THE ENVIRONMENTAL TECHNOLOGY VERIFICATION PROGRAM

U.S. Environmental Protection Agency

ETV Joint Verification Statement

TECHNOLOGY TYPE:	**Rapid Polymerase Chain Reaction**
APPLICATION:	**DETECTING BIOLOGICAL AGENTS AND PATHOGENS IN WATER**
TECHNOLOGY NAME:	**R.A.P.I.D.® System**

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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 bacteria in water. This verification statement provides a summary of the test results for the Idaho Technology Inc.'s (ITI's) Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.®) System for the detection of *Francisella tularensis* (*F. tularensis*), *Yersinia pestis* (*Y. pestis*), *Bacillus anthracis* (*B. anthracis*), *Brucella suis*, and *Escherichia coli (E. coli)*.

VERIFICATION TEST DESCRIPTION

The R.A.P.I.D.® System was evaluated between May 27 and July 8, 2004, using *F. tularensis* LVS [American Type Culture Collection (ATCC)# 29684], *Y. pestis* CO92, *B. anthracis* Ames strain, *Brucella suis* (ATCC#23444), and *E. coli* O157:H7. The performance of the R.A.P.I.D.® System 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 system 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 system 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 were also 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 system LOD). QC samples included method blank samples and positive and negative controls, as supplied with the R.A.P.I.D.® System. For all contaminants, plate enumerations were performed in triplicate to confirm the concentration of the stock solution of each bacteria prior to testing.

For the purposes of this test, 1,000 colony forming units per milliliter (cfu/mL) were used to calculate the concentration levels spiked into the PT and DW samples.. 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 R.A.P.I.D.® System. This concentration level is referred to as the "system LOD." The system LOD incorporates the sensitivities and uncertainties of the entire R.A.P.I.D.® System, in particular the ITI 1-2-3 Flow Kit deoxyribonucleic acid (DNA) purification step, as well as the ITI freeze-dried reagents; and, as such, it 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 for use with the R.A.P.I.D.® System in this verification test, and it should be noted that Idaho Technology Inc. does not claim this to be the true LOD of the R.A.P.I.D.® System. Detection limits for individual components of the R.A.P.I.D.® System and the system as a whole may differ and were not verified in this test.

Samples were spiked individually with each bacteria (*F. tularensis*, *Y. pestis*, *B. anthracis*, *Brucella suis*, and *E. coli*) at 2×10^3 cfu/mL, 5×10^3 cfu/mL, 1×10^4 cfu/mL, and 5×10^4 cfu/mL for PT samples and 1×10^4 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*, *B. anthracis*, *Brucella suis*, and *E. coli* were 4×10^5 cfu/mL, 0.28 cfu/mL, 200 cfu/mL, 40 cfu/mL, and 0.2 cfu/mL respectively. Samples were prepared in 5 mL quantities and tested blindly by trained Battelle operators who had prior PCR experience. To test a liquid sample for the presence of *F. tularensis*, *Y. pestis*, *B. anthracis*, *Brucella suis*, or *E. coli*, DNA was extracted and purified using the R.A.P.I.D.® System's ITI 1-2-3 Flow Kit, samples were prepared using the R.A.P.I.D.® System's freeze-dried reagents, and the samples were analyzed using the R.A.P.I.D.® 7200 instrument and software. Each reconstituted, freeze-dried reagent sample was split into two glass capillaries for analysis in duplicate on the R.A.P.I.D.® 7200 instrument. For *F. tularensis*, *Y. pestis*, and *B. anthracis*, more than one freeze-dried reagent target was used for the analysis of each bacteria. The system was only tested for one bacteria at a time. All samples were analyzed in quadruplicate from the same batch of purified DNA. The R.A.P.I.D.® System was evaluated for qualitative results only by monitoring the positive and negative controls along with the amplification curves and crossing points for each sample using the R.A.P.I.D.® 7200 software Second Derivative Maximum method. Only positive, negative, and inconclusive results were recorded. Inconclusive results occurred when the results from the two capillaries for the sample didn't agree with each other (i.e., one was positive and one was negative).

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 R.A.P.I.D.® System was provided by the vendor and was not subjected to verification in this test.

