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Environmental Technology Verification Report

APPLIED BIOSYSTEMS TAQMAN® *E. COLI* O157:H7 DETECTION SYSTEM

Prepared by
Battelle

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Environmental Technology Verification Report

ETV Advanced Monitoring Systems Center

Applied Biosystems TaqMan® *E. coli* O157:H7 Detection System

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Notice

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development, has financially supported and collaborated in the extramural program described here. This document has been peer reviewed by the Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation by the EPA for use.

Foreword

The U.S. Environmental Protection Agency (EPA) is charged by Congress with protecting the nation's air, water, and land resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the EPA's Office of Research and Development provides data and science support that can be used to solve environmental problems and to build the scientific knowledge base needed to manage our ecological resources wisely, to understand how pollutants affect our health, and to prevent or reduce environmental risks.

The Environmental Technology Verification (ETV) Program has been established by the EPA to verify the performance characteristics of innovative environmental technology across all media and to report this objective information to permitters, buyers, and users of the technology, thus substantially accelerating the entrance of new environmental technologies into the marketplace. Verification organizations oversee and report verification activities based on testing and quality assurance protocols developed with input from major stakeholders and customer groups associated with the technology area. ETV consists of six verification centers. Information about each of these centers can be found on the Internet at http://www.epa.gov/etv/.

Effective verifications of monitoring technologies are needed to assess environmental quality and to supply cost and performance data to select the most appropriate technology for that assessment. Under a cooperative agreement, Battelle has received EPA funding to plan, coordinate, and conduct such verification tests for "Advanced Monitoring Systems for Air, Water, and Soil" and report the results to the community at large. Information concerning this specific environmental technology area can be found on the Internet at http://www.epa.gov/etv/centers/center1.html.

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List of Abbreviations

AMS	Advanced Monitoring Systems
ASTM	American Society of Testing and Materials
ATEL	AquaTech Environmental Laboratories, Inc.
ATCC	American Type Culture Collection
BSL	Biosafety Level
Ca	calcium
cfu	colony forming unit
cm	centimeter
DI	deionized water
DNA	deoxyribonucleic acid
DW	drinking water
EPA	U.S. Environmental Protection Agency
ETV	Environmental Technology Verification
ID	identification
IPC	internal positive control
L	liter
LOD	limit of detection
MB	method blank
Mg	magnesium
mg	milligram
µL	microliter
mL	milliliter
MWD	Metropolitan Water District
NAC	No Amplification Control
NTC	No Template Control
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PT	performance test
QA	quality assurance
QC	quality control
QMP	Quality Management Plan
SOP	standard operating procedure
TSA	technical systems audit

Chapter 1
Background

The U.S. Environmental Protection Agency (EPA) supports the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative environmental technologies through performance verification and dissemination of information. The goal of the ETV Program is to further environmental protection by accelerating the acceptance and use of improved and cost-effective technologies. ETV seeks to achieve this goal by providing high-quality, peer-reviewed data on technology performance to those involved in the design, distribution, financing, permitting, purchase, and use of environmental technologies.

ETV works in partnership with recognized testing organizations; with stakeholder groups consisting of buyers, vendor organizations, and permitters; and with the full participation of individual technology developers. The program evaluates the performance of innovative technologies by developing test plans that are responsive to the needs of stakeholders, conducting field or laboratory tests (as appropriate), collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure that data of known and adequate quality are generated and that the results are defensible.

The EPA's National Exposure Research Laboratory and its verification organization partner, Battelle, operate the Advanced Monitoring Systems (AMS) Center under ETV. The AMS Center recently evaluated the performance of Applied Biosystems' TaqMan® *E. coli* O157:H7 Detection System for the detection of *Escherichia coli* O157:H7 (*E. coli*).

Chapter 2
Technology Description

The objective of the ETV AMS Center is to verify the performance characteristics of environmental monitoring technologies for air, water, and soil. This verification report provides results for the verification testing of the TaqMan® *E. coli* O157:H7 Detection System, which includes the TaqMan® *E. coli* O157:H7 Detection Kit, the PrepMan™ Ultra Sample Preparation Reagent, and the ABI Prism® 7000 Sequence Detection System and associated software. The following is a description of the TaqMan® *E. coli* O157:H7 Detection System based on information provided by the vendor. The information provided below was not subjected to verification in this test.

The TaqMan® *E. coli* O157:H7 Detection Kit is part of an integrated system that includes polymerase chain reaction (PCR) chemistry, instrumentation, and data analysis software. Each TaqMan® Detection Kit contains TaqMan® probes and primers in the PCR mix along with $MgCl_2$, dNTPs, and an internal positive control (IPC) system. The ABI Prism® 7000 Sequence Detection System is a real-time PCR fluorescence detection instrument that combines a thermal cycler with an optical detection system. During amplification, it can distinguish between the fluorogenic labels for the assay target (i.e., *E. coli* O157:H7) and an IPC. The ABI Prism® 7000 uses a microplate capable of running 96 samples. The PrepMan™ Ultra Sample Preparation Reagent removes the inhibitory and interfering substances that potentially affect PCR amplification and lead to inconclusive results.

Figure 2-1. Applied Biosystems' ABI Prism® 7000 Sequence Detection System

The basis for TaqMan® pathogen detection assays is the specific amplification of a deoxyribonucleic acid (DNA) target located within a bacterial genome and unique to the target organism. AmpliTaq Gold® DNA polymerase, which is used exclusively in the detection kit, prevents the formation of non-specific PCR products that decrease performance of the enzyme and assay. A fluorogenic signal is not generated unless both the PCR primers and the 5' nuclease probe hybridize to the PCR target. The Sequence Detection System software offers an Absolute Quantitation (real-time) and Plus/Minus (endpoint) assay for PCR and data analysis. The Absolute Quantitation assay can provide qualitative and quantitative information, while the Plus/Minus assay provides a "yes/no" result for each sample. The Absolute Quantitation assay was used in this test.

All three components of the TaqMan® *E. coli* O157:H7 Detection System were used in this verification test for the analysis of test samples. The ABI Prism® 7000 Sequence Detection System, excluding the computer, measures approximately 20 inches [51 centimeters (cm)] deep by 21 inches (53 cm) high by 15 inches (39 cm) wide, and weighs 75 pounds (34 kilograms). The ABI Prism® 7000 Sequence Detection System costs $47,250. The T ® *E. coli* O157:H7 Detection Kit costs $800 for approximately 100 assays, and the PrepMan™ Ultra Sample Preparation Reagent costs $100 for 50 to 100 sample DNA extractions.

Chapter 3
Test Design and Procedures

3.1 Introduction

The purpose of this verification test of rapid PCR technologies was to evaluate the ability of these technologies to detect the presence of specific bacteria in water and to determine the technologies' performance when specific interferents were added to pure water and when interferents were inherently present in several drinking water matrices. The technologies for this verification test operate based on the PCR process, which involves enzyme-mediated reactions that allow for target DNA (that from the bacteria of interest) replication and amplification through a series of temperature cycles. Before the target DNA can be amplified, however, it must first be extracted from the bacteria and then purified.

Because rapid PCR technologies are anticipated to serve mostly as screening tools in water monitoring scenarios, providing rapid results as to whether or not a pathogen or biological agent is present in the water, this verification test involved only qualitative results. This verification test of the TaqMan® *E. coli* O157:H7 Detection System was conducted according to procedures specified in the *Test/QA Plan for Verification of Rapid PCR Technologies*.[1] The performance of the TaqMan® *E. coli* O157:H7 Detection System was verified in terms of the following parameters:

- Accuracy
- Specificity
- False positive/negative responses
- Precision
- Interferences
- Other performance factors.

The performance of the TaqMan® *E. coli* O157:H7 Detection System was verified by challenging it with various concentration levels of *E. coli* O157:H7 in American Society of Testing and Materials (ASTM) Type II deionized (DI) water, ASTM Type II DI water spiked with various interferents, and concentrated drinking water (DW) samples obtained from four water utilities from different geographical locations in the United States. Each source of DW represented a unique water treatment process. In addition, the interferent and DW samples were analyzed without adding any contaminant to evaluate the potential for false positive results.

