THE ENVIRONMENTAL TECHNOLOGY VERIFICATION PROGRAM

ETV Joint Verification Statement

TECHNOLOGY TYPE: **Rapid Polymerase Chain Reaction**

APPLICATION: **DETECTING BIOLOGICAL AGENTS AND PATHOGENS IN WATER**

TECHNOLOGY NAME: **PathAlert™ Detection Kit**

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The U.S. Environmental Protection Agency (EPA) supports the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative or improved 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. Information and ETV documents are available at www.epa.gov/etv.

ETV works in partnership with recognized standards and testing organizations, with stakeholder groups (consisting of buyers, vendor organizations, and permitters), and with 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 Advanced Monitoring Systems (AMS) Center, one of six verification centers under ETV, is operated by Battelle in cooperation with EPA's National Exposure Research Laboratory. The AMS Center has recently evaluated the performance of rapid polymerase chain reaction (PCR) systems to detect biological agents and pathogens in water. This verification statement provides a summary of the test results for the Invitrogen Corporation's PathAlert™ Detection Kits for the detection of *Francisella tularensis* (*F. tularensis*), *Yersinia pestis* (*Y. pestis*), and *Bacillus anthracis* (*B. anthracis*).

VERIFICATION TEST DESCRIPTION

The PathAlert™ Detection Kits were evaluated between June 9 and June 30, 2004, using *F. tularensis* LVS [American Type Culture Collection (ATCC)# 29684], *Y. pestis* CO92, and *B. anthracis* Ames strain. The performance of each PathAlert™ Detection Kit was verified in terms of its accuracy, specificity, number of false positive/negative responses, precision, interferences, ease of use, and sample throughput. Performance test (PT) samples, drinking water (DW) samples, and quality control (QC) samples were used in the verification test for each bacteria. PT samples included individual bacteria spiked into American Society of Testing and Materials (ASTM) Type II deionized (DI) water at 2, 5, 10, and 50 times the vendor-stated method limit of detection (LOD), as well as the infective/lethal dose for each contaminant. PT samples also included potential interferent samples containing a single concentration (10 times the method LOD) of the contaminant of interest in the presence of fulvic and humic acids [at 0.5 milligram (mg)/liter (L) each and 2.5 mg/L each] spiked into ASTM Type II DI water. Interferent samples also were analyzed without the addition of any bacteria. DW samples consisted of chlorinated filtered surface water, chloraminated filtered surface water, chlorinated filtered groundwater, and chlorinated unfiltered surface water collected from four geographically distributed municipal sources. DW samples were analyzed without adding contaminant and after fortification with each individual bacteria at a single concentration level (10 times the vendor-stated method LOD). QC samples included method blank samples and positive (both internal and external) and negative controls, as supplied with each PathAlert™ Detection Kit. For all contaminants, plate enumerations were performed in triplicate to confirm the concentrations of the stock solutions of each bacteria prior to testing.

For the purposes of this test, 1×10^4 colony forming units per milliliter (cfu/mL) were used to calculate the concentration levels of *F. tularensis* and *B. anthracis* spiked into the PT and DW samples; 100 cfu/mL were used to calculate levels of *Y. pestis* spiked in the PT and DW samples. These vendor-provided concentration levels were anticipated to be the levels for the entire experimental process at which quantifiably reproducible positive results could be obtained from a raw water sample. These concentration levels are referred to as the "method LOD" for a particular assay. The method LOD incorporates the sensitivities and uncertainties of not only the PathAlert™ Detection Kit, but also the deoxyribonucleic acid (DNA) purification step; and, as such, it is an experimental detection limit rather than an instrument or reagent-specific detection limit. As mentioned previously, the method LOD provided by the vendor was used specifically as a guideline in calculating sample concentration ranges for use with the PathAlert™ Detection Kit and all other components used in this verification test to analyze a sample, and it should be noted that Invitrogen Corporation does not claim this to be the true LOD of the PathAlert™ Detection Kit alone. The vendor claims the absolute LOD (the least amount of target DNA that would generate a positive result) for the PathAlert™ Detection Kit alone is as low as 1 to 10 copies of DNA, depending on the assay. This information was not verified in this test.