The R.A.P.I.D.® System is an integrated three-part system for the timely detection and identification of pathogens and biowarfare agents, including anthrax, plague, salmonella, and botulism, in water. The system allows for rapid and specific presumptive identification of threat pathogens in hours rather than days. The system components consist of the ITI 1-2-3 Flow Kit for the purification of DNA, ITI target-specific freeze-dried reagents containing all the necessary ingredients for specific pathogen DNA detection, and the R.A.P.I.D.® 7200 instrument. The operator prepares the DNA from the environmental sample, hydrates the freeze-dried reagents with the DNA sample, runs the R.A.P.I.D.® 7200 instrument, and then reads the auto-analyzed results using the R.A.P.I.D.® software.

The ITI 1-2-3 Flow Kit is a three-step DNA extraction and purification kit that contains the components for purifying DNA from water or other environmental matrix. It removes inhibitors from a sample that would adversely affect a reaction and has been optimized for purifying DNA from difficult-to-process anthrax spores, as well as non-spore forming bacteria. Each kit is optimized and validated for the R.A.P.I.D.® 7200 instrument and contains all the ingredients necessary for DNA purification (one 30 mL, one 20 mL, and one 25 mL buffer; 50 bead tubes with lysis buffer; 50 spin filters; 200 receiver tubes; and 50 swabs). The ITI freeze-dried reagents (hybridization probe reagents) are freeze-dried in a single tube and require no refrigeration or freezing. There are multiple gene targets for assay confirmation, with the additional backup of melting curve analysis to confirm any suspected positive test.

The R.A.P.I.D.® 7200 instrument is a field-hardened, air-driven, real-time thermocycler with concurrent fluorescence monitoring that is capable of automatically analyzing samples for the presence of any given DNA sequence. The R.A.P.I.D.® 7200 instrument is based on LightCycler technology and is capable of 45 PCR cycles in 30 minutes. It has three color optics, can run on either 110 or 220 volt power, and is watertight in its case. R.A.P.I.D.® software allows the user to automatically collect and interpret data and report results. The software has two user levels, with simple push-button software and auto-analysis or with all the features of the original laboratory instrument for advanced real-time analysis. The R.A.P.I.D.® 7200 instrument is a 50-pound (22.7-kg), portable commercial off-the-shelf system. It operates in various environmental conditions (heat, humidity, salt spray) and has passed a one-meter drop test. The R.A.P.I.D.® instrument includes a backpack, laptop computer, microcentrifuge, and sample capillaries. Its dimensions are 19.4 inches [49.3 centimeters (cm)] by 14.3 inches (36.3 cm) by 10.5 inches (26.7 cm), and its cost is $55,000 U.S., including the centrifuge and laptop. Sample preparation using the ITI 1-2-3 Flow Kit costs approximately $8 per sample, and testing the samples using the freeze-dried reagents costs approximately $17 per sample.

VERIFICATION OF PERFORMANCE

Accuracy: Accuracy was assessed by evaluating how often the R.A.P.I.D.® 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. 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 Target 1	2×10^3 to 4×10^5	100% (20/20)
F. tularensis Target 2	2×10^3 to 4×10^5	100% (20/20)
Y. pestis Target 1	2×10^3 to 5×10^4	100% (16/16)
Y. pestis Target 2	2×10^3 to 5×10^4	100% (16/16)
B. anthracis Target 1	2×10^3 to 5×10^4	94% (15/16)
B. anthracis Target 2	2×10^3 to 5×10^4	94% (15/16)
B. anthracis Target 3	2×10^3 to 5×10^4	100% (16/16)
Brucella suis	2×10^3 to 5×10^4	88% (14/16)
E. coli	2×10^3 to 5×10^4	100% (16/16)

For *F. tularensis* (both targets), *Y. pestis* (both targets), *B. anthracis* Target 3, and *E. coli*, all samples at concentration levels above the vendor-stated system LOD generated positive responses for each set of replicates, resulting in 100% agreement for the overall accuracy of the R.A.P.I.D.® System for each bacteria. One replicate at 2×10^3 cfu/mL for *B. anthracis* Target 1 reagents resulted in an inconclusive response; one replicate at 2×10^3 cfu/mL for *B. anthracis* Target 2 reagents resulted in a negative response; and two replicates at 2×10^3 cfu/mL for *Brucella suis* reagents resulted in inconclusive responses; all other replicates at each level for each bacteria produced positive results. The infective/lethal doses for *Y. pestis* (0.28 cfu/mL), *B. anthracis* (200 cfu/mL), *Brucella suis* (40 cfu/mL), and *E. coli* (0.2 cfu/mL) were below the system LOD and not included in the accuracy calculations for those bacteria.