Contaminant concentrations included the infective/lethal dose concentration given in Table 3-1 for *E. coli* and approximately 2, 5, 10, and 50 times the vendor-reported system limit of detection (LOD) for this technology. The infective/lethal dose of *E. coli* was determined by calculating the concentration at which ingestion of 250 milliliters (mL) of water is likely to cause the death of a 70-kilogram (approximately 154 pounds) person based on human LD_{50} or ID_{50} data.[2] The results from quadruplicate analysis of the contaminant performance test (PT) samples and comparison with the known sample compositions provided information on the accuracy and precision of the TaqMan® *E. coli* O157:H7 Detection System. The interferent PT samples contained humic and fulvic acids at two concentrations, both spiked and unspiked with *E. coli*. Each was analyzed in quadruplicate and provided information on potential matrix interferences. As necessary, additional concentration levels were analyzed to more thoroughly evaluate the performance of the TaqMan® *E. coli* O157:H7 Detection System.

For the purposes of this test, 10 colony forming units (cfu)/mL were used to calculate the concentration levels spiked in the PT samples per the original test/QA plan design. This vendor-provided concentration level was anticipated to be the level at which quantifiably reproducible positive results could be obtained from a raw water sample using the TaqMan® *E. coli* O157:H7 Detection System. This concentration level is referred to in this report as the "system LOD." The system LOD incorporates the sensitivities and uncertainties of the entire TaqMan® *E. coli* O157:H7 Detection System, in particular the PrepMan™ Ultra Sample Preparation Reagent, as well as the TaqMan® *E. coli* O157:H7 Detection Kit itself; and, as such, the system LOD is a method detection limit rather than an instrument or reagent-specific detection limit. As mentioned previously, the system LOD provided by the vendor was used specifically as a guideline in calculating sample concentration ranges per the original test/QA plan (2, 5, 10, and 50 times the system LOD for PT samples) for use with the TaqMan® *E. coli* O157:H7 Detection System in this verification test. It should be noted that the concentration level provided by the vendor was one that was known for the system in detecting *E. coli* in pre-enriched food samples (see Section 6.1). Applied Biosystems does not claim that this is the true LOD of the TaqMan® *E. coli* O157:H7 Detection System. Detection limits for individual components of the TaqMan® E. *coli* O157:H7 Detection System and the system as a whole may differ and were not verified in this test.

The verification test was conducted at Battelle from June 2, 2004, through June 24, 2004. Aqua Tech Environmental Laboratories, Inc. (ATEL) of Marion, Ohio, performed physicochemical characterization for each DW sample, including turbidity, dissolved and total organic carbon, specific conductivity, alkalinity, pH, magnesium (Mg), calcium (Ca), hardness, total organic halides, trihalomethanes, and haloacetic acids. Battelle cultured the bacteria, provided the stock solutions used in this test, and then confirmed the presence and quantity of *E. coli* bacteria in the stock solutions using plate enumeration. The stock solutions of *E. coli* were stored frozen as 1 mL aliquots. A new 1 mL vial of stock solution was thawed and used for each day of testing. All test samples were prepared from the stock solutions on the day of analysis. All purified DNA was used the same day it was extracted and purified. Each set of replicates for a sample came from the same batch of purified DNA.

Table 3-1. Infective/Lethal Dose of Target Contaminant

Contaminant	Infective/Lethal Dose Concentration
E. coli O157:H7	0.2 cfu/mL

3.2 Test Samples

Test samples used in this verification test included PT samples, DW samples, and quality control (QC) samples. Each type of test sample, including QC samples, is described further below.

3.2.1 Performance Test Samples

Table 3-2 lists the PT samples analyzed in this verification test for *E. coli*. PT samples were prepared in ASTM Type II DI water. The first type of PT sample consisted of ASTM Type II DI water spiked at various concentration levels of *E. coli.* In accordance with the test/QA plan, contaminant PT samples with concentrations ranging from the infective/lethal dose concentration to 50 times the vendor-stated system LOD were analyzed using the TaqMan® *E. coli* O157:H7 Detection System. The infective/lethal dose concentration was analyzed to document the response of the TaqMan® *E. coli* O157:H7 Detection System at that important concentration level. Four concentration levels at 2, 5, 10, and 50 times the vendor-reported system LOD in addition to the infective/lethal dose concentration were analyzed. Each concentration level for the PT samples was analyzed in quadruplicate. Preliminary data for these samples indicated that *E. coli* was not detectable or that results were highly inconsistent at these levels. To more thoroughly assess the system and determine the best contaminant level to use in the interferent PT and DW samples, a dilution series of contaminant-only PT samples was analyzed, ranging from $1x10^6$ cfu/mL to 10 cfu/mL of *E. coli* in order of magnitude increments. This testing and the subsequent results are fully described in Section 6.1.

Table 3-2. Performance Test Samples

Type of PT Sample	Sample Characteristics	Approximate Concentrations (cfu/mL)
Contaminant-only	*E. coli* in DI water	0.2 to 1×10^6
Interferent	*E. coli* in 0.5 milligram per liter (mg/L) humic acid and 0.5 mg/L fulvic acid	1×10^4
	E. coli in 2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^4

The second type of PT sample was potential interferent samples. Four replicates of each interferent PT sample were analyzed to determine the performance of the TaqMan® *E. coli* O157:H7 Detection System in the presence of humic and fulvic acids. The interferent PT

samples contained humic and fulvic acids isolated from Elliot Soil near Joliett, IL, (obtained from the International Humic Substances Society) spiked into ASTM Type II DI water. Each of these interference mixtures was prepared at two concentration levels. One concentration was near the upper limit of what would be expected in DW (5 mg/L) and one was at a mid-low range of what would be expected (1 mg/L). The 1 mg/L interferent mixture was prepared as 0.5 mg/L humic acid and 0.5 mg/L fulvic acid. Similarly, the 5 mg/L interferent solution was prepared as 2.5 mg/L humic acid and 2.5 mg/L fulvic acid. These interferent levels were confirmed through analysis of aliquots by ATEL. Also, *E. coli* was added to these samples, along with the potential interferent, at a concentration level (1×10^4 cfu/mL) as determined in the dilution series analysis described above. This concentration level was set at approximately 10 times the lowest level in the dilution series where consistent results were obtained for all replicates. The samples were analyzed in quadruplicate.

In all cases, four replicates for each PT sample, DW sample, and QC sample were taken from the extracted and purified product (unspiked) or DNA (spiked) of one sample solution. That is, only one spiked or unspiked sample solution was prepared for each set of replicates and taken through the DNA extraction and purification procedure. Four replicates were then taken from the same purified product or DNA. In an effort to characterize the efficacy of the extraction and purification procedure in the presence of inhibitory substances (humic and fulvic acids), four solutions of humic and fulvic acids each at 0.5 mg/L spiked with *E. coli* at $1x10^4$ cfu/mL were prepared in addition to the samples listed in Table 3-2. Each solution was put through the DNA extraction and isolation procedure, and then four replicates from each of the four purified DNA solutions were analyzed on the TaqMan® *E. coli* O157:H7 Detection System.

3.2.2 Drinking Water Samples

Table 3-3 lists the DW samples analyzed for *E.coli* in this test. DW samples were collected from four geographically distributed municipal sources (Ohio, California, Florida, and New York) to evaluate the performance of the TaqMan® *E. coli* O157:H7 Detection System with various sample matrices. These samples varied in their source and treatment and disinfection process. All samples had undergone either chlorination or chloramination prior to receipt. Samples were collected from utility systems with the following treatment and source characteristics:

- Chlorinated filtered surface water
- Chloraminated filtered surface water
- Chlorinated filtered groundwater
- Chlorinated unfiltered surface water.

