replicates) at T_{24} showed approximately 2×10^8 CFU/sample for the 0.1 g MG sample. This was less than the control although accurate counts could not be obtained from either treatment since the correct dilution was not plated and colonies were too numerous to count accurately. No counts from the DE samples were obtained due to the presence of contaminating colonies (background organisms).

A follow-up plating experiment with MG material showed that it was sterile. Therefore, the MG material represented the best candidate material for the MF approach to concentrate *Y. pestis* and possibly other vegetative cells prior to RV-PCR analysis. However, MF was not pursued further

mostly due to variation in SAP behavior from one experiment to the next with regard to physical structure and filtration properties. It was difficult to reproducibly prepare the materials to allow rapid filtration with the current setup; therefore, use of this approach for cell concentration could add significantly to the sample processing time. Slight variation in weights (or particle size distributions of the materials) applied to the Autovials led to gel formation and poor filtration whereas other times SAPs remained more dispersed and showed rapid filtration. The variability in filtration behavior would make it difficult to employ this approach operationally, such that more investigation would be required. It is also possible that the material amount and method could not be sufficiently standardized for high throughput sample processing.

Table 13. RV-PCR Analysis* of *Y. pestis* CO92 pgm$^-$ Cells (~4 × 10^4) Collected by a Modified Filtration Approach

Modified Filtration Treatment**	PCR Replicate	YC2 Assay Ave. C_T (SD)*		ΔC_T ($T_2 - T_{24}$)
		T_0	T_{24}	
0.1 g DE	1	31.4	19.4	12.1
	2	31.2	18.9	
	3	31.1	19.2	
	Ave (SD)	**31.3 (0.1)**	**19.2 (0.2)**	
0.2 g DE	1	32.4	23.2	9.5
	2	32.8	23.2	
	3	33.2	23.4	
	Ave (SD)	**32.8 (0.4)**	**23.3 (0.1)**	
0.1 g DE + 0.1 g MG	1	30.7	18.3	12.5
	2	30.8	18.4	
	3	30.9	18.3	
	Ave (SD)	**30.8 (0.1)**	**18.3 (0.1)**	
0.1 g MG	1	29.5	15.7	13.7
	2	29.5	15.8	
	3	29.6	15.7	
	Ave (SD)	**29.5 (0.1)**	**15.8 (0.1)**	
0.1 g H-300 + 0.1 g H-400	1	31.8	18.0	14.1
	2	32.2	18.2	
	3	32.3	17.8	
	Ave (SD)	**32.1 (0.2)**	**18.0 (0.2)**	
Control	1	30.2	14.9	15.4
	2	30.5	14.9	
	3	30.2	14.9	
	Ave (SD)	**30.3 (0.2)**	**14.9 (0.1)**	

* 10-fold diluted DNA extracts were analyzed. Universal Master Mix and Standard cycling conditions were used.
** Average (Ave) and standard deviation (SD) from triplicate PCR analyses. DE = diatomaceous earth; MG = Miracle Gro® (100% acrylamide); H-300 and H-400 = superabsorbent polymers (JRM Chemical, Inc.).

4.2.8 Evaluation of Immunomagnetic Separation for Concentration of Y. pestis Cells from Larger Volume Water Samples

This proof-of-principle study was conducted using IMS to capture of *Y. pestis* cells from PBS solutions as a surrogate for water samples. The Pathatrix system was used to concentrate *Y. pestis* cells from ~30 mL prior to RV-PCR analysis. Anti-*Y. pestis* polyclonal antibody from the Critical Reagents Program (CRP) through BEI Resources, Inc. (BEI Cat. No. DD-514; CRP Cat. No. AB-G-YERS) was used to coat the beads. Pathatrix beads were coated following the manufacturer's directions using recommended antibody concentrations. Two experiments were conducted using RV-PCR analysis of IMS beads containing *Y. pestis* cells captured from suspensions and limited plating was conducted to estimate cell recovery efficiency using IMS.

Y. pestis CO92 pgm^- cells were grown overnight in YPEB, washed in PBS and prepared to ~ 10^7 CFU/mL. Ten-fold serial dilutions were made in PBS and cells were added at three levels (~50, ~500, and ~5000 CFU determined from reference plating) to 30 mL PBS samples (as a surrogate for actual water samples). Counts were actually 54–5400 and 56–5600 for the first and second experiments, respectively. The cell levels were tested in triplicate and a negative control IMS sample without cells was also processed using 30 mL PBS. The recommended amount of antibody-coated Pathatrix beads was used (50 µL) for each sample or control. The recovered beads from IMS were resuspended in 100 µL PBS with 20 µL used to prepare dilutions for plating analysis, and the remaining 80 µL used in an RV-PCR experiment. Specifically, this aliquot (80 µL) was added to 3 mL YPEB in a 48-well plate for RV-PCR analysis. Controls had 3 mL of the original cell suspension added to wells, corresponding to the same number of cells processed by IMS. In addition, two negative controls were included in the 48-well plate by adding 3 mL PBS. At T_0, the well contents were pipet-mixed, and a 0.5 mL aliquot was removed and processed for DNA and subsequent real-time PCR analysis. After 24 hr incubation, another 0.5 mL aliquot was obtained and processed for PCR analysis. DNA was extracted using the modified Promega Magnesil protocol and extracts were analyzed using the YC2 (chromosomal) assay without dilution.

The RV-PCR results from the first and second replicate experiments are shown in Table 14 and Table 15, respectively. For both experiments, 3 of 3 were positive for viable *Y. pestis* for ~500 and ~5000 cells processed by IMS, however, either 2 of 3 or 1 of 3 replicates were positive for ~50 cells processed by IMS for the first and second replicate experiments, respectively. This is contrasted with the control treatments (which were not diluted to 33 mL and processed by IMS prior to RV-PCR), which had 3 of 3 positive by RV-PCR for all three cell levels. Similar trends were observed between the two experiments. In general, there was about a two C_T difference in ΔC_T values between IMS-treated cells and control cells at the ~500 and ~5000 cell levels (the difference was < 1 cycle for the 500-cell level for the first experiment), although the T_{24} C_T values differed by ~4–7 cycles for treated and control cells. There were greater differences between the IMS and control treatments for the ~50 cell level.

Limited culture analysis of Pathatrix beads on TBA plates was performed in parallel in order to obtain some data on cell recovery using IMS. For the first experiment, culture results from the ~5000 cell level showed variable recoveries ranging from ~2–37%. For the second experiment, more data were obtained showing that recoveries ranged from ~3–14% for both the ~500 and ~5000 cell levels. However, when the remaining cell suspension from the Pathatrix instrument was plated (representing cells not captured on beads) and used to determine the cells captured (by

difference), the recoveries were higher based on this analysis with an average of 36.3 ± 10.9% recovered for the different cell levels. Based on this data, it is possible that plating of beads underestimated the actual cell recovery; however, there were clearly significant losses of cells based on plating the remaining suspension. Furthermore, when starting with ~50 cells all three replicates were not detected, whereas starting cell levels down to 10 CFU per sample have previously been detected by RV-PCR at 24 hr. It is also possible that cells captured onto beads propagated more slowly (with a possible lag period) compared to cells not treated with this method.

Table 14. RV-PCR Analysis of Samples Containing Different Levels of *Y. pestis* CO92 pgm⁻ Cells Processed by IMS – YC2 Assay

Sample Type	Sample Replicate	Ave. C_T (SD)*		ΔC_T	Positive Replicates
		T_0	T_{24}		
~5000 cells IMS	1	NDT	24.6 (0.1)	20.4	**3 of 3**
	2	39.1 (0.4)	22.4 (0.1)	16.7	
	3	NDT	23.2 (0.1)	21.8	
	Ave (SD)	**43.0 (3.0)**	**23.4 (1.0)**	**19.6 (2.6)**	
~5000 cells control	1	34.5 (0.3)	18.6 (0.2)	16.0	**3 of 3**
	2	34.9 (0.4)	18.3 0.2)	16.6	
	3	35.8 (0.4)	19.7 (0.1)	16.1	
	Ave (SD)	**35.1 (0.6)**	**18.9 (0.7)**	**16.2 (0.4)**	
~500 cells IMS	1	NDT	28.2 (0.2)	16.8	**3 of 3**
	2	NDT	26.4 (0.1)	18.6	
	3	41.9 (0.2)**	27.5 (0.1)	14.3	
	Ave (SD)	**44.0 (1.6)**	**27.4 (0.8)**	**16.6 (2.2)**	
~500 cells control	1	37.8 (0.9)	20.5 (0.1)	17.3	**3 of 3**
	2	37.5 (0.8)	20.5 (0.1)	17.0	
	3	38.7 (0.9)	20.7 (0.1)	18.0	
	Ave (SD)	**38.0 (0.9)**	**20.6 (0.1)**	**17.4 (0.6)**	
~50 cells IMS	1	NDT	NDT	0.0	**2 of 3**
	2	NDT	31.3 (0.1)	13.7	
	3	41.7 (0.2)**	28.9 (0.1)	12.8	
	Ave (SD)	**43.4 (2.3)*****	**30.1 (1.6)*****	**13.3 (0.7)*****	
~50 cells control	1	NDT	22.8 (0.2)	22.2	**3 of 3**
	2	NDT	24.4 (0.1)	20.6	
	3	NDT	24.6 (0.1)	20.4	
	Ave (SD)	**NDT**	**23.9 (0.9)**	**21.1 (1.0)**	
Negative Control for IMS		NDT	NDT	0.0	**0 of 1**
Negative control for 48-well plate	1	NDT	NDT	0.0	**0 of 2**
	2	41.0 (0.7)**	NDT	-4.0	
	Ave (SD)	**43.0 (2.2)**	**NDT**	**-2.0 (2.8)**	

* Average (Ave) and standard deviation (SD) are from triplicate PCR analyses per sample replicate. Universal Master Mix and Standard cycling conditions were used.
** Values are from two PCR replicates; the third replicate was non-detect.
*** Values are from two sample replicates; the third replicate was non-detect (negative).
NDT = Non-Detect. NDT set to 45 to calculate ΔC_T.