Samples were spiked with *F. tularensis* and *B. anthracis* at 2×10^4 colony-forming units (cfu)/milliliter (mL), 5×10^4 cfu/mL, 1×10^5 cfu/mL, and 5×10^5 cfu/mL for PT samples and 1×10^5 cfu/mL for interferent and DW samples. Samples were spiked with *Y. pestis* at 2×10^2 cfu/mL, 5×10^2 cfu/mL, 1×10^3 cfu/mL, and 5×10^3 cfu/mL for PT samples and 1×10^3 cfu/mL for interferent and DW samples. The infective/lethal dose of each contaminant was determined by calculating the concentration at which ingestion of 250 mL of water is likely to cause the death of a 70-kilogram person based on human LD_{50} or ID_{50} data. The infective/lethal doses for *F. tularensis*, *Y. pestis*, and *B. anthracis* were 4×10^5 cfu/mL, 0.28 cfu/mL, and 200 cfu/mL, respectively. Samples were prepared in 1 mL quantities and tested blindly by trained Battelle operators who had prior PCR experience. To test a 1 mL liquid sample for the presence or absence of *F. tularensis, Y. pestis*, or *B. anthracis*, DNA was extracted and purified from the sample using the Roche High Pure PCR Template Preparation Kit, assays were prepared using the PathAlert™ Detection Kit reagents, PCR was performed using a MJ Research DNA Engine® (PTC-200™) Peltier Thermal Cycler, and the amplified products were analyzed using the Agilent 2100 Bioanalyzer instrument along with the 2100 Bioanalyzer DNA 500 chips and reagent kit and associated 2100 Bioanalyzer software. The kit was only tested for one bacteria at a time. All samples were analyzed in quadruplicate from the same batch of purified DNA. The PathAlert™ Detection Kit was evaluated for qualitative results only by monitoring the internal positive control (IPC) along with the bacteria-specific peaks in the 2100 Bioanalyzer electropherogram output. Only

positive, negative, and inconclusive results were recorded. Inconclusive results occurred when not all of the bacteria-specific peaks were present in the electropherogram.

QA oversight of verification testing was provided by Battelle and EPA. Battelle QA staff conducted a technical systems audit and a data quality audit of 10% of the test data. This verification statement, the full report on which it is based, and the test/QA plan for this verification are all available at www.epa.gov/etv/centers/center1.html.

TECHNOLOGY DESCRIPTION

The following description of the PathAlert™ Detection Kit was provided by the vendor and was not subjected to verification in this test.

The PathAlert™ Detection Kit is a multiplex PCR reagent system capable of detecting *F. tularensis*, *Y. pestis*, *B. anthracis*, or smallpox in individual assays. The PathAlert™ Detection Kit comprises an optimized PCR SuperMix specific to the pathogen of interest, as well as an external positive control (EPC) template for system validation. The kit includes Taq polymerase, pre-complexed with antibodies to maintain "hot start" PCR (for specificity and sensitivity); uracil DNA glycosylase and deoxyuridane triphosphate to eliminate post-PCR cross-contamination; and an IPC to identify potential PCR inhibition from sample contaminants or environmental samples. Included in the kit is an EPC that has been engineered to produce different amplicon sizes than either the IPC or the pathogen-specific loci. As a result, pathogen-specific results can be read with minimal interference if contamination by the external control should occur.

The PathAlert™ Detection Kit is an endpoint assay; post-amplification products may be analyzed using any platform capable of distinguishing amplicon size, such as the Agilent Bioanalyzer 2100, Agilent ALP (high throughput), transgenomic WAVE high-performance liquid chromatography, gel electrophoresis, and Caliper AMS 90. The Agilent Bioanalyzer 2100 and ALP are recommended by Invitrogen Corporation for use with the PathAlert™ Detection Kit because all field testing to date has been performed with these systems, and Agilent and Invitrogen Corporation have established a co-marketing relationship for the complete system. The list price of each PathAlert™ Detection Kit assay is $12 to $16. Additional discounts based on volume and concept of operations are available through the vendor. PathAlert™ Detection Kits can perform up to 320 assays per kit.