All samples were collected in pre-cleaned high density polyethylene containers. After sample collection, to characterize the DW matrix, an aliquot of each DW sample was sent to ATEL to determine the following water quality parameters: turbidity, organic carbon, conductivity, alkalinity, pH, Ca, Mg, hardness, total organic halides, concentration of trihalomethanes, and haloacetic acids. The DW samples were dechlorinated with sodium thiosulfate pentahydrate to prevent the degradation of some of the contaminants by chlorine. Because real-world applications of PCR technologies to screen water samples rely on pre-concentration of the water

sample to be analyzed, approximately 100 L of each of the above sources of DW were dechlorinated and then concentrated through ultrafiltration techniques to a final volume of 250 mL by the Metropolitan Water District (MWD) of Southern California. As shown in Table 3-3, each DW sample was analyzed without adding any contaminant (i.e., unspiked), as well as after fortification with *E. coli* at a single concentration level (the same as determined for the interferent PT samples).

Table 3-3. Drinking Water Samples

Drinking Water Sample Description			Approximate Contaminant Concentrations (cfu/mL)
Water Utility	**Water Treatment**	**Source Type**	***E. coli***
Columbus, Ohio (OH)	chlorinated filtered	surface	unspiked and 1×10^4
MWD of Southern California (CA)	chloraminated filtered	surface	unspiked and 1×10^4
Orlando, Florida (FL)	chlorinated filtered	ground	unspiked and 1×10^4
New York City, New York (NY)	chlorinated unfiltered	surface	unspiked and 1×10^4

3.2.3 Quality Control Samples

QC samples included method blank (MB) samples consisting of ASTM Type II DI water and positive and negative controls, as provided by the vendor. A positive control reaction was also analyzed in quadruplicate with each batch of samples in a given day. All of the MB QC samples were exposed to sample preparation and analysis procedures identical to the test samples. The positive control reaction sample was simply DI water spiked with *E. coli* at 1×10^6 cfu/mL, per the PrepMan™ Ultra Sample Preparation Reagent protocol. External positive and negative controls were prepared and used according to the protocol provided by the vendor. At least two positive controls and 12 negative controls were prepared with each 96-well plate of samples placed in the ABI Prism® 7000. The negative controls consisted of six No Amplification Control (NAC) reactions and six No Template Control (NTC) reactions. The MB samples were used to confirm negative responses in the absence of any contaminant and to ensure that no sources of contamination were introduced into handling and analysis procedures. At least 10% of the test samples (eight replicates) were MB samples. The vendor-provided control samples indicated to the technician whether the TaqMan® *E. coli* O157:H7 Detection System was functioning properly. If the controls failed for any reason, that batch of samples would be discarded and the extracts re-analyzed. To the extent practicable, the test samples were analyzed blindly by having the technician label the vials with only a sample number prior to the DNA purification step, such

that the samples were tracked through the purification, PCR, and detection steps by only a sample number.

3.3 Reference Methods

3.3.1 Plate Enumeration

Plate enumeration was used to quantify *E. coli* to confirm the concentration of the aliquoted stock solution of this contaminant. The Battelle standard operating procedure (SOP) followed was SOP No. MREF X-054, *Standard Operating Procedure (SOP) for the Enumeration of BSL-2 and BSL-3 Bacteria Samples Via the Spread Plate Technique.*

Prior to testing, the *E. coli* were grown and then suspended in phosphate buffered saline (PBS). Twenty-five or more individual 1 mL aliquots of stock solution were prepared from the original PBS stock solution. Three 1 mL aliquots were randomly taken for enumeration, while the others were frozen for later use in sample preparation. The enumerations were done on each of the three selected 1 mL aliquot to confirm the determined concentration.

3.3.2 Drinking W

Because *E. coli* can occur naturally in water, and because rapid PCR technologies cannot distinguish between live and dead organisms, each unspiked concentrated DW sample was plate enumerated to verify, to the extent practicable, the presence or absence of the contaminant of interest. The samples were plated onto tryptic soy agar plates with 5% sheep blood and incubated at 30 to 35°C. After 20 hours of incubation, the unspiked OH, CA, and NY DW samples produced lawns of bacteria with a level of contamination estimated to be greater than 1×10^3 cfu/mL. The unspiked FL DW sample showed only 10 to 100 cfu/mL estimated concentration levels after 20 hours. After further incubation, the FL DW sample produced bacteria at a concentration estimated to be greater than 1×10^3 cfu/mL. Each DW sample had at least three distinct types of bacteria growing. Gram stains were performed on any distinct colony types visible in each sample to gain further insight into the colony morphology. For OH and CA DW, three Gram negative bacteria colonies were identified. For NY, four Gram negative colonies were identified; and, for FL, both Gram negative and positive colonies were present. Because no confirmed positive responses for *E. coli* were detected in any of the unspiked DW samples, further identification tests were not conducted.

3.4 Test Procedure

3.4.1 Sample Handling

All testing for this verification test was conducted within Battelle laboratories staffed with technicians trained to safely handle *E. coli* bacteria. The technician operating the TaqMan® *E. coli* O157:H7 Detection System had prior PCR experience. *E. coli* samples were tested in a

Biosafety Level 2 (BSL-2) laboratory. Appropriate safety guidelines associated with the laboratory were followed throughout the verification test. Each day, fresh samples were prepared in either DI water, an interferent matrix, or a DW matrix from a thawed aliquot of frozen stock solution. Concentration levels for spiked samples from the original test/QA plan design at various multiples of the TaqMan® *E. coli* O157:H7 Detection System's LOD (2, 5, 10, and 50 times the system LOD for PT samples) were calculated from the system LOD provided by the vendor. Sample solutions were prepared to these concentrations, and other concentrations used in the verification test, based on the concentration of the bacteria stock solution, which was determined through triplicate plate enumeration prior to testing. Each sample was prepared in its own container and labeled only with a sample identification (ID) number that also was recorded in a laboratory record book along with details of the sample preparation. Samples were diluted to the appropriate concentration using volumetric pipettes and glassware. Each sample was prepared in 1 mL quantities. The entire 1 mL quantity was taken through the DNA extraction and purification step. For the dilution series contaminant-only PT samples used in the expanded testing discussed in Section 3.2.1, 900 microliters (µL) of each sample from $1x10^6$ cfu/mL to 100 cfu/mL were used to accommodate the processing of the order of magnitude dilution samples and ensure that each sample was within the series.

Despite rigorous sample preparation efforts, solutions consisting of low bacterial concentrations, such as the *E. coli* infective/lethal dose, may have no DNA present in a given sample or aliquot.[3,4] The rationale for this is based on the Poisson statistical distribution, where there is some probability that a sample taken will contain no particles (i.e., bacteria or target DNA) and thus yield a negative result.[3,4] As a practical example, assume that 1 mL contains exactly five particles (i.e., bacteria or target DNA) of interest. If one takes ten 0.1 mL samples and analyzes them, the maximum number of positives will be five out of the ten samples. From this it follows that there will be at least five negatives. Random variation in the sampling will cause this ratio to change. This verification test was not designed to differentiate between the stochastic nature of the low concentration samples and the capabilities of the assays, but this phenomenon should be noted.

3.4.2 Sample Preparation and Analysis

Three steps were carried out to test a liquid sample for the presence of *E. coli* bacteria: (1) DNA extraction using the PrepMan™ Ultra Sample Preparation Reagent, (2) PCR setup using the TaqMan® *E. coli* O157:H7 Detection Kit, and (3) PCR and analysis using the ABI Prism® 7000 Sequence Detection System and software. To perform these steps, the laboratory work area was separated into three distinct areas: DNA extraction and plating the purified DNA onto the 96-well plate was done in one area; preparing the Pathogen PCR Cocktail and putting it on the 96-well plate was performed in a separate, DNA-free area; and loading the instrument (the ABI Prism® 7000) was done in another area. These steps are described below.