Table 15. RV-PCR Analysis of Samples Containing Different Levels of *Y. pestis* CO92 pgm⁻ Cells Processed by IMS – YC2 Assay (Replicate Experiment)

Sample Type	Sample Replicate	Ave. C_T (SD)*		ΔC_T	Positive Replicates
		T_0	T_{24}		
~5000 cells IMS	1	NDT	23.0 (0.2)	22.0	**3 of 3**
	2	NDT	21.7 (0.2)	23.3	
	3	NDT	21.7 (0.1)	23.3	
	Ave (SD)	**NDT**	**22.1 (0.7)**	**22.9 (0.8)**	
~5000 cells control	1	34.9 (0.7)	17.7 (0.1)	17.2	**3 of 3**
	2	39.2 (0.9)	18.7 (0.1)	20.5	
	3	40.1 (0.9)	18.7 (0.1)	21.4	
	Ave (SD)	**38.1 (2.5)**	**18.4 (0.5)**	**19.7 (2.2)**	
~500 cells IMS	1	NDT	28.3 (0.2)	16.7	**3 of 3**
	2	NDT	28.3 (0.2)	16.7	
	3	NDT	23.2 (0.1)	21.8	
	Ave (SD)	**NDT**	**26.6 (2.6)**	**18.4 (3.0)**	
~500 cells control	1	38.7 (0.6)	21.2 (0.1)	17.5	**3 of 3**
	2	37.4 (0.3)	21.3 (0.1)	16.1	
	3	38.8 (2.1)	21.4 (0.1)	17.4	
	Ave (SD)	**38.3 (1.3)**	**21.3 (0.1)**	**17.0 (1.0)**	
~50 cells IMS	1	NDT	NDT	0.0	**1 of 3**
	2	NDT	NDT	0.0	
	3	NDT	32.0 (0.1)	13.0	
	Ave (SD)	**NDT**	**NA**	**NA**	
~50 cells control	1	NDT	23.9 (0.1)	21.1	**3 of 3**
	2	NDT	23.9 (0.1)	21.1	
	3	NDT	24.0 (0.1)	21.0	
	Ave (SD)	**NDT**	**23.9 (0.1)**	**21.1 (0.1)**	
Negative control for IMS		NDT	NDT	0.0	**0 of 1**
Negative control for 48-well plate	1	NDT	NDT	0.0	**0 of 2**
	2	NDT	NDT	0.0	
	Ave (SD)	**NDT**	**NA**	**0.0**	

***** Average (Ave) and standard deviation (SD) are from triplicate PCR analyses per sample replicate. Universal Master Mix and Standard cycling conditions were used.
NA = Not Applicable; NDT = Non-Detect. NDT set to 45 to calculate ΔC_T.

This preliminary analysis of IMS integrated with RV-PCR showed relatively poor recovery of cells such that additional experiments with IMS were not conducted. It is possible that use of affinity-purified antibody could have enabled better cell recoveries (although these were not available for this effort). The cost, availability, and reproducibility of the IMS approach also need to be considered, especially for operational use for detection of viable *Y. pestis* from water samples. In this case, IMS would also increase the overall time to results from about 36 hr to 40 hr for 48 samples, since each batch of five samples (up to 60 mL) takes about 25 min (15 min on the Pathatrix instrument and 10 min sample preparation and recovery).

4.3 TASK 3: Further development and optimization of RV-PCR protocols for *Y. pestis*

4.3.1 Objectives

With the optimized protocol resulting from improvements in cell growth procedures (Task 2) and DNA purification/concentration procedures (Task 1), a shorter incubation period of 24-hr was proposed. The main objective of this task was then to evaluate whether the shorter incubation resulted in positive detection of viable *Y. pestis* cells even for the types of complex samples expected. The RV-PCR protocol for *Y. pestis* had not previously been tested with challenges including potential PCR and growth inhibitors. Furthermore, method evaluation in a background of dead, target cells was required to determine how the method would work in real-world decontamination or natural degradation scenarios. With these challenges, it was important to evaluate the incubation period and LOD in order to assess method performance with regard to the types of water samples that could be analyzed using this method. Therefore, the optimized protocols from Tasks 1 and 2 were used to confirm the RV-PCR method incubation and establish the method LOD for different challenges including potential chemical and biological interferences.

4.3.2 Evaluation of RV-PCR Method Performance with Complex Water Samples

In this task, water samples with iron sulfate and humic acids as chemical interferences were evaluated. These were selected since iron and humics are often mentioned as PCR inhibitors and the goal was to ensure that their presence did not negatively impact either *Y. pestis* growth or PCR analysis of DNA extracted from cells. 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 is included in Materials and Methods Section 2.4.

The *Y. pestis* RV-PCR method was evaluated with water samples (using phosphate-buffered saline) containing complex backgrounds of live non-target spores/cells (non-autoclaved AZ Test Dust, 4 mg/mL), or humic acids (50 µg/mL) plus iron (10 µg/mL Fe as $FeSO_4$). These samples were compared with controls lacking the challenge material to determine the effect of biological and chemical challenges on RV-PCR method performance for *Y. pestis*. An overnight culture of *Y. pestis* CO92 was harvested by centrifugation, washed with PBS, and diluted to 0.1 OD_{600} with PBS (approximately 1×10^7 CFU/mL). *Y. pestis* CO92 cells were serially diluted and added at ~18 or 180 CFU/mL (from reference plating). Cells in PBS were reconstituted by adding 10X YPEB and 3 mL were added to each well. A 0.5 mL T_0 aliquot was removed, centrifuged at 20,800 RCF for 10 min at 4°C, 300 µL were removed, and the resulting pellet was stored at -20°C until pellets were extracted for DNA. The remaining culture was incubated at 30°C with shaking at 180 rpm for 24 hr, after which a 0.5 mL T_{24} aliquot was removed and processed as for the T_0 aliquot. Aliquots were extracted for DNA using Promega Magnesil reagents and PCR analysis was conducted using the YC2 assay.

For the 100-cell level (Table 16), the data showed that there was no PCR inhibition for the control treatment with similar ΔC_T values for undiluted and 10-fold diluted extracts; however, the treatment with iron and humic acid showed inhibition for 1 of the 3 replicates, while the 10-fold diluted extract had similar data to the control treatment (i.e., elimination of PCR inhibition). For

the treatment with native (un-autoclaved) test dust, about 30% lower ΔC_T values were observed (Average [Ave.] $\Delta C_T = 8.8 \pm 3.6$) than those for the other treatments (Ave. $\Delta C_T = 13.1 \pm 0.3$ and 12.5 ± 0.5 for the control and Fe/Humic treatments, respectively). This was likely due to growth inhibition from the indigenous organisms in the test dust, which includes faster growing genera such as *Bacillus*. Plating could not be conducted at the end of incubation since *Y. pestis* colonies would not easily be detected in the background colony growth. The ΔC_T values were ≥ 6 (the criteria set-up for detection of live cells in samples) for only 2 of the 3 replicates for native ATD suggesting a longer incubation period may be needed for detection of low cell levels for these types of samples.

The same trends were observed for the lower cell level (~18 CFU/mL; Table 17) with lower ΔC_T values for the native test dust treatment. In this case, however, the ΔC_T values were significantly lower than 6, ranging from 1.3 to 4.4 for the 10-fold diluted extracts. Therefore, positive detection could not be achieved for these low starting cell levels in the presence of background organisms, at least after 24 hr. As for the 100-cell level samples, the 10-fold dilution gave consistently higher ΔC_T values (that met the requirement for positive detection) than the undiluted extracts for the Fe/Humic treatment, showing PCR inhibition in undiluted samples.

A replicate evaluation was conducted for the *Y. pestis* RV-PCR method with water samples (using PBS) containing challenge materials at the same concentrations, AZ Test Dust (4 mg/mL), or humic acids (50 µg/mL) plus iron (10 µg/mL Fe as $FeSO_4$). These treatments (in triplicate) were also compared with triplicate controls lacking the challenge material. *Y. pestis* cells were prepared as described for the previous experiment, and added at ~10 or 100 CFU/mL per sample well (determined from reference plating). Aliquots were removed and subjected to DNA extraction and PCR analysis as described previously.

For the 100-cell level (Table 18), as for the initial experiment the data showed that there was little to no PCR inhibition for the control treatment with similar ΔC_T values for undiluted and 10-fold diluted extracts; however, the treatment with iron and humic acid showed inhibition for 1 of the 3 replicates for undiluted extracts, while the 10-fold diluted extract had similar data to the control treatment. However, contrary to the first replicate experiment (Table 17), the treatment with native (un-autoclaved) test dust showed similar ΔC_T values to the control treatment for either undiluted or 10-fold diluted extracts. Unlike the first replicate experiment, higher T_0 C_T values were observed, which led to greater ΔC_T values since T_{24} C_T values were comparable between experiments. Although, the replicate experiments were conducted the same with regard to *Y. pestis* cell preparation, it appeared that the first experiment had a higher concentration of dead cells that led to lower T_0 C_T values. The first experiment also used slightly higher starting cell concentrations although the difference was only about 2-fold higher.