VERIFICATION OF PERFORMANCE

Accuracy: Accuracy was assessed by evaluating how often the PathAlert™ Detection Kit results were positive in the presence of a concentration of contaminant above the method 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 method LOD. The results are presented in the table below.

Bacteria	Concentration Range of Samples Used in Accuracy Calculations (cfu/mL)	Overall Accuracy (Positive Results Out of Total Replicates)
F. tularensis	2×10^4 to 5×10^5	100% (20/20)
Y. pestis	2×10^2 to 5×10^3	100% (16/16)
B. anthracis	2×10^4 to 5×10^5	100% (16/16)

For *F. tularensis*, *Y. pestis*, and *B. anthracis*, all samples at concentration levels above the vendor-stated method LOD generated positive responses for each set of replicates, resulting in 100% agreement for the overall accuracy of the PathAlert™ Detection Kit for each bacteria. The infective/lethal doses for *Y. pestis* (0.28 cfu/mL) and

B. anthracis (200 cfu/mL) were below the method LOD and not included in the accuracy calculations for those bacteria.

Specificity: The ability of the PathAlert™ Detection Kit 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. Unspiked interferent PT samples and unspiked DW samples were used to assess specificity. The results are presented in the table below. For *F. tularensis* and *Y. pestis*, one unspiked DW replicate for each bacteria produced an inconclusive response.

Bacteria	Overall Specificity (Negative Results Out of Total Replicates)
F. tularensis	96% (23/24)
Y. pestis	96% (23/24)
B. anthracis	100% (22/22)

False positive/negative responses: A false positive response was defined as a detectable or positive PathAlert™ Detection Kit response when the interferent PT samples or DW samples were not spiked. The false positive rate was reported as the frequency of false positive results out of the total number of unspiked samples. The false negative response was defined as a negative response when the sample was spiked with a contaminant at a concentration greater than the method LOD. Spiked PT (contaminant and interferent) samples and spiked DW samples were included in the analysis. The false negative rate was reported as the frequency of false negative results out of the total number of spiked samples for a particular contaminant. The results are presented in the table below. No false positives or false negatives were found for any of the sample matrices for any bacteria. One replicate for unspiked DW in two different DW samples showed an inconclusive result for *F. tularensis* and one for *Y. pestis*. Two inconclusive results were reported (one for *F. tularensis* in one DW sample and one for *Y. pestis* in a different DW sample).

Bacteria	False Positive Rate	False Negative Rate
F. tularensis	0/24	0/60
Y. pestis	0/24	0/56
B. anthracis	0/22	0/56

Precision: The precision of the PathAlert™ Detection Kit was assessed by calculating the overall percentage of consistent responses for all the sample sets. Responses were considered consistent if all responses of the four replicates were the same.

For *F. tularensis* replicates, 95% of the sample sets (20 out of 21) showed consistent results. Similarly, 95% of the sample sets (20 out of 21) showed consistent results for *Y. pestis*. For both bacteria, the inconsistency resulted from an inconclusive result for a DW replicate for each bacteria. As with *F. tularensis* and *Y. pestis*, 95% of the sample sets for *B. anthracis* (20 out of 21) showed consistent results among the replicates. For this bacteria, the one sample set with inconsistent results was the infective dose PT sample. In this set of replicates, three of the four samples had inconclusive results, while the fourth sample was positive for *B. anthracis*. The infective dose of *B. anthracis* was below the method LOD for this bacteria.

Other performance factors: A Battelle technician with prior PCR experience who was trained by the vendor operated the PathAlert™ Detection Kit. The kit was straightforward and easy to use. All components necessary for

the PCR process (excluding the sample DNA) were contained in one solution, which had to be stored at -20°C. Three separate work areas were needed for testing: a "clean" area free of DNA, a "medium" area with moderate DNA presence, and a "dirty" area, with high DNA presence. SuperMix prep took approximately 15 minutes and loading the sample DNA into the PCR tubes took between 15 and 30 minutes for each set of samples. Sample throughput for this verification test (from DNA purification to amplified product detection) was 36 to 48 samples per day.

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