First, the DNA was isolated and purified from the sample. The entire 1 mL sample was taken through this isolation procedure. The PrepMan™ Ultra Sample Preparation Reagent general extraction procedures for pathogens from food samples were followed. This procedure calls for a pre-enrichment step that was not followed because the samples used in this test were already

prepared to be at levels specified by the vendor (or determined through additional testing) to be detectable. After the extraction step was complete, the PCR reaction setup using the TaqMan® *E. coli* O157:H7 Detection Kit was performed in the DNA-free area. This process involved first making the Pathogen PCR Cocktail, which consists of the TaqMan *E. coli* O157:H7 mix and the AmpliTaq Gold® DNA Polymerase (as provided in the TaqMan® *E. coli* O157:H7 Detection Kit). Appropriate quantities of the PCR Cocktail were prepared for each day of testing as it should be made within one hour of use. A volume of 45 µL of PCR Cocktail mix was added to the wells on a 96-well plate to be used for samples, positive controls, and NTCs. A volume of 44 µL of TaqMan *E. coli* O157:H7 mix only was added to the six NAC wells. A volume of 5 µL of Negative Control was then added to the NTC wells, and the wells were capped with optical caps. The remaining wells were lightly capped and moved to another area.

In a separate area, 5 µL of purified DNA were added to the appropriate sample wells and then those wells were capped. A volume of 5 µL of Positive Control was added to the two positive control wells and 5 µL of Negative Control were added to each NAC well. Once all wells were filled and capped, the 96-well PCR plate was spun down to ensure that everything was in solution and to remove any bubbles from the bottom of the well. The plate was then loaded onto the ABI Prism® 7000 Sequence Detection System per the instructions provided in the TaqMan® Pathogen Detection Kits User Manual with the following vendor-made modifications: an Absolute Quantitation Assay (a real-time assay) instead of a Plus/Minus Assay (an endpoint assay) was used, a VIC detector (fluorescent reporter dye) was used for the IPC in the Sequence Detection System software (version 1.0), and no pre-read or post-read procedures were conducted. A FAM detector (fluorescent reporter dye) was used for the sample wells. After the instrument had completed its PCR program run, which consisted of 40 cycles on the thermal cycler, the results were analyzed using the Sequence Detection System 7000 software. The resulting amplification plots (plots of relative fluorescence units versus the thermal cycler cycle number) were used to determine the results for each sample. Whether the sample was positive or negative was based on the sample fluorescence crossing a set threshold. A default threshold of 0.2 ΔR_n (relative unit of fluorescence proportional to the amplification of the target template) was set by the software for each PCR run. The threshold is defined as a point above the mean background fluorescence signal present in all wells on the plate for a particular PCR run. As needed, this threshold value can be adjusted by the operator to be more specific to the data being analyzed. The adjustment of the threshold is based on the operator's experience with and understanding of PCR amplification plots. The threshold was set based on the location of the exponential phase data (amplification curves) to fall within the linear portion of the plots and generally be set above the noise in the amplification plot. For this test, the default threshold value of 0.2 ΔR_n was appropriate and was used with the FAM detector for all sample wells. Any amplification for a given sample above this threshold indicated a positive result (*E. coli* is present in the sample) and was assigned a C_t value or threshold cycle value by the software. The C_t value is the fractional cycle number where the exponential phase fluorescence or amplification curve crosses the threshold. When the amplification did not cross the threshold, the sample was considered negative (no *E. coli* detected in the sample) and no C_t value was assigned by the software. The negative controls were considered successful if no amplification on the FAM detector was present. The positive controls were considered successful if amplification was noted in the FAM detector. The IPC was monitored using the VIC detector

screen to ensure that amplification of the IPC was present in each sample. As with the FAM detector, a default threshold of 0.2 ΔR_n was set by the software for the IPC determination. This threshold was incorrect for the IPC amplification results and was set to 0.08 ΔR_n in all cases for the determination of IPC amplification. The technician recorded the sample identification number on a sample data sheet along with the qualitative results (positive or negative) for each sample.

3.4.3 Drinking Water Characterization

An aliquot of each DW sample, collected as described in Section 3.2.2, was sent to ATEL prior to concentration to determine the following water quality parameters: turbidity; concentration of dissolved and total organic carbon; conductivity; alkalinity; pH; concentration of Ca and Mg; hardness; and concentration of total organic halides, trihalomethanes, and haloacetic acids. Table 3-4 lists the methods used to characterize the DW samples, as well as the characterization data from the four water samples used in this verification test. Water samples were collected and water quality parameters were measured by ATEL in January 2004. Some of the water quality parameters may have changed slightly prior to verification testing.

Table 3-4. ATEL Water Quality Characterization of Drinking Water Samples

			Sources of Drinking Water Samples			
Parameter	**Unit**	**Method**	**Columbus, Ohio (OH DW)**	**MWD, California (CA DW)**	**Orlando, Florida (FL DW)**	**New York City, New York (NY DW)**
Turbidity	NTU	EPA 180.1[5]	0.2	0.1	0.5	1.3
Dissolved organic carbon	mg/L	SM 5310[6]	1.9	2.3	1.7	1.5
Total organic carbon	mg/L	SM 5310[6]	1.6	2.1	1.8	2.1
Specific conductivity	microSiemens	SM 2510[6]	357	740	325	85
Alkalinity	mg/L	SM 2320[6]	55	90	124	4
pH		EPA 150.1[7]	7.33	7.91	7.93	6.80
Ca	mg/L	EPA 200.8[8]	42	35	41	5.7
Mg	mg/L	EPA 200.8[8]	5.9	1.5	8.4	19
Hardness	mg/L	EPA 130.2[7]	125	161	137	28
Total organic halides	µg/L	SM 5320[6]	360	370	370	310
Trihalomethanes	µg/L/analyte	EPA 524.2[9]	26.9	79.7	80.9	38.4
Haloacetic acids	µg/L/analyte	EPA 552.2[10]	23.2	17.6	41.1	40.3

NTU = nephelometric turbidity unit
µg = microgram

Chapter 4
Quality Assurance/Quality Control

Quality assurance/quality control procedures were performed in accordance with the quality management plan (QMP) for the AMS Center[11] and the test/QA plan for this verification test.[1]

4.1 Sample Chain-of Custody Procedures

Sample custody was documented throughout collection, shipping, and analysis of the samples. Sample chain-of-custody procedures were generally those provided in the guidelines in ASAT.II-007, *Standard Operating Procedure for Chain of Custody for Dioxin/Furan Analysis*. The chain-of-custody forms summarized the samples collected and analyses requested and were signed by the person relinquishing samples once that person had verified that the custody forms were accurate. The original sample custody forms accompanied the samples; the shipper kept a copy. Upon receipt at the sample destination, sample custody forms were signed by the person receiving the samples once that person had verified that all samples identified on the custody forms were present in the shipping container.

4.2 Equipment Calibration

The TaqMan® *E. coli* O157:H7 Detection System, and all associated reagents and supplies specific for the detection of *E. coli* were provided to Battelle by the vendor. This system required no calibration. The performance of the system was monitored through positive controls, IPC, and negative controls. For DW characterization and confirmation of the possible interferent, analytical equipment was calibrated by ATEL according to the procedures specified in the appropriate standard methods. Pipettes used during the verification test were calibrated according to Battelle SOP VI-025, *Operation, Calibration, and Maintaining Fixed and Adjustable Volume Pipettes.*

4.3 Characterization of Contaminant Stock Solution

E. coli was grown and prepared by Battelle. The bacteria were plate enumerated in triplicate for confirmation of the concentration of the 1 mL aliquots of stock solution. The Battelle SOP No. MREF X-054, *Standard Operating Procedure (SOP) for the Enumeration of BSL-2 and BSL-3 Bacteria Samples Via the Spread Plate Technique* were followed for the plate enumerations of

E. coli. The results of the plate enumerations are presented in Table 4-1. The plate enumeration was conducted prior to testing. The average of triplicate enumerations was used to calculate and prepare all spiked sample solutions.