Similar trends were observed for the lower cell level (~10 CFU/mL; Table 19); however, in this case there were lower ΔC_T values for the native test dust treatment compared to the control treatment especially for the 10-fold diluted extracts. It should also be noted that 1 of 3 control replicates showed high T_{24} C_T values (~32-33) likely due to poor growth and/or operator error in DNA extraction or PCR analysis for this replicate. Unlike the previous experiment, the ΔC_T values for the native test dust treatment still met the criterion for positive detection for all but one replicate for 10-fold dilution only (for the undiluted extract, the ΔC_T value was 12.2). For the Fe/humics

treatment, only 1 of 3 replicates showed PCR inhibition although this was resolved with 10-fold dilution. Together these results suggest that debris such as the reference test dust could cause growth inhibition due to the presence of indigenous organisms such as faster growing *Bacillus* spp. The test dust was shown to contain *Bacillus* and other bacteria as well as fungal spores (Rose et al., 2011). However, at these low cell levels, the RV-PCR method still showed the ability to accurately detect live cells in complex backgrounds, with consistent detection at the 100-cell level. The negative controls were non-detect for all replicates at both time points (data not shown).

The first experiment had lower T_0 C_T values, which impacted the ΔC_T values; the T_{24} C_T values were comparable between experiments. Although the experiments were conducted the same way with regard to cell propagation and preparation of the inoculum, the first experiment appeared to have a higher concentration of dead cells, which likely contributed to lower T_0 C_T values, although this was not measured directly. The cells used as inoculum were obtained from an overnight culture that was washed to remove spent broth, but dead cells could not be removed. Regardless, the experiments showed that Fe/humic acid did not impact the method incubation period and LOD; however, for low cell numbers (~10–20 cells per sample) especially with high dead cell concentrations, detection after 24-hr incubation could be inconsistent such that a longer incubation period (i.e., 36 hr) could be warranted.

Table 16. RV-PCR Results for *Y. pestis* CO92 (~180 CFU/Sample) in the Presence of Chemical or Biological Backgrounds

Treatment	Sample Replicate - PCR Replicate	YC2 Assay Ave. C_T (SD)*				ΔC_T ($T_0 - T_{24}$)	
		Undiluted		10-Fold Dilution		Undiluted	10-Fold Dilution
		T_0	T_{24}	T_0	T_{24}		
Control	1 – 1	34.6	22.4	37.9	25.3	**12.2**	**12.9**
	1 – 2	34.0	22.1	38.2	25.0		
	1 – 3	34.6	22.0	38.1	25.2		
	Ave (SD)	**34.4 (0.4)**	**22.2 (0.2)**	**38.1 (0.2)**	**25.2 (0.2)**		
	2 – 1	34.0	22.1	37.5	24.8	**12.1**	**13.1**
	2 – 2	33.8	21.7	38.2	24.8		
	2 – 3	33.9	21.6	38.1	24.9		
	Ave (SD)	**33.9 (0.1)**	**21.8 (0.2)**	**38.0 (0.4)**	**24.9 (0.1)**		
	3 – 1	33.8	21.6	37.3	24.3	**12.2**	**13.4**
	3 – 2	33.3	21.2	37.7	24.2		
	3 – 3	33.5	21.2	38.2	24.3		
	Ave (SD)	**33.5 (0.2)**	**21.3 (0.2)**	**37.7 (0.4)**	**24.3 (0.1)**		
	Overall Ave (SD)	**33.9 (0.5)**	**21.8 (0.4)**	**37.9 (0.4)**	**24.8 (0.4)**	**12.1 (0.1)**	**13.1 (0.3)**
Fe/Humics	1 – 1	39.2	27.4	38.2	25.3	**12.5**	**12.8**
	1 – 2	38.5	26.0	38.2	25.3		
	1 – 3	38.8	25.5	38.0	25.3		
	Ave (SD)	**38.8 (0.3)**	**26.3 (1.0)**	**38.1 (0.1)**	**25.3 (0.1)**		
	2 – 1	36.6	40.9	40.2	27.4	**-3.0**	**11.9**
	2 – 2	35.9	39.0	38.9	27.5		
	2 – 3	35.8	37.4	39.2	27.5		
	Ave (SD)	**36.1 (0.5)**	**39.1 (1.8)**	**39.4 (0.7)**	**27.5 (0.1)**		
	3 – 1	36.1	26.1	37.7	25.1	**10.6**	**12.8**
	3 – 2	36.3	25.3	38.0	25.1		
	3 – 3	35.9	25.1	38.0	25.1		
	Ave (SD)	**36.1 (0.2)**	**25.5 (0.5)**	**37.9 (0.2)**	**25.1 (0.1)**		
	Overall Ave (SD)	**37.0 (1.4)**	**30.3 (6.7)**	**38.5 (0.8)**	**26.0 (1.2)**	**6.7 (8.5)**	**12.5 (0.5)**
Native ATD	1 – 1	34.6	31.2	37.7	32.2	**3.9**	**5.7**
	1 – 2	35.0	31.0	37.9	32.2		
	1 – 3	34.8	30.6	38.4	32.4		
	Ave (SD)	**34.8 (0.2)**	**30.9 (0.3)**	**38.0 (0.4)**	**32.3 (0.1)**		
	2 – 1	37.1	33.6	39.9	32.4	**4.7**	**12.7**
	2 – 2	37.2	32.2	NDT	32.2		
	2 – 3	37.3	31.7	NDT	32.3		
	Ave (SD)	**37.2 (0.1)**	**32.5 (1.0)**	**NDT**	**32.3 (0.1)**		
	3 – 1	36.2	33.2	39.9	32.0	**3.8**	**7.9**
	3 – 2	36.6	32.3	38.4	32.1		
	3 – 3	36.2	31.9	41.9	32.4		
	Ave (SD)	**36.3 (0.2)**	**32.5 (0.7)**	**40.1 (1.8)**	**32.2 (0.2)**		
	Overall Ave (SD)	**36.1 (1.1)**	**32.0 (1.0)**	**41.0 (3.3)**	**32.3 (0.1)**	**4.1 (0.5)**	**8.8 (3.6)**

* Average (Ave) and standard deviation (SD) are from Universal reagents and Standard cycling conditions.
ATD = Arizona Test Dust. NDT = Non-detect.

Table 17. RV-PCR Results for *Y. pestis* CO92 (~18 CFU/Sample) in the Presence of Chemical or Biological Backgrounds

Treatment	Sample Replicate - PCR Replicate	YC2 Assay Ave. C_T (SD)*				ΔC_T ($T_0 - T_{24}$)	
		Undiluted		10-Fold Dilution		Undiluted	10-Fold Dilution
		T_0	T_{24}	T_0	T_{24}		
Control	1 – 1	38.4	25.4	38.1	28.1	**12.8**	**10.4**
	1 – 2	38.1	25.3	38.1	27.9		
	1 – 3	37.9	25.1	39.3	28.2		
	Ave (SD)	**38.1 (0.3)**	**25.3 (0.2)**	**38.5 (0.7)**	**28.1 (0.2)**		
	2 – 1	34.1	25.3	37.7	27.7	**9.1**	**10.1**
	2 – 2	34.0	24.8	37.8	27.7		
	2 – 3	34.0	24.7	38.2	27.9		
	Ave (SD)	**34.0 (0.1)**	**24.9 (0.3)**	**37.9 (0.3)**	**27.8 (0.1)**		
	3 – 1	33.6	27.0	38.1	29.7	**6.9**	**9.2**
	3 – 2	33.5	26.6	37.3	29.6		
	3 – 3	33.5	26.4	41.3	29.7		
	Ave (SD)	**33.6 (0.1)**	**26.7 (0.3)**	**38.9 (2.1)**	**29.7 (0.1)**		
	Overall Ave (SD)	**35.2 (2.2)**	**25.6 (0.9)**	**38.4 (1.2)**	**28.6 (0.9)**	**9.6 (3.0)**	**9.8 (0.6)**
Fe/Humics	1 – 1	35.1	28.2	37.4	27.6	**7.0**	**10.3**
	1 – 2	34.9	28.4	38.8	27.6		
	1 – 3	35.3	27.7	37.6	27.6		
	Ave (SD)	**35.1 (0.2)**	**28.1 (0.4)**	**37.9 (0.7)**	**27.6 (0.1)**		
	2 – 1	41.0	40.3	38.4	27.1	**2.4**	**11.5**
	2 – 2	40.0	37.8	39.7	27.1		
	2 – 3	40.0	35.7	38.0	27.2		
	Ave (SD)	**40.3 (0.6)**	**37.9 (2.3)**	**38.7 (0.9)**	**27.2 (0.1)**		
	3 – 1	35.5	28.6	39.0	28.8	**7.0**	**10.4**
	3 – 2	34.9	28.1	40.4	28.7		
	3 – 3	35.3	27.8	38.1	28.8		
	Ave (SD)	**35.2 (0.3)**	**28.2 (0.4)**	**39.2 (1.1)**	**28.8 (0.1)**		
	Overall Ave (SD)	**36.9 (2.6)**	**21.4 (5.0)**	**38.6 (1.0)**	**27.9 (0.7)**	**5.5 (2.7)**	**10.7 (0.7)**
Native ATD	1 – 1	35.9	33.8	38.5	35.0	**2.4**	**3.3**
	1 – 2	35.3	33.2	38.3	35.2		
	1 – 3	36.2	33.2	38.2	35.0		
	Ave (SD)	**35.8 (0.5)**	**33.4 (0.3)**	**38.3 (0.1)**	**35.0 (0.1)**		
	2 – 1	35.8	35.8	37.2	37.4	**0.1**	**1.3**
	2 – 2	35.8	35.5	39.1	37.1		
	2 – 3	35.4	35.5	38.8	36.8		
	Ave (SD)	**35.7 (0.2)**	**35.6 (0.2)**	**38.4 (1.0)**	**37.1 (0.3)**		
	3 – 1	36.0	32.9	39.8	34.6	**3.3**	**4.4**
	3 – 2	35.6	32.5	38.8	34.3		
	3 – 3	36.1	32.3	38.0	34.5		
	Ave (SD)	**35.9 (0.3)**	**32.6 (0.3)**	**38.9 (0.9)**	**34.5 (0.2)**		
	Overall Ave (SD)	**35.8 (0.3)**	**33.9 (1.4)**	**38.5 (0.7)**	**35.5 (1.2)**	**1.9 (1.7)**	**3.0 (1.6)**

* Average (Ave) and standard deviation (SD) are from Universal reagents and Standard cycling conditions.
ATD = Arizona Test Dust.

Table 18. RV-PCR Results for *Y. pestis* CO92 (~100 CFU/Sample) in the Presence of Chemical or Biological Backgrounds

Treatment	Sample Replicate - PCR Replicate	YC2 Assay Ave. C_T (SD)*				ΔC_T ($T_0 - T_{24}$)	
		Undiluted		10-Fold Dilution		Undiluted	10-Fold Dilution
		T_0	T_{24}	T_0	T_{24}		
Control	1 – 1	38.6	24.2	NDT	28.3	15.2	16.8
	1 – 2	39.6	23.3	NDT	28.1		
	1 – 3	38.3	23.3	40.2	28.4		
	Ave (SD)	**38.8 (0.7)**	**23.6 (0.5)**	**NDT**	**28.2 (0.2)**		
	2 – 1	37.0	23.7	39.7	21.2	12.7	16.2
	2 – 2	36.7	25.2	NDT	24.2		
	2 – 3	37.1	24.0	39.1	24.3		
	Ave (SD)	**37.0 (0.2)**	**24.3 (0.8)**	**39.4 (0.4)****	**23.2 (1.8)**		
	3 – 1	38.0	22.6	38.1	25.8	14.9	12.9
	3 – 2	37.1	23.1	NDT	26.8		
	3 – 3	38.6	23.4	41.0	27.2		
	Ave (SD)	**37.9 (0.8)**	**23.0 (0.4)**	**39.5 (2.1)****	**26.6 (0.7)**		
	Overall Ave (SD)	**37.9 (0.9)**	**23.6 (0.8)**	**41.3 (3.0)**	**26.0 (2.4)**	**14.3 (1.4)**	**15.3 (2.1)**
Fe/ Humics	1 – 1	NDT	28.4	NDT	30.0	17.3	14.6
	1 – 2	41.0	27.5	39.8	31.0		
	1 – 3	NDT	27.2	NDT	30.4		
	Ave (SD)	**NDT**	**27.7 (0.6)**	**NDT**	**30.4 (0.5)**		
	2 – 1	44.7	33.4	NDT	31.4	12.7	13.1
	2 – 2	43.3	NDT	41.7	26.2		
	2 – 3	42.8	28.8	41.1	27.4		
	Ave (SD)	**43.8 (1.3)****	**31.1 (3.3)****	**41.4 (0.4)****	**28.3 (2.8)**		
	3 – 1	42.1	31.1	40.1	28.3	3.3	13.4
	3 – 2	41.6	42.8	40.3	26.6		
	3 – 3	41.7	41.8	NDT	25.7		
	Ave (SD)	**41.8 (0.3)**	**38.5 (6.5)**	**40.2 (0.1)****	**26.8 (1.3)**		
	Overall Ave (SD)	**43.5 (1.5)**	**32.4 (6.0)**	**42.2 (2.2)**	**28.5 (2.2)**	**11.1 (7.1)**	**13.7 (0.8)**
Native ATD	1 – 1	NDT	31.6	40.6	32.5	13.7	12.2
	1 – 2	41.8	31.0	NDT	32.4		
	1 – 3	NDT	31.4	NDT	33.5		
	Ave (SD)	**NDT**	**31.3 (0.3)**	**NDT**	**32.8 (0.4)**		
	2 – 1	43.1	27.4	NDT	29.6	15.9	15.3
	2 – 2	NDT	26.7	40.5	29.5		
	2 – 3	42.7	26.8	NDT	30.1		
	Ave (SD)	**42.9 (0.3)****	**27.0 (0.4)**	**NDT**	**29.7 (0.3)**		
	3 – 1	NDT	30.0	NDT	32.5	15.5	12.6
	3 – 2	NDT	29.0	NDT	32.1		
	3 – 3	NDT	29.6	NDT	32.6		
	Ave (SD)	**NDT**	**29.5 (0.5)**	**NDT**	**32.4 (0.2)**		
	Overall Ave (SD)	**44.3 (1.1)**	**29.2 (1.9)**	**NDT**	**31.6 (1.5)**	**15.1 (1.2)**	**13.4 (1.7)**

* Average (Ave) and standard deviation (SD) are from Universal reagents and Standard cycling conditions.
** Values are from two PCR replicates; the third replicate was non-detect.
NDT = Non-detect. NDT set to 45 to calculate ΔC_T.ATD = Arizona Test Dust.

Table 19. RV-PCR Results for *Y. pestis* CO92 (~10 CFU/Sample) in the Presence of Chemical or Biological Backgrounds

Treatment	Sample Replicate - PCR Replicate	YC2 Assay Ave. C_T (SD)*				ΔC_T ($T_0 - T_{24}$)	
		Undiluted		10-Fold Dilution		Undiluted	10-Fold Dilution
		T_0	T_{24}	T_0	T_{24}		
Control	1 – 1	39.1	24.6	NDT	29.0	**14.7**	**15.2**
	1 – 2	39.4	24.3	NDT	30.0		
	1 – 3	38.9	24.3	NDT	30.3		
	Ave (SD)	**39.1 (0.3)**	**24.4 (0.2)**	**NDT**	**29.8 (0.7)**		
	2 – 1	40.9	33.2	NDT	34.1	**7.9**	**11.1**
	2 – 2	40.5	32.4	NDT	33.7		
	2 – 3	40.3	32.5	NDT	33.9		
	Ave (SD)	**40.6 (0.3)**	**32.7 (0.5)**	**NDT**	**33.9 (3.3)**		
	3 – 1	NDT	23.9	NDT	27.9	**21.4**	**17.0**
	3 – 2	NDT	23.5	NDT	27.9		
	3 – 3	NDT	23.3	NDT	28.3		
	Ave (SD)	**NDT**	**23.6 (0.3)**	**NDT**	**28.0 (0.2)**		
	Overall Ave (SD)	**41.6 (2.7)**	**26.9 (4.4)**	**NDT**	**30.6 (3.1)**	**14.7 (6.8)**	**14.4**
Fe/Humics	1 – 1	42.4	34.8	NDT	31.2	**9.1**	**13.9**
	1 – 2	42.2	32.4	NDT	31.0		
	1 – 3	NDT	32.3	NDT	31.1		
	Ave (SD)	**42.3 (0.1)****	**33.2 (1.4)**	**NDT**	**31.1 (0.1)**		
	2 – 1	44.1	37.7	NDT	30.3	**11.1**	**14.7**
	2 – 2	NDT	31.8	NDT	30.3		
	2 – 3	NDT	32.2	NDT	30.2		
	Ave (SD)	**NDT**	**33.9 (0.2)**	**NDT**	**30.3 (0.1)**		
	3 – 1	NDT	NDT	43.5	28.2	**0.0**	**14.0**
	3 – 2	NDT	NDT	41.1	28.2		
	3 – 3	NDT	39.7	NDT	28.3		
	Ave (SD)	**NDT**	**NDT**	**42.3 (1.7)****	**28.3 (0.1)**		
	Overall Ave (SD)	**44.1 (1.4)**	**37.3 (5.8)**	**44.1 (1.6)**	**29.9 (1.3)**	**6.7 (5.9)**	**14.2**
Native ATD	1 – 1	NDT	34.2	40.0	36.2	**12.2**	**4.1**
	1 – 2	NDT	33.7	NDT	36.1		
	1 – 3	43.5	33.5	40.1	35.3		
	Ave (SD)	**NDT**	**33.8 (0.4)**	**40.0 (0.1)****	**35.9 (0.5)**		
	2 – 1	NDT	34.5	NDT	36.9	**10.8**	**7.4**
	2 – 2	NDT	34.1	NDT	38.5		
	2 – 3	NDT	34.0	NDT	37.5		
	Ave (SD)	**NDT**	**34.2 (0.2)**	**NDT**	**37.6 (0.8)**		
	3 – 1	41.3	32.9	NDT	35.8	**7.8**	**9.5**
	3 – 2	39.1	32.9	41.6	35.4		
	3 – 3	41.3	32.3	NDT	35.4		
	Ave (SD)	**40.5 (1.3)**	**32.7 (0.4)**	**NDT**	**35.5 (0.3)**		
	Overall Ave (SD)	**43.5 (2.3)**	**33.6 (0.7)**	**43.3 (2.5)**	**36.3 (1.1)**	**10.3 (2.2)**	**7.0 (2.7)**

* Average (Ave) and standard deviation (SD) are from Universal reagents and Standard cycling conditions.
** Values are from two PCR replicates; the third replicate was non-detect.
NDT = Non-detect. NDT set to 45 to calculate ΔC_T. ATD = Arizona Test Dust.