Table 4-1. *E. coli* Triplicate Plate Enumeration Data

Bacteria	Plate 1 Concentration (cfu/mL)	Plate 2 Concentration (cfu/mL)	Plate 3 Concentration (cfu/mL)	Average (cfu/mL)	Relative Standard Deviation
E. coli	7.3×10^8	5.0×10^8	9.0×10^8	7.1×10^8	28%

4.4 Quality Control Samples

MB samples consisting of ASTM Type II DI water, and positive and negative control (NTC and NAC) samples, as provided in the TaqMan® *E. coli* O157:H7 Detection System, were analyzed to help identify potential cross-contamination issues as well as verify that the PCR process was functioning properly. Positive control reaction samples as prepared by the Battelle technician were also analyzed as part of each day of testing. Positive control reaction samples were DI water samples spiked with *E. coli* at 1×10^6 cfu/mL and put through the same analysis procedures (DNA extraction, PCR setup, etc.) as regular samples. These positive control reaction samples were also handled blindly, as the regular test samples were. IPCs were part of each sample that was analyzed and provided further checks on the performance of the system, especially in identifying the presence of potential inhibitory substances. Two positive control samples, 12 negative control samples (six NTC and six NAC), and four positive control reaction samples were run with each set of samples placed on the 96-well plate. Eight MB replicates were analyzed over the course of the verification test.

All eight MB replicates for this verification test returned negative results. All positive control reaction samples returned positive results. A threshold of approximately 0.8 ΔR_n was chosen for the IPC samples based on the amplification plots. IPC peaks were present in all samples except for one positive control, one positive control reaction replicate, and one unspiked NY DW replicate. The suppression of the IPC in the unspiked NY DW likely indicates the presence of inhibitors in the sample. For the positive control samples, all other IPCs within the sample set produced clear amplification, including all other positive controls for that plate. No IPC amplification was present in any NAC samples, as expected. No positive or negative controls failed.

4.5 Audits

4.5.1 Technical Systems Audit

The Battelle Quality Manager conducted a technical systems audit (TSA) on June 23, 2004, to ensure that the verification test was performed in accordance with the test/QA plan[1] and the AMS Center QMP.[11] As part of the audit, the Battelle Quality Manager reviewed the standards and methods used, compared actual test procedures to those specified in the test/QA plan, and reviewed data acquisition and handling procedures. Observations and findings from this audit were documented and submitted to the Verification Test Coordinator for response. No findings were documented that required any significant action. The records concerning the TSA are stored for at least seven years with the Battelle Quality Manager.

4.5.2 Audit of Data Quality

At least 10% of the data acquired during the verification test was audited. Battelle's Quality Manager traced the data from the initial acquisition, through reduction and statistical analysis, to final reporting, to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

4.6 QA/QC Reporting

Each assessment and audit was documented in accordance with Sections 3.3.4 and 3.3.5 of the QMP for the ETV AMS Center.[11] Once the assessment report was prepared, the Verification Test Coordinator ensured that a response was provided for each adverse finding or potential problem and implemented any necessary follow-up corrective action. The Battelle Quality Manager ensured that follow-up corrective action was taken. The results of the TSA were sent to the EPA.

4.7 Data Review

Records generated in the verification test were reviewed before these records were used to calculate, evaluate, or report verification results. Table 4-2 summarizes the types of data recorded. The review was performed by a Battelle technical staff member involved in the verification test, but not the staff member that originally generated the record. The person performing the review added his/her initials and the date to a hard copy of the record being reviewed.

Table 4-2. Data Recording Process

Data to Be Recorded	Where Recorded	How Often Recorded	Disposition of Data[a]
Dates and times of test events	ETV data sheets	Start/end of test and at each change of a test parameter	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
Sample collection and preparation information, including chain-of-custody	ETV data sheets and chain-of-custody forms	At time of sample collection and preparation	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
TaqMan® *E. coli* O157:H7 Detection System procedures and sample results	ETV data sheets and data acquisition system	Throughout test duration	Manually incorporated in data spreadsheets
Enumeration data	Enumeration data forms and ETV data sheets	With every enumeration	Used to organize/check test results
Reference method procedures and sample results	Data acquisition system, as appropriate	Throughout sample analysis process	Transferred to spreadsheets

[a] All activities subsequent to data recording were carried out by Battelle, except for the reference method analyses (DW characterization), which were carried out by ATEL.

Chapter 5
Data Analysis

The TaqMan® *E. coli* O157:H7 Detection System was evaluated for qualitative results (i.e., positive/negative responses to samples) based on the expected application of rapid PCR technologies as rapid screening tools. All data analyses were based on these qualitative results. QC and MB samples were not included in any of the analyses.

5.1 Accuracy

Accuracy was assessed by evaluating how often the TaqMan® *E. coli* O157:H7 Detection System results were positive in the presence of a concentration of contaminant above the system LOD. Contaminant-only PT samples were used for this analysis. An overall percent agreement was determined by dividing the number of positive responses by the overall number of analyses of contaminant-only PT samples above the system LOD.

5.2 Specificity

The ability of the TaqMan® *E. coli* O157:H7 Detection System to provide a negative response when the contaminant was absent was assessed. The specificity rate was determined by dividing the number of negative responses by the total number of unspiked samples.

5.3 False Positive/Negative Responses

A false positive response was defined as a detectable or positive TaqMan® *E. coli* O157:H7 Detection System response when the ASTM Type II DI water (including interferent samples) or DW samples were not spiked. A false positive rate was reported as the frequency of false positive results out of the total number of unspiked samples.

A false negative response was defined as a non-detectable response or negative response when the sample was spiked with a contaminant at a concentration greater than the system LOD. Spiked PT (contaminant and interferent) samples and spiked DW samples were included in the analysis. A false negative rate was evaluated as the frequency of false negative results out of the total number of spiked samples for a particular contaminant.

5.4 Precision

The precision of the four replicates of each sample set were assessed. Responses were considered consistent if all four replicates gave the same result. The precision of the TaqMan® *E. coli* O157:H7 Detection System was assessed by calculating the overall number of consistent responses for all the sample sets.

5.5 Interferences

The potential effect of the DW matrix on the TaqMan® *E. coli* O157:H7 Detection System performance was evaluated qualitatively by comparing the results for the spiked and unspiked DW samples to those for the PT samples. Similarly, the potential effect of interferent PT samples containing humic and fulvic acids at two levels, both spiked and not spiked with bacteria, were evaluated.

5.6 Other Performance Factors

Aspects of the TaqMan® *E. coli* O157:H7 Detection System performance such as ease of use and sample throughput are discussed in Section 6. Also addressed are qualitative observations of the technician pertaining to the performance of the TaqMan® *E. coli* O157:H7 Detection System.

Chapter 6
Test Results

The results for the TaqMan® *E. coli* O157:H7 Detection System were evaluated based on the responses provided by the Sequence Detection System software Absolute Quantitation Assay amplification plot output. An example amplification plot for *E. coli* is presented in Figure 6-1. The plot displays the change in relative fluorescence versus the thermal cycler cycle number, which is relative to time. In this example, all of the samples are displayed at once and assigned a different color. Using the Sequence Detection System 7000 software, the number of samples displayed on the amplification plot can be controlled by the operator. Only qualitative (positive/negative) responses were recorded for each sample. To determine the results of each sample, the threshold cycle or C_t value for each curve on the amplification plot was monitored for the FAM detector. To do this, a threshold had to be defined. This threshold is meant to help the user distinguish between noise and actual DNA amplification. A default threshold is set by the software at 0.2 ΔR_n. Based on the amplification plots for the samples from this test, the default value was determined to be appropriate and was used as the threshold when analyzing all samples. Amplification curves that crossed this threshold were assigned a C_t value by the software at the fractional cycle at which the amplification curve crossed the threshold. *E. coli* was considered present in the sample if the amplification curve crossed the threshold and showed actual amplification (i.e., the crossing of the threshold was real amplification and not simply a spike in the curve). The software reports an "Undetected" in place of a C_t value when amplification does not cross the defined threshold, meaning that no amplification occurred within the 40 cycles of the PCR. The *E. coli* was considered not present, and thus a negative response was recorded, if no amplification was apparent for the FAM detector for that sample and the curve did not cross the threshold. For the purposes of this test, amplification curves that crossed the threshold ΔR_n value were considered positive, regardless of the cycle number at which they crossed. In some cases, many C_t values were quite high (i.e., close to 40 cycles). Often, such high C_t values, even those as low as 37 cycles, might lead to suspect amplification and might not necessarily be considered by some to be a positive sample without further analyses. Often, comparison to the results of the other replicate samples can help in determining if the amplification is real or not. Positive controls and negative controls were monitored with each day's sample set. No controls were unsuccessful throughout the testing process.