4.3.3 Evaluation of RV-PCR Method Performance in a Dead Y. pestis Cell Background

The *Y. pestis* RV-PCR method with 24-hr incubation period was evaluated for application to post-decontamination scenarios where low levels of live target cells must be detected in samples with high concentrations of dead target cells. Initially, methods for generating a background of dead cells were investigated such that the cells remained intact. Intact, dead cells represented the most challenging case for testing the RV-PCR method. Different disinfection methods included autoclaving, antibiotic exposure, and isopropanol exposure. After generating dead cell populations, sterility was confirmed by plating analysis using 30% volume of the cell suspension (300 µL from total volume of 1 mL with ~5×10^7 CFU). The dead cell concentrations ranged from 10^2 to 10^6 cells per sample.

As described in Materials and Methods Section 2.6, the pgm$^-$ strain was used for IPA exposure, using protocols from generation of dead cells for microscopy and flow cytometry as referenced (i.e., Live/Dead® BacLight™ Bacterial Viability Kit, Molecular Probes, Inc.). Initially, a 1-hr exposure to 70% IPA was tested with ~ 1×10^9 CFU/mL; however, only about 6-log kill was achieved based on serial dilution and plating. The culture conditions were changed to be more controlled (synchronized) and exposures were conducted with fewer cells, ~5×10^7 CFU/mL (early log phase) for both 1 and 2 hr with mixing every 30 min. These conditions produced completely dead cell suspensions for both exposure times based on plating triplicate 0.1 mL aliquots (detection limit of ~3 – 4 CFU/mL).

Prior to RV-PCR experiments, IPA-killed cells were tested for DNA content by heat lysis and PCR analysis. Since previous PCR analysis showed that the pgm$^-$ strain was also reactive with the YC2 (as well as YpP1, and YpMT1) assays, this allowed use of the YC2 assay to evaluate whether the disinfection method led to loss of DNA from cells. A flow chart for generation of dead cells and PCR analysis of different components during the preparation of the IPA-killed cell suspension is shown in Figure 8. Control cell suspensions treated with PBS instead of IPA were processed and analyzed for DNA content in parallel.

The C_T values were comparable between IPA-killed cells and control cells and even showed lower average C_T values for IPA-killed cells (Table 20). This trend was consistent for heat lysates from cell suspensions and cell pellets as well as from supernatants. The PCR data from supernatants showed loss of DNA for both treated and untreated cells although values suggested about 2-log less DNA in supernatants compared to the other fractions. Similar analysis was conducted 9 days later. The data showed no significant differences between PCR results for the different components for IPA-killed and control cell suspensions with p-values ranging from 0.1 – 0.6 (Student's two-tailed, paired T-test). In addition, cells tested in the same way up to 40 days after initial preparation showed similar results for heat lysates of cell suspensions and supernatants. These data suggested that dead cells remained intact and stable over time while stored at 4°C, thus providing the most challenging test case for a dead cell background for RV-PCR (highest levels of DNA from dead cells). The 2-hr IPA-exposed cells were used in RV-PCR experiments and tested by PCR for loss of DNA prior to each experiment (i.e., cell used up to 40-days after exposure and preparation).

This page intentionally left blank

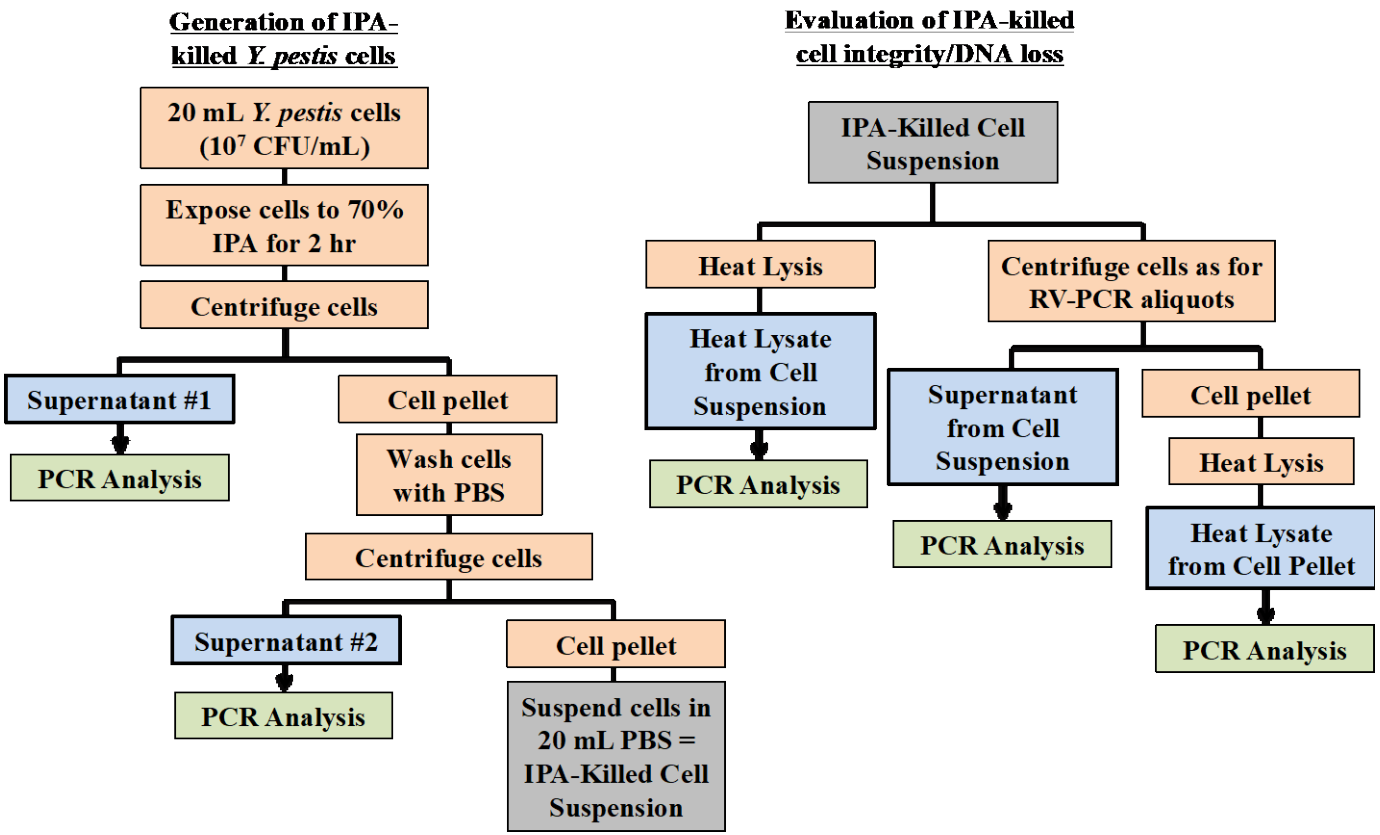

Figure 8. Flow chart for preparation and analysis of IPA-killed cell suspensions. Data from analysis (blue boxes) is shown in Table 20.

Table 20. PCR Analysis of Components Generated During the Preparation of IPA-Killed *Y. pestis* Cell Suspensions to Assess DNA Content for Loss

Sample Type	PCR Replicate	C_T by Component From IPA-Killed Cell Suspension Preparation*								
		Supernatant (#1)		Supernatant Wash (#2)		Heat Lysate from Cell Suspension		Heat Lysate Pellet		Supernatant from Cell Suspension
		10-Fold Dilution	50-Fold Dilution	Undiluted	10-Fold Dilution	Undiluted	10-Fold Dilution	Undiluted	10-Fold Dilution	Undiluted
IPA-exposed	1	40.0	40.1	29.9	26.9	28.2	23.3	25.0	22.0	30.4
	2	38.5	38.5	30.4	27.1	27.6	22.9	24.1	21.9	29.3
	Ave (SD)	**39.3 (1.1)**	**39.3 (1.1)**	**30.1 (0.3)**	**27.0 (0.2)**	**27.9 (0.4)**	**23.1 (0.2)**	**24.5 (0.6)**	**21.9 (0.1)**	**29.9 (0.8)**
Control	1	31.2	28.8	32.2	29.7	29.8	25.7	26.0	23.6	34.5
	2	31.5	28.9	32.8	29.7	28.7	25.5	25.0	23.5	33.2
	Ave (SD)	**31.4 (0.2)**	**28.9 (0.1)**	**32.5 (0.4)**	**29.7 (0.1)**	**29.2 (0.8)**	**25.6 (0.1)**	**25.5 (0.7)**	**23.6 (0.1)**	**33.8 (0.9)**

* Cell suspensions were exposed to 70% isopropanol (IPA) for 2 hr. C_T data are from the YC2 assay using Universal reagents and Standard cycling conditions. Data correspond to the blue boxes in Figure 8.

RV-PCR experiments were conducted using IPA-killed *Y. pestis* cells as background with 10 and 100 CFU/mL live cell concentrations. Two sets of experiments were conducted. The first experiment included 10^2 and 10^4 dead cells/mL with 120 ± 30 or 12 ± 3 live cells (from reference plating), while the second experiment used 10^5 and 10^6 dead cells/mL with 170 ± 20 or 17 ± 2 live cells (from reference plating). In both cases, control treatments without dead cells were processed in parallel. Aliquots were processed for DNA at T_0 and T_{24} and analyzed using the YC2 chromosomal assay with both 10-fold and 100-fold dilutions to check for PCR inhibition and ensure that the reaction was not saturated with DNA template from high levels of dead *Y. pestis* cells. Although the same data trends were evident, the 10-fold dilutions consistently showed more positive detection compared with 100-fold dilution; the latter showing more non-detect results. The potential issue of DNA saturation was not evident in these experiments so data from 10-fold dilutions are shown.

Results from the first experiment for 10-fold diluted extracts showed that for the 100 live cell level (for 10-fold diluted extracts), both dead cell backgrounds did not impact RV-PCR positive results with average ΔC_T values of 17.2 and 14.7 for 10^2 and 10^4 dead cells, respectively; these values were similar to the 0 dead cell level (Ave ΔC_T = 15.6; Table 21). For these treatments, 3 of 3 replicates were positive by RV-PCR. For the 10 live cell level with a 10^4 dead cell concentration an average ΔC_T of 6.1 was achieved; however, individual ΔC_T values were 6.0, 7.9, and 4.5 showing that only 2 of 3 replicates met the criteria for positive detection. Positive PCR results were obtained for the 10^2-dead cell concentrations with the average ΔC_T of 15.6, while the control without dead cells had an average ΔC_T of 16.7. In Table 21, cases are highlighted where fewer than 3 of 3 replicates were positive based on $\Delta C_T \geq 6$ (with the 24 hrs incubation period) since these would represent false negative results.

For the second experiment with higher levels of dead cells, the 10^2 live cell level still had average ΔC_T values ≥ 6 for all dead cell backgrounds (for 10-fold diluted extracts), although for 10^6 dead cells the individual ΔC_T values were 7.1, 5.6, and 5.1 showing only 1 of 3 positive. It should be noted however that these dead cell levels are quite high and would likely not be expected from native water samples without some concentration method applied. For the 10 live cell level (10-fold diluted extracts), the dead cell concentrations of 10^5 and 10^6 prevented positive detection, since in all cases ΔC_T values were < 6. For these high levels of dead cells, the control treatments with 0 live cells showed negative results as expected with 0 of 3 positive; the data showed that the method did not produce false positive results for high concentrations of dead cells.

These experiments served to bracket the conditions RV-PCR analysis could be used for detection of live cells in post-decontamination scenarios. Based on these results, to improve the limit of detection and reduce the false negative rate, a longer incubation could be warranted; an incubation period of 36 hrs could provide sufficient growth above the dead cell DNA background to detect live cells at either the 10 or 100 live cell level. It is also recommended to analyze both undiluted and 10-fold diluted DNA extracts to minimize false positive/false negative rates.

Table 21. RV-PCR Results for 10- and 100-Cell Levels of Live *Y. pestis* Cells With Different IPA-Killed Target Cell Concentrations – 10-Fold Diluted DNA Extracts

Sample Type		Ave C_T (SD)*		Ave ΔC_T (SD)†	Positive Replicates
Live Cell Level	Dead Cell Level	T_0	T_{24}		
100	**0**	41.8 (2.5)	26.2 (1.0)	15.6 (3.9)	3 of 3
	10^2	NDT	27.8 (3.3)	17.2 (3.8)	3 of 3
	10^4	39.9 (4.2)**	25.2 (0.7)	14.7 (4.9)	3 of 3
	10^5	34.1 (0.7)	26.3 (1.2)	7.8 (1.9)	3 of 3
	10^6	31.3 (0.5)	25.4 (0.6)	6.0 (1.0)†	1 of 3
10	**0**	NDT	28.3 (0.7)	16.7 (0.9)	3 of 3
	10^2	NDT	29.4 (0.6)	15.6 (0.7)	3 of 3
	10^4	39.0 (2.7)	32.9 (2.8)	6.1 (1.7) †	2 of 3
	10^5	33.0 (1.1)	29.3 (0.9)	3.7 (1.3)	0 of 3
	10^6	29.7 (0.2)	30.1 (0.4)	-0.4 (0.3)	0 of 3
0	**0**	NDT	NDT	0 (NA)	0 of 3
	10^2	NDT	NDT	0 (NA)	0 of 3
	10^4	38.2 (1.8)	39.8 (1.1)	-1.4 (0.8)	0 of 3
	10^5	34.7 (0.8)	34.8 (0.2)	-0.1 (1.0)	0 of 3
	10^6	30.8 (0.3)	31.2 (0.4)	-0.4 (0.3)	0 of 3

***** Average (Ave) and standard deviation (SD) based on triplicate PCR analyses.
** Average (SD) based on two replicates; NDT = Non-detect. Cases where less than 3 of 3 are positive based the requirement that $\Delta C_T \geq 6$ are highlighted and delineated by a heavy border.
† Denotes case where average ΔC_T value exceeds the criterion for positive detection, however individual replicate ΔC_T values were not all ≥ 6.

A replicate experiment was conducted using the conditions that produced data at the border between 3 of 3 positive and < 3 of 3 positive, namely $10^4 – 10^6$ dead cells with 100 live cells and $10^4 – 10^5$ dead cells with 10 live cells. From reference plating, the actual live cells were 150 ± 30 for the 100 live cell level and 15 ± 3 for the 10 live cell level. The experiment was conducted as that described previously and the results are shown in Table 22 for 10-fold DNA extracts. Based on the results of the first two experiments where only 10-fold and 100-fold diluted DNA extracts were analyzed, in this case undiluted extracts were also analyzed while the 100-fold dilutions were

not. The results from the replicate experiment were consistent with those shown in Table 21 however, in this case all replicates for the 10^5 dead cell level with 100 live cells were not positive (although the average $\Delta C_T > 6$), showing ΔC_T values of 7.5, 4.9, and 6.8. The replicate ΔC_T values for the 10^6 dead cell/100 live cell treatment were 11.0, 5.5, and 3.7. For the 10-cell level, the results were consistent with the first replicate experiment except that the 10^4 dead cell/10 live cell treatment showed 3 of 3 positive in this case.

Table 22. RV-PCR Results for 10- and 100-Cell Levels of Live *Y. pestis* Cells With Different IPA-Killed Target Cell Concentrations – YC2 Assay With 10-Fold Diluted DNA Extracts (Replicate Experiment)

Sample Type		Ave C_T (SD) *		Ave ΔC_T (SD)	Positive Replicates
Live Cell Level	Dead Cell Level	T_0	T_{24}		
100	0	NDT**	26.5 (1.6)	17.5 (0.4)**	2 of 2
	10^4	37.2 (1.3)	26.7 (1.8)	10.5 (1.6)	3 of 3
	10^5	32.7 (2.2)	26.3 (1.1)	6.4 (1.4) †	2 of 3
	10^6	31.8 (2.7)	25.1 (1.1)	6.7 (3.8) †	1 of 3
10	0	NDT	27.7 (0.7)	17.3 (0.8)	3 of 3
	10^4	37.1 (0.8)	29.3 (1.0)	7.8 (1.0)	3 of 3
	10^5	33.2 (0.4)	28.3 (0.6)	4.9 (0.5)	0 of 3
0	0	NDT	NDT*	0.0 (NA)	0 of 3
	10^4	35.9 (1.6)	36.4 (0.4)	-0.5 (2.1)**	0 of 2
	10^5	33.8 (0.7)	33.1 (0.7)	0.7 (0.4)	0 of 3
	10^6	29.5 (0.7)	29.2 (0.1)	0.3 (0.7)	0 of 3

* Average (Ave) and standard deviation (SD) based on triplicate PCR analyses.
** Average (SD) based on two replicates; NDT = Non-detect. NDT set to 45 to calculate ΔC_T.
Cases where less than 3 of 3 are positive based the requirement that $\Delta C_T \geq 6$ are highlighted and delineated by a heavy border.
† Denotes case where average ΔC_T value exceeds the criterion for positive detection, however individual replicate ΔC_T values were not all ≥ 6.

The results for analysis of undiluted DNA extracts for the replicate experiment are shown in Table 23. The data between undiluted and 10-fold diluted DNA extracts were similar as expected, although undiluted extracts showed more positive results than 10-fold diluted extracts. It would be advantageous to analyze both undiluted and 10-fold diluted extracts for critical samples—especially where decontamination or natural degradation may have contributed to high dead cell concentrations—to minimize the false negative rate for this analysis. As mentioned, the incubation period could also be extended to 36 hrs to ensure more accurate results. It should also be noted that the negative controls with 0 live cells and high levels of dead cells did not show false positive results (Table 23).