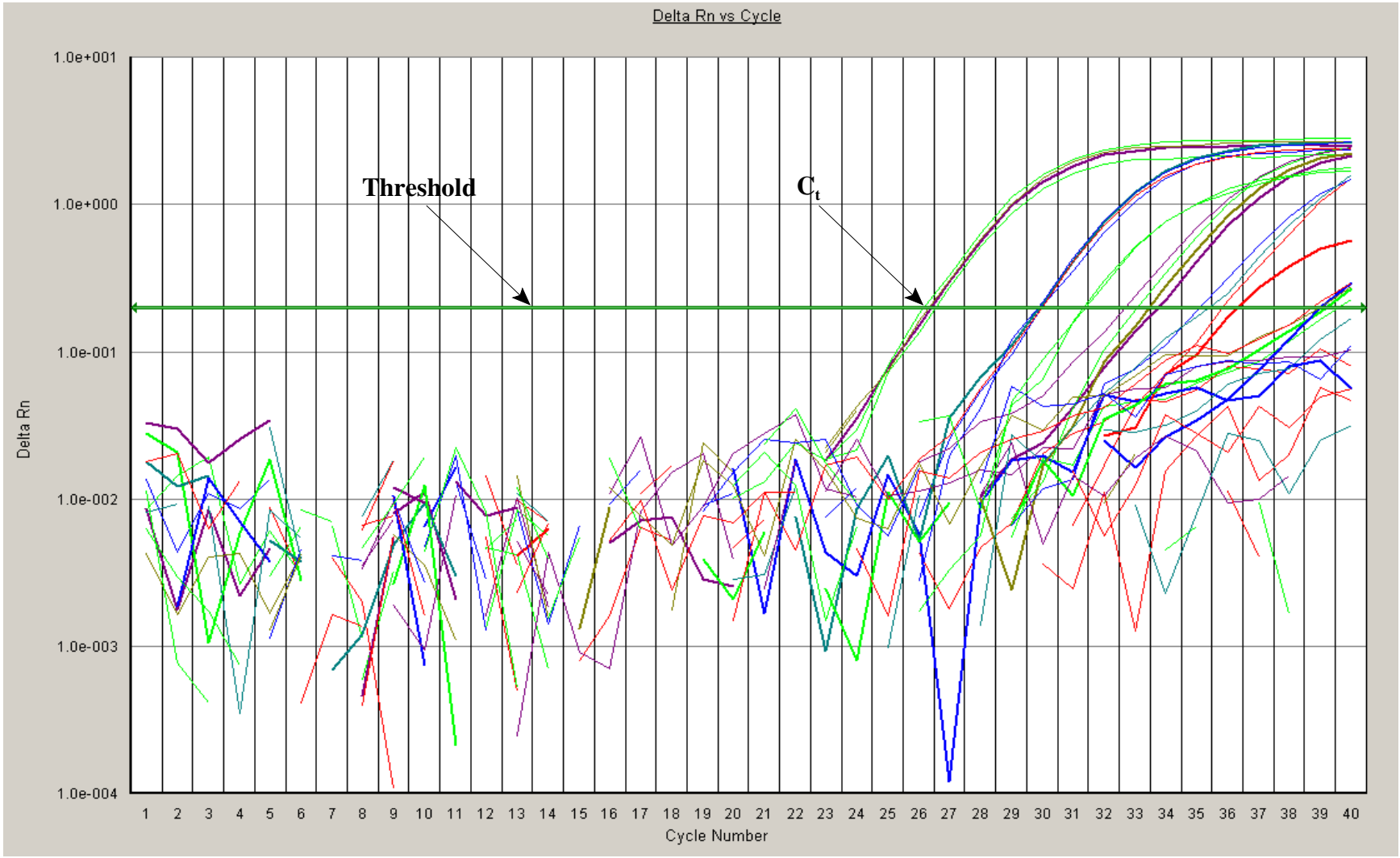

Figure 6-1. ΔR_n vs. Cycle Number ABI Prism® 7000 Sequence Detection System Amplification Plot for *E. coli* Samples and Controls

6.1 Accuracy

The results for the TaqMan® *E. coli* O157:H7 Detection System using the contaminant-only PT samples containing *E. coli* are discussed in this section. The infective/lethal dose samples are included in the contaminant-only PT samples. The infective dose for *E. coli* (see Table 3-1) was below the vendor-stated system LOD. Although the results for *E. coli* at the infective/lethal dose are presented in Table 6-1, they are not included in the overall accuracy calculations.

The results obtained for the PT samples containing *E. coli* are given in Table 6-1. The first five concentration levels listed reflect the original test/QA plan samples (infective/lethal dose, 2, 5, 10, and 50 times the system LOD) that were initially analyzed. The results indicated large inconsistencies among replicates in samples that were at levels above the system LOD originally supplied by the vendor. In most instances where a positive result was obtained, the amplification curve crossed the threshold at a very high cycle number (usually around 38 or 39) and barely crossed above the threshold line on the amplification plot. Only the 500 cfu/mL sample gave consistent positive results (4/4) for all replicates. Because the positive control reaction replicates (1×10^6 cfu/mL) were all positive, the positive and negative controls did not fail, and the IPC curves in the VIC detector appeared to amplify correctly, operator error or failure of the TaqMan® *E. coli* O157:H7 Detection System to function properly were not suspected. After discussions with Applied Biosystems, a possible explanation for these results was considered. The system LOD originally provided by Applied Biosystems was one that was known for *E. coli* in food samples. Because the TaqMan® *E. coli* O157:H7 Detection System had not been tested extensively with water samples, it was possible that the LOD for food was not appropriate for this test with water matrices. Given this, and that the concentrations of the original set of PT samples were low-bacterial concentrations, the vendor suggested that Poisson distribution effects were playing a part in the results for these samples.

Additional testing beyond that described in the test/QA plan was performed to gain more information about the performance of the TaqMan® *E. coli* O157:H7 Detection System. For the purposes of this verification test, a system LOD is anticipated to be the level at which quantifiably reproducible positive results are obtained. It represented more of a method detection limit than an instrument detection limit. Results from the additional testing were meant to help estimate the system LOD per this definition. Results from this expanded testing were also used to determine the appropriate level to spike the interferent PT and DW samples. To do this, a dilution series of contaminant-only PT samples were tested from 1×10^6 cfu/mL to 10 cfu/mL, with each solution being 10 times less than the one before (see Table 6-1). A concentration of 1×10^6 cfu/mL was chosen as the highest level analyzed because it is the same concentration as the positive control reaction sample, which was consistently positive when analyzed with the original set of test/QA plan PT samples. A concentration of 10 cfu/mL was chosen as the lowest level in the dilution series because it was the original system LOD provided by the vendor. Four positive results out of four replicates were found for the samples containing 1×10^6, 1×10^5, 1×10^4, and 1×10^3 cfu/mL of *E. coli*. All of the replicates from each sample showed clear and strong amplification. Three out of four samples were positive for the 100 cfu/mL replicates, and two out of four samples were positive for the 10 cfu/mL replicates. The crossing point for the positive samples at the lowest dilutions were around 39 cycles and were generally only slightly above the threshold.

Clearly the TaqMan® *E. coli* O157:H7 Detection System is capable of detecting *E. coli* in DI water at 10 cfu/mL, but consistent results are not obtained below samples at 500 cfu/mL. Based on these results, a system LOD (as defined above) of 500 cfu/mL was used for all subsequent calculations concerning samples above the system LOD. An overall accuracy was calculated using the replicate results from the 500 cfu/mL, 1×10^3 cfu/mL, and 1×10^4 cfu/mL samples to best replicate the contaminant-only PT samples defined in the test/QA plan (2, 5, 10, and 50 times the system LOD). Additional test samples above 1×10^4 cfu/mL were considered outside of the original sample range and not included in any calculations.