Table 23. RV-PCR Results for 10- and 100-Cell Levels of Live *Y. pestis* Cells With Different IPA-Killed Target Cell Concentrations – YC2 Assay With Undiluted DNA Extracts (Replicate Experiment)

Sample Type		Ave C_T (SD)*		Ave ΔC_T (SD)	Positive Replicates
Live Cell Level	Dead Cell Level	T_0	T_{24}		
100	0	38.3 (1.8)	21.6 (0.6)	16.7 (1.2)	3 of 3
	10^4	33.4 (0.3)	22.5 (0.7)	10.9 (1.1)	3 of 3
	10^5	30.6 (0.9)	23.2 (0.6)	7.4 (0.8)	3 of 3
	10^6	26.4 (0.3)	21.8 (0.9)	4.6 (0.8)	0 of 3
10	0	42.7 (3.3)*	24.3 (0.8)	19.2 (2.5)	3 of 3
	10^4	33.8 (0.4)	26.3 (0.7)	7.5 (0.8)	3 of 3
	10^5	30.3 (0.5)	24.6 (0.6)	5.7 (0.5)	1 of 3
0	0	NDT	NDT**	0 (NA)	0 of 3
	10^4	34.5 (0.3)**	33.5 (0.4)	1.1 (0.3)	0 of 3
	10^5	30.2 (0.6)	29.9 (0.5)	0.4 (0.6)	0 of 3
	10^6	26.2 (0.9)	26.3 (0.5)	-0.1 (0.5)	0 of 3

* Average (Ave) and standard deviation (SD) based on triplicate PCR analyses.

** Average (SD) based on two replicates; NDT = Non-detect. NDT set to 45 to calculate ΔC_T.

Cases where less than 3 of 3 are positive based the requirement that $\Delta C_T \geq 6$ are highlighted and delineated by a heavy border.

5.0 Conclusions

In this effort, an RV-PCR method for detection of viable *Y. pestis* cells from water samples was developed which also served as a model for vegetative cells of other pathogens. The RV-PCR method was developed and optimized by improving procedures for high throughput culturing and DNA extraction/purification. The overall method was then evaluated with regard to detection limit and performance with complex water samples and high concentrations of dead target cells, representing a range of possible real-world sample conditions.

Optimization of culturing procedures included use of YPEB in place of BHI broth for more consistent growth in liquid culture. Experiments showed that 10X YPEB could be added to the water sample to yield a 1X broth concentration, thereby not significantly diluting the growth medium. This broth consistently produced ~4-log cell growth over 24 hrs for starting cell levels of ~10^1 –10^4 CFU/mL. In addition, TBA base (without blood) plates provided more reproducible *Y. pestis* colony growth compared with BHI agar plates to more accurately quantify resulting cell suspensions.

Optimization of DNA extraction and purification protocols included streamlining the existing Promega Magnesil kit procedure used for *B. anthracis* (Gram-positive) for the more readily-lysed *Y. pestis* (Gram-negative) cells; namely, two steps were removed (a lysis buffer wash and alcohol wash step). The modification did not appear to have any negative effect on DNA yields obtained since estimated CFU/mL from real-time PCR results were typically ≥ 0.2-log higher compared with CFU/mL from plate counts. Although PCR cannot be used to determine absolute CFU/mL due to uncertainties in gene copy number per cell or per mass and variability in pipetting, PCR efficiency, among others, the results suggested that cell lysis and DNA recovery were optimal for this application. In addition, chemical lysis using Promega reagents provided more consistent, lower C_T values (more DNA recovery and/or lack of PCR inhibition) compared with heat lysis. With regard to PCR analysis, there were no differences between Fast reagents with Fast cycling conditions and Universal reagents with Standard cycling conditions for the chromosomal assay (YC2), although the Fast/Fast combination showed lower C_T values for the pPCP1 assay (YpP1) compared to the Universal/Standard combination.

The results of this effort indicated that a 24 hr incubation period was optimal for the 10-cell level (10-99 cells) LOD for *Y. pestis*. The same incubation period allowed maintenance of the 10-cell level LOD even in the presence of high levels of insoluble and soluble potential chemical interferences, high concentrations of live, non-target cells and spores, and high concentrations of dead *Y. pestis* cells (10^2 – 10^4). However, for more complex samples or with >10^4 dead *Y. pestis* cell concentration (e.g., post-decontamination or clearance samples), the incubation time may be extended to 36 hrs to maintain the 10-cell level LOD. The RV-PCR method is expected to have an advantage over traditional culture methods since isolated *Y. pestis* colonies may be difficult to detect in samples containing high concentration of non-target cells and spores.

The results from preliminary investigation into methods for cell concentration prior to RV-PCR analysis (included modified filtration and immunomagnetic separation) did not provide an advantage since the cell recovery was poor. In addition, the sample processing time was significantly extended resulting in a longer time to results. Because of these results and the

perceived challenges for operational use, these cell concentration methods (as tested in this effort) are not desirable for a rapid detection method. Other methods to concentrate cells prior to RV-PCR analysis such as ultrafiltration could show better results in terms of LOD and reproducibility.

Together these findings showed that the RV-PCR method could be readily applied to *Y. pestis* in water samples, demonstrating good sensitivity and method performance with complex sample matrices. Though traditional culture methods are still the gold standard, the time to confirmed-results can be 72 hrs or more. In contrast, the RV-PCR method could provide results in less than half this time. Compared to traditional culture methods, the RV-PCR method can also significantly reduce the waste generated and the footprint for analysis. For example, just for growth, RV-PCR uses a single 48-well plate for 48 samples (and controls) compared with the culture method that uses 11 plates, dilution tubes, an enrichment culture tube, and additional plates for isolation and subsequent confirmation by PCR.

Other existing manual or automated DNA extraction platforms can also be employed for RV-PCR analysis. The advantage with the DNA extraction procedure used in this effort is that it minimizes the use of centrifugation. The real-time PCR assays used in this effort consistently demonstrated < 10 genome equivalent LODs; however, other assays in use for detection of *Y. pestis* could be readily integrated into the RV-PCR method as well.

The RV-PCR method for *Y. pestis* can be used as a model for developing methods for additional pathogens of concern including both bioterrorism threats and public health threats. This rapid viability method enhances the capabilities of the ERLN to respond to bioattacks, unintentional, or natural outbreak scenarios. More rapid results with the same or improved accuracy compared to plating methods will aid decision makers in planning decontamination efforts and determining if they are successful, thereby enabling safe, timely restoration and reuse.

6.0 References

Amoako, K.K., M.J. Shields, N. Goji, C. Paquet, M.C. Thomas, T.W. Janzen, C.I.B. Kingombe, A.J. Kell, and K.R. Hahn. 2012. Rapid detection and identification of *Yersinia pestis* from food using immunomagnetic separation and pyrosequencing. *Journal of Pathogens*, 2012: Article ID 781652, 6 pages. doi:10.1155/2012/781652.

Buchrieser, C., C. Rusniok, L. Frangeul, E. Couve, A. Billault, F. Kunst, E. Carniel, and P. Glaser. 1999. The 102-kilobase *pgm* locus of *Yersinia pestis*: Sequence analysis and comparison of selected regions among different *Yersinia pestis* and *Yersinia pseudotuberculosis* strains. *Infection and Immunity*, 67(9):4851-61.

Derbise, A., V. Chenal-Francisque, C.`le Huon, C. Fayolle, C. E. Demeure, B. Chane-Woon-Ming, C. Me´digue, B. J. Hinnebusch, and E. Carniel. 2010. Delineation and analysis of chromosomal regions specifying *Yersinia pestis*. *Infection and Immunity*, 78(9):3930–3941.

Doran, T., Hanes, D., Weagant, S., Torosian, S., Burr, D., Yoshitomi, K., Jinneman, K., Penev, R., Adeyemo, O., Williams-Hill, D., Morin, P. 2013. FERN *Yersinia pestis* Screening Method, SOP No: FERN-MIC.0004.02, Issuing Authority: Food Emergency Response Network (FERN).

US EPA, 2005. Method 415.3 Determination of Total Organic Carbon and Specific UV Absorbance at 254 nm in Source Water. Revision 1.1. EPA/600/R-05/055.

US EPA, 2017. Sampling Guidance for Unknown Contaminants in Drinking Water. EPA-817-R-08-003.

Gilbert, S.E., L.J. Rose, M. Howard, M.D. Bradley, S. Shah, E. Silvestri, F.W. Schaefer III, and J. Noble-Wang. 2014. Evaluation of swabs and transport media for the recovery of *Yersinia pestis*. *Journal of Microbiological Methods*, 96:35-41.

Hernandez, E., M. Girardet, F. Ramisse, D. Vidal, and J.-D. Cavallo. 2003. Antibiotic susceptibilities of 94 isolates of *Yersinia pestis* to 24 antimicrobial agents. *Journal of Antimicrobial Chemotherapy*, 52(6):1029-31.

Himathongkham, S., M.L. Dodd, J.K. Yee, D.K. Lau, R.G. Bryant, A.S. Badoiu, H.K. Lau, L.S. Guthertz, L. Crawford-Miksza, and M.A. Soliman. 2007. Recirculating immunomagnetic separation and optimal enrichment conditions for enhanced detection and recovery of low

levels of *Escherichia coli* O157:H7 from fresh leafy produce and surface water. *Journal of Food Protection*, 70(12):2717-24.

National Research Council (NRC). 1979. Iron. Baltimore, MD, University Park Press.

Rose, L.J., L. Hodges, H. O'Connell, and J. Noble-Wang. 2011. National validation study of a cellulose sponge wipe-processing method for use after sampling *Bacillus anthracis* spores from surfaces. *Applied and Environmental Microbiology,* 77(23):8355-8359.

US EPA. 2012. Protocol for Detection of *Bacillus anthracis* in Environmental Samples During the Remediation Phase of an Anthrax Incident. Cincinnati, Ohio: U.S. Environmental Protection Agency. EPA/ 600/R-12/577.

World Health Organization (WHO). 1996. Guidelines for Drinking-Water Quality, 2nd ed. Vol. 2. Health Criteria and Other Supporting Information. Geneva: World Health Organization. WHO/SDE/WSH/03.04/08.