Table 6-1. *E. coli* Contaminant-Only PT Sample Results

Sample Type	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates
Original test/QA plan PT samples	0.2[b]	0/4
	20	1/4
	50	2/4
	100	2/4
	500[d]	4/4
Additional dilution series PT samples[c]	1×10^6	4/4
	1×10^5	4/4
	1×10^4[d]	4/4
	1×10^3[d]	4/4
	1×10^2	3/4
	1×10^1	2/4
Overall Accuracy		**100% (12/12)[d]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated system LOD for the original test/QA plan PT samples from stock solutions based on the enumeration data (see Table 4-1).
[b] Infective/lethal dose—below vendor-stated system LOD.
[c] Additional testing samples only contained 900 µL of samples, except for 10 cfu/mL, which contained 1 mL of sample.
[d] Only samples in grayed areas were used to calculate the overall accuracy.

6.2 Specificity

Specificity assesses the TaqMan® *E. coli* O157:H7 Detection System's ability to provide a negative response when the contaminant was absent. The results from all unspiked interferent PT samples and unspiked DW samples are presented in this section. Negative results out of total replicates are presented in each table.

The results obtained for the unspiked samples are given in Table 6-2. For the unspiked interferent PT samples, all of the 2.5 mg/L humic and fulvic acid replicates showed all negative responses. One of the four 0.5 mg/L humic and fulvic acid replicates showed a positive response. This replicate had a C_t value of 38.88. OH, CA, FL, and NY unspiked DW samples showed all negative responses also, indicating that the bacteria were not present in these samples, as would be expected.

An overall specificity rate was determined by dividing the number of negative responses to the overall number of analyses of unspiked samples. This resulted in 96% agreement for the overall specificity of the TaqMan® *E. coli* O157:H7 Detection System.

Table 6-2. *E. coli* Specificity Results

Sample Type	Sample	Negative Results Out of Total Replicates
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid, unspiked	3/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid, unspiked	4/4
DW Samples	OH DW, unspiked	4/4
	CA DW, unspiked	4/4
	FL DW, unspiked	4/4
	NY DW, unspiked	4/4
Overall Specificity		**96% (23/24)**

6.3 False Positive/Negative Responses

Contaminant-only PT samples (as determined with additional testing, see Section 6.1 above), interferent PT samples, and DW samples were evaluated to determine false positive and false negative results for the TaqMan® *E. coli* O157:H7 Detection System. Included in the calculations were the 16 additional interferent samples (0.5 mg/L humic and fulvic acids) tested to determine

the effects of the DNA extraction and isolation on the results. A false positive response was defined as a positive result when bacteria were not spiked into the sample. A false negative response was defined as a negative result when the sample was spiked with a contaminant at a concentration greater than the TaqMan® *E. coli* O157:H7 Detection System LOD for that bacteria.

The contaminant-only PT samples at 10 times the vendor-supplied system LOD showed inconsistent results among the replicates. For the interferent and DW samples, a level that showed consistent positive results in spiked DI water needed to be evaluated to test the ability of the TaqMan® *E. coli* O157:H7 Detection System to perform in the presence of inhibitors. Since the DI water samples at 10 times the vendor-provided system LOD were inconsistent, results from any interferent or DW samples spiked at this level that might be attributable to inhibitors present in these samples could not be distinguished from the normal performance of the system at that concentration level. Based on the results of the additional dilution series contaminant-only PT samples that were tested, 1×10^4 cfu/mL was chosen as an appropriate spiking level for the interferent and DW samples. This concentration provided consistent positive results for all replicates. Furthermore, because 500 cfu/mL was the lowest level tested to provide consistent positive results, and given that data were available for the performance of the system at 1×10^4 cfu/mL, 1×10^4 cfu/mL served as a good surrogate for the 10 times the system LOD called for in the test/QA plan, assuming that the system LOD in this case was one that provided consistent positive responses for spiked samples.

It should be noted that false positive responses cannot be absolutely confirmed as false because there is a possibility of cross-contamination. All appropriate steps were taken throughout the verification test to avoid this issue, such as using three work areas and following daily clean-up procedures. However, cross-contamination is always a possibility in any PCR process.[12] No appropriate reference method was available to cross-check the amplified PCR product to confirm the TaqMan® *E. coli* O157:H7 Detection System responses.

Table 6-3 presents the results for the *E. coli* samples. The number of positive samples out of the total replicates analyzed is presented in the table. No false negative samples were found in any of the sample matrices. One replicate for unspiked 0.5 mg/L humic acid and 0.5 mg/L fulvic acid did show a positive result for the presence of *E. coli*. The crossing point for this replicate was 38.88 cycles (out of 40 total).

Table 6-3. *E. coli* False Positive/Negative Results

Sample Type	Sample	Concentration[(a)] (cfu/mL)	Positive Results Out of Total Replicates
Contaminant-Only PT Samples	DI water	500	4/4
	DI water	1×10^3	4/4
	DI water	1×10^4	4/4
Interferent PT Samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	1/4
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^4	20/20
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^4	4/4
DW Samples	OH DW	Blank	0/4
	OH DW	1×10^4	4/4
	CA DW	Blank	0/4
	CA DW	1×10^4	4/4
	FL DW	Blank	0/4
	FL DW	1×10^4	4/4
	NY DW	Blank	0/4
	NY DW	1×10^4	4/4
False Positive Rate			**1/24**
False Negative Rate			**0/52**

[(a)] Sample solutions were prepared at these levels from stock solutions based on the enumeration data (see Table 4-1).

6.4 Precision

The performance of the TaqMan® *E. coli* O157:H7 Detection System within sample sets of four replicates was consistent for the DW and interferent PT samples. Only one set of replicates, that for unspiked 0.5 mg/L humic and fulvic acids, was inconsistent, with one of the replicates showing a positive result while the other samples were negative. All other DW and interferent PT samples showed the same results within a set of replicates.

For the contaminant-only PT samples, all samples at or above 500 cfu/mL showed consistent results within each set or replicates. For those samples below 500 cfu/mL, the results for sample sets were inconsistent, with mixed positive and negative results for all five sets of replicates. The one exception to the inconsistent results for sample sets below 500 cfu/mL was the sample set for *E. coli* at the infective/lethal dose, which showed negative results for all replicates. Out of the 27 sample sets analyzed, six were inconsistent, resulting in 78% (21/27) of the sample sets showing consistent results.

6.5 Interferences

6.5.1 Interferent PT Samples

In both the 0.5 mg/L and 2.5 mg/L humic and fulvic acid solutions spiked with *E. coli* and the 2.5 mg/L unspiked sample, the TaqMan® *E. coli* O157:H7 Detection System provided expected results. In the absence of the bacteria, the samples tested negative; in the presence of the bacteria, the samples tested positive. For the 0.5 mg/L unspiked humic and fulvic acid sample, one of the four replicates tested positive for the presence of *E. coli*. In all cases for the interferent PT samples, the IPC was present and showed clear amplification in the amplification plot for the VIC detector. This would indicate that the humic and fulvic acids were not acting as inhibitory substances for the TaqMan® system.

As discussed in section 3.2.1, four solutions of humic and fulvic acids each at 0.5 mg/L spiked with *E. coli* at 1×10^4 cfu/mL were prepared in addition to the initial 0.5 mg/L and 2.5 mg/L humic and fulvic acid solutions. Each solution was put through the DNA extraction and isolation procedure, and then four replicates from each of the four purified DNA solutions were analyzed using the TaqMan® *E. coli* O157:H7 Detection System. These samples were included in the verification test in an effort to evaluate the efficacy of the DNA extraction and isolation procedure in the presence of inhibitory substances. These samples also contribute to the precision evaluations of the TaqMan® *E. coli* O157:H7 Detection System. All of the samples tested resulted in positive responses. Thus, 20 out of the 20 spiked 0.5 mg/L each humic and fulvic acid samples tested resulted in positive responses. No IPC suppression was apparent in any of these samples.

6.5.2 Drinking Water Samples

The TaqMan® *E. coli* O157:H7 Detection System DW sample results are presented in Table 6-3. The TaqMan® *E. coli* O157:H7 Detection System showed positive results for each set of replicates for the spiked DW samples and negative results for each set of replicates for the unspiked samples. IPC suppression was unapparent in all but one of the DW samples, indicating that the DW matrices were generally not acting as inhibitors for the TaqMan® system. Only one replicate for unspiked NY DW did not have a C_t value for the IPC. All PT samples spiked with 1×10^4 cfu/mL of *E. coli* showed consistent positive responses and no IPC suppression.

6.6 Other Performance Factors

The TaqMan® *E. coli* O157:H7 Detection System was operated by the same Battelle technician throughout the verification test. This technician had prior PCR experience as well as prior ABI Prism® 7000 Sequence Detection System experience and was trained by Applied Biosystems in the operation of the TaqMan® *E. coli* O157:H7 Detection System before testing began. The Battelle technician was already familiar with general DNA extraction and isolation techniques, PCR 96-well plating techniques, and ABI Prism® 7000 Sequence Detection System operation, as well as general PCR theory, prior to training. The overall operation of the TaqMan® *E. coli* O157:H7 Detection System was straightforward, and the experienced technician found the system easy to use with slight difficulties in data interpretation. The PrepMan™ Ultra Sample Preparation Reagent procedure was short and simple, requiring very little reagent. The PCR setup was also straightforward. The aseptic procedure while handling the PCR Cocktail and adding the sample DNA is essential to ensure reliable sample results. The TaqMan® *E. coli* O157:H7 Detection Kit required very little reagent to make the PCR Cocktail and was straightforward. The amount of sample DNA needed to run the PCR was also small. Loading the 96-well plate into the ABI Prism® 7000 Sequence Detection System and starting the instrument were very straightforward. Setting up the real-time PCR run and conducting data analysis, however, were a little more complicated and would require either prior experience or training to do properly. The user's manual is straightforward in helping to set the thermal cycler conditions for a run, but it might be difficult to optimize an assay without further training on the instrument and software. Data analysis using the Absolute Quantitation Assay can be somewhat complicated and relies on the operator to make appropriate choices. Though the software produced the amplification plots and gave C_t values for all amplification curves above the threshold, it was up to the technician to determine the appropriate threshold and then further determine if the sample was positive or negative, even if the curve crossed the threshold. An understanding of the software, what the C_t value represents, and a general understanding of the amplification plots were necessary for the data interpretations. In some instances, even the experienced technician had trouble determining if a sample was positive and showing true amplification when the sample had spikes at the last few temperature cycles. Overall, the Sequence Detection System software was easy to navigate through, and the amplification plot analysis section controls were user friendly.

All testing was performed in a laboratory setting because the TaqMan® *E. coli* O157:H7 Detection System is not field portable. Three distinct and separate testing areas were required in each

laboratory to operate the TaqMan® *E. coli* O157:H7 Detection System. The PrepMan™ Ultra Sample Preparation Reagent components were stable at room temperature. The TaqMan® *E. coli* O157:H7 Detection Kit had to be stored at -15 to -25°C and thawed before use. The Pathogen PCR Cocktail must be made within one hour of use and can be stored at room temperature until its use. The PCR reactions were assembled at room temperature. The Prism® has a footprint of 20 inches by 15 inches and stands 21 inches high. It comes with a laptop.

E. coli samples were tested in a BSL-2 laboratory. Because live bacteria were being handled, special safety requirements and protocols had to be implemented in the laboratory. Some of these requirements may have impacted the analysis time for the TaqMan® *E. coli* O157:H7 Detection System and are inherently present in any throughput estimations for this verification test. Thus, such performance factors mentioned here also incorporate the safety and facility requirements necessary for this test.

More than 128 samples (including method blanks and positive control reactions) were tested for *E. coli* using the TaqMan® *E. coli* O157:H7 Detection System. The DNA extraction and isolation step for between seven and nine sample solutions took 40 minutes to an hour. The PCR steps, including the Pathogen PCR Cocktail set up, took 30 minutes to an hour, depending on the number of samples. The loading of the ABI Prism® 7000 Sequence Detection System and the completion of the thermal cycle program to amplify the sample DNA took a little over two hours. For this study, the technician analyzed on average 28 to 36 replicate samples in a day. The plates used in the instrument can hold up to 96 samples and controls.

For the purposes of this test, the Absolute Quantitation Assay was used to determine the results for each sample. This assay is a real-time PCR assay, meaning that results can be monitored in real time because the DNA present in each sample is amplified. Although not used in this test, this assay can also be used in conjunction with standards of known concentration to determine the concentration of the unknown samples being analyzed. The Sequence Detection System 7000 software also offers the use of a Plus/Minus Assay, which is an endpoint assay that identifies samples as positive or negative. In this assay, the software sets the threshold for the for data analysis using the IPC and negative control results and then assigns a positive (+), negative (-), or undetermined (?) call to the sample based on this analysis. An undetermined or inconclusive result is returned if neither the target nor the IPC is detected in the sample. A ΔR_n value is also given for each sample. Using this assay, no manual data interpretation is needed because the software sets and uses consistent criteria to produce positive and negative results for each sample.

Chapter 7
Performance Summary

Table 7-1 . *E. coli* Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	Original test/QA plan samples— DI water	0.2 cfu/mL[(a)]	0/4
			20 cfu/mL	1/4
			50 cfu/mL	2/4
			100 cfu/mL	2/4
			500 cfu/mL	4/4
		Additional testing samples— DI water[(b)]	1×10^{6} cfu/mL	4/4
			1×10^{5} cfu/mL	4/4
			1×10^{4} cfu/mL	4/4
			1×10^{3} cfu/mL	4/4
			1×10^{2} cfu/mL	3/4
			1×10^{1} cfu/mL	2/4
	Interferent PT samples	Humic and fulvic acids	1×10^{4} cfu/mL	24/24
	DW samples	Concentrated DW	1×10^{4} cfu/mL	16/16
Accuracy		100% (12 out of 12) of the contaminant-only PT samples between (and including) 500 cfu/mL and 1×10^{4} cfu/mL were positive.		
Specificity		96% (23 out of 24) of the unspiked interferent and DW samples were negative. One unspiked humic and fulvic acids replicate at 0.5/mg/L of each acid returned a positive result.		
False positives		One false positive resulted from the analysis of the unspiked 0.5 mg/L humic acid and 0.5 mg/L fulvic acid. No false positives resulted for any other interferent or DW samples.		
False negatives		No false negative results were obtained from the analysis of the interferent and DW samples spiked with detectable levels of *E. coli*.		
Precision		78% (21 out of 27) of the sample sets showed consistent results among the individual replicates within that set.		

[(a)] Infective/lethal dose. Below the vendor-stated system LOD.

[(b)] Additional testing samples only contained 900 µL of samples, except for 10 cfu/mL, which contained 1 mL of sample.

Other performance factors: A technician with prior PCR and ABI Prism® Sequence Detection System experience operated the TaqMan® *E. coli* O157:H7 Detection System at all times. The PrepMan™ Ultra Sample Preparation Regent and TaqMan® *E. coli* O157:H7 Detection Kit were straightforward and easy to use. The ABI Prism® Sequence Detection System data analysis and PCR setup were more complicated and required experience and understanding to properly use. Three separate work areas were needed for testing. Small amounts of reagents were required for DNA extraction and PCR setup and small amounts of sample DNA were needed for PCR. Reagents for the various steps of the system had different storage requirements. Sample throughput for this verification test was 28 to 36 replicate samples per day. The approximate operational times were less than one hour for DNA extraction/purification, less than one hour for PCR setup, and two hours for PCR. The cost is around $100 for the PrepMan™ Ultra Sample Preparation Reagent (50 to 100 DNA extractions), $800 for the TaqMan® *E. coli* O157:H7 Detection Kit (100 assays) and approximately $47,250 for the ABI Prism® Sequence Detection System (20 inches x 21 inches x15 inches, 75 pounds).

Chapter 8
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