Annex 1 Standard Operational Procedure
Protocol for Rapid Viability Polymerase Chain Reaction (RV-PCR) for Analysis of *Yersinia pestis* 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 24 hr (to allow *Yersinia pestis* cell propagation prior to DNA extraction and analysis); however, for post-decontamination, field samples (with potentially high concentrations of dead *Y. pestis* cells) the incubation period may be extended to 36 hr to ensure that low concentrations of live cells can be detected in these samples.

Acronyms

BSC ..... Biosafety Cabinet
DI ..... deionized
MOPs ..... 3-(4-morpholino)propane sulfonic acid
PBS ..... phosphate buffered saline
PMPs ..... paramagnetic particles
PES ..... polyethersulfone
RCF ..... relative centrifugal force

Trademarked Products

Trademark	Holder	Location
AeraSeal™	Excel Scientific, Inc.	Victorville, CA
Bacto™	Difco Laboratories	Franklin Lakes, NJ
Biopur® Safe-Lock®	Eppendorf	Hamburg, Germany
Dynamag™	Life Technologies	Carlsbad, CA
MagneSil®	Promega	Madison, WI
MasterPure®	Epicentre Biotechnologies	Madison, WI
Millipore®, Milli-Q™	Millipore Corp.	Billerica, MA
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 + 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" × 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 (DI) 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 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. 187950001 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 (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).

Equipment

- Biological Safety 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)
- 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 micro-centrifuge 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 28–30 °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
- 10X PBS buffer (Teknova Cat. No. P0195 or equivalent)
- Phosphate buffered saline (PBS) buffer (Teknova Cat. No. P0261 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
- TaqMan® Universal PCR Master Mix (Life Technologies, Cat. No. 4304437)
- Primers and probe for YC2 PCR assay targeting a hypothetical gene on the chromosome of *Y. pestis*
    - Forward Primer (YP-EPA-YC2F) – 5'-CAACGACTAGCCAGGCGAC-3'
    - Reverse Primer (YP-EPA-YC2R) – 5'-CATTGTTCGCACGAAACGTAA -3'
    - Probe (YP-EPA-YC2P) – 5'-6FAM-TTTTATAACGATGCCTACAACGGCTCTGCAA-BHQ1-3'
- Primers and probe for YpMT1 targeting a putative F1 operon positive regulatory protein on the pMT1 plasmid of *Y. pestis*
    - Forward Primer (YP-EPA-MT1F) – 5'-GGTAACAGATTCGTGGTTGAAGG-3'
    - Reverse Primer (YP-EPA-MT1R) – 5'-CCCCACGGCAGTATAGGATG-3'
    - Probe (YP-EPA-MT1P) – 5'-6FAM-TCCCTTCTACCCAACAAACCTTTAAAGGACCA-BHQ1-3'
- Primers and probe for YpP1 targeting the *pla* outer membrane protease gene on the pPCP1 plasmid of *Y. pestis*
    - Forward Primer (YP -EPA-YP1F) – 5'-TGGGTTCGGGCACATGATA-3'
    - Reverse Primer (YP -EPA-YP1R) – 5'-CCAGCGTTAATTACGGTACCATAA-3'
    - Probe (YP-EPA-YP1P) – 5'-6FAM-CTTACTTTCCGTGAGAAGACATCCGGCTC-BHQ1-3'

Tryptose Blood Agar (TBA) plates (without blood)

1. Weigh 33 g Bacto™ Tryptic Blood Agar Base powder into 1-L flask or bottle.
2. Add 500 mL MilliQ® H_2O.
3. Place mixture on hotplate and gently mix with spin bar.
4. Autoclave per manufacturer's directions.
5. Place on hotplate and gently mix with spin bar. Allow agar to cool down to 45°C before pouring.
6. Pour 20 mL of solution in each petri dish using a serological pipette. Pouring is performed in a sterile BSC.

1X *Yersinia pestis* Enrichment Broth (1X YPEB)

1. Weigh out the following:
   25 g Bacto Heart Infusion Broth powder
   6 g Yeast extract
   3 g Soytone
   0.5 g Ferric Ammonium Sulfate
   8.77 g MOPS buffering agent
2. Add 500 mL MilliQ H_2O.
3. Place mixture on stir plate and gently mix with spin bar.
4. Bring volume to 1000 mL.
5. Filter-sterilize using 1-L 0.22 µm cellulose acetate filtering system with disposable bottle.

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

1. Weigh out the following:
   125 g Bacto Heart Infusion Broth powder
   30 g Yeast extract
   15 g Soytone
   2.5 g Ferric Ammonium Sulfate
   43.85 g MOPS
2. Add 250 mL MilliQ H_2O.
3. Place mixture on stir plate and gently mix with spin bar.
4. Bring volume to 500 mL.
5. Filter-sterilize using 0.5-L or 1-L 0.22 µm cellulose acetate filtering system with disposable bottle.

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.

Sample Processing and Plating for Water Samples

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 4500 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 48-well plate.
2. Add concentrated growth medium and process for RV-PCR analysis.
   a. Add 333 µL of 10X YPEB to each well of the 48-well plate using a 1000 µL pipettor (Final YPEB ~ 1X). Mix well.
   b. For each well, transfer 500 µL from each 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-lock bag and seal bag.
   c. Incubate 48-well plate at 28ºC on a shaker incubator at 180 rpm for 24 hour (h).
4. Process T_0 aliquot for DNA extraction
   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_{24} (after 24 hr incubation), transfer 500 µL from each sample well to an appropriately labeled 2-mL screw cap tube. Ensure that the T_{24} 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_{24} aliquot for each sample.

Note: For post-decontamination, field samples (with potentially high concentrations of dead Y. pestis cells), the incubation period may be extended to 36 hr.
   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_{24} aliquots by the **DNA Extraction/Purification Procedure** below**.**

RV-PCR Analysis: DNA Extraction/Purification Procedure

Note: T_0 and T_{24} extractions can be completed separately.

1. Thaw T_0 and T_{24} 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_{24} 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_{24} lysate (containing 1 mL sample), hereafter referred to as "T_0 and T_{24} 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_{24} tubes.
7. Vortex each T_0 and T_{24} 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_{24} 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 of the T_0 and T_{24} 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_{24} 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_{24} 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_{24} 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_{24} 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_{24} tube and discard used tips in a sharps container. Recap the tube. Repeat for all T_0 and T_{24} tubes.
16. 2nd Salt Wash: Repeat Salt Wash for all T_0 and T_{24} tubes.
17. 1st Alcohol Wash: Uncap each T_0 and T_{24} 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 of the T_0 and T_{24} tubes, vortex each tube for 5 − 10 seconds (low speed) and place on the magnetic stand. After all the T_0 and T_{24} 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_{24} 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_{24} tubes.
21. 3rd Alcohol Wash: Repeat Alcohol Wash for all T_0 and T_{24} 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_{24} tubes and air dry for 2 minutes.
23. Heat the open T_0 and T_{24} 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_{24} tube, and close tube.
25. Vortex for 10 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_{24} 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_{24} DNA extract tubes "referred to as T_0 and T_{24} 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.

Cleanup Procedure

- Dispose of all biological materials in autoclave bags (double bagged) 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 *Y. pestis* 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_{24} 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 All Selected *Y. pestis* Assays

Reagent	**Volume (µL)**	**Final Concentration**
TaqMan® 2X Universal Master Mix	12.5	1X
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	6.1	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_{24} DNA extracts of each sample. Subtract the average C_T of the T_{24} 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_{24} 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_{24} (ΔC_T) indicates a positive result suggesting the presence of viable *Y. pestis* cells in the sample. A ΔC_T criterion of ≥ 6 (an approximate two log difference in DNA concentration) and a corresponding T_{24} C_T of ≤ 39 was set. If an incubation time longer than 24 hours was used for the RV-PCR, instead of T_{24}, appropriate T_f (incubation time) should be used (i.e., 36 hr for post-decontamination, field samples with high concentrations of dead *Y. pestis* cells). However, (ΔC_T) ≥ 6 algorithm should still be used for a positive result. A minimum of two out of three T_0 PCR replicates must result in C_T values ≤ 44 (in a 45-cycle PCR) to calculate the average T_0 C_T. A minimum of two out of three T_{24} PCR replicates must result in C_T values ≤ 39 to calculate the average C_T for a sample result to be considered positive. Negative controls (No-Template Controls, NTCs) should not yield any measurable C_T values above the background level. If C_T values are obtained as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat analysis. In addition, field blank samples should not yield any measurable C_T values. If C_T values are observed as a result of a possible contamination or cross-contamination, a careful interpretation of the C_T values for the sample DNA extracts and field blanks must be done to determine if the data is considered valid or if the PCR analyses must be repeated.

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

1. Inoculate TBA plates with 100 µL of each sample (each dilution is plated in triplicate).
2. Using one Lazy-L cell spreader 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 TBA plates at 28°C for 3 days.
5. Place sealed sample tubes in a secondary container (re-sealable bag); store tubes at 4°C.
6. After 3 days, confirm growth for *Y. pestis*. Confirm that a subset of the colonies is characterized as *Y. pestis* based on real-time PCR analysis using YC2 *Y. pestis*-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)

This page intentionally left blank

This page intentionally left blank

United States
Environmental Protection
Agency

PRESORTED STANDARD
POSTAGE & FEES PAID
EPA
PERMIT NO. G-35

Office of Research and Development (8101R)
Washington, DC 20460

Official Business
Penalty for Private Use
$300