Specificity: The ability of the R.A.P.I.D.® 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. Unspiked interferent PT samples and unspiked DW samples were used to assess specificity. The results are presented in the table below. For *F. tularensis* Target 1, three of the replicates for unspiked 2.5 mg/L humic and fulvic acids produced inconclusive results. For *F. tularensis* Target 2, one unspiked DW replicate produced an inconclusive response. The results for all other bacteria were negative for all unspiked samples.

Bacteria	Overall Specificity (Negative Results Out of Total Replicates)
F. tularensis Target 1	88% (21/24)
F. tularensis Target 2	96% (23/24)
Y. pestis Target 1	100% (24/24)
Y. pestis Target 2	100% (24/24)
B. anthracis Target 1	100% (24/24)
B. anthracis Target 2	100% (24/24)
B. anthracis Target 3	100% (24/24)
Brucella suis	100% (24/24)
E. coli	100% (24/24)

False positive/negative responses: A false positive response was defined as a detectable or positive R.A.P.I.D.® System 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 system 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. Inconclusive results were considered neither positive nor negative and were thus not counted as false positives or false negatives. The results are presented in the table below.

Bacteria	False Positive Rate	False Negative Rate
F. tularensis Target 1	0/24	0/60
F. tularensis Target 2	0/24	4/60
Y. pestis Target 1	0/24	0/56
Y. pestis Target 2	0/24	0/56
B. anthracis Target 1	0/24	2/56
B. anthracis Target 2	0/24	2/56
B. anthracis Target 3	0/24	0/56
Brucella suis	0/24	0/56
E. coli	0/24	0/52

No false positives were found for any of the sample matrices for any bacteria. Three unspiked interferent PT sample replicates showed inconclusive results for *F. tularensis* Target 1. Inconclusive results were also found for one replicate in unspiked DW using *F. tularensis* Target 2.

Four false negatives were found for *F. tularensis* Target 2: one in a set of replicates for spiked interferent PT samples, and three in a set of replicates for spiked DW. Inconclusive results were also found for one replicate in spiked DW for this target. One replicate for spiked DW in each of two different DW samples produced a false negative result for *B. anthracis* Target 1. One replicate each for two different spiked DW samples and spiked DI water at 2×10^3 cfu/mL produced inconclusive results for *B. anthracis* Target 1. Samples analyzed using *B. anthracis* Target 2 also had two false negatives: one replicate of 2×10^3 cfu/mL *B. anthracis* in DI water and one replicate of spiked DW. Two inconclusive results were also found for spiked DW replicates using *B. anthracis* Target 2 reagents. *B. anthracis* Target 3 reported no false negative results, although four spiked DW replicates from one DW sample generated inconclusive results. Two inconclusive results were obtained for DI water spiked at 2×10^3 cfu/mL for *Brucella suis*.

Precision: The precision of the R.A.P.I.D.® System 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* Target 1 replicates, 95% of the sample sets (20 out of 21) showed consistent results; and for *F. tularensis* Target 2 replicates, 86% of the sample sets (18 out of 21) showed consistent results. The inconsistencies for the *F. tularensis* Target 1 resulted from inconclusive results for three interferent PT sample replicates; Target 2 inconsistencies resulted from an inconclusive DW replicate and a negative response for an

interferent PT sample replicate. For *Y. pestis* Targets 1 and 2, 100% of the samples sets (21 out of 21) showed consistent results among the replicates.

For B. *anthracis*, 76% of the Target 1 sample sets (16 out of 21) showed consistent results, 86% of the Target 2 sample sets (18 out of 21) showed consistent results, and 95% of the Target 3 sample sets (20 out of 21) showed consistent results. For *B. anthracis* Target 1, the five sample sets with inconsistent results included two PT samples (one inconclusive replicate at 2×10^3 cfu/mL and three inconclusive and one negative result at the infective/lethal dose) and three different spiked DW samples (one inconclusive replicate in each of two different DW samples and one negative replicate in each of two different DW samples). For *B. anthracis* Target 2, the three sample sets with inconsistent results included two PT samples (one negative replicate at 2×10^3 cfu/mL and two inconclusive and two negative results at the infective/lethal dose) and one spiked DW sample (two inconclusive, one positive, and one negative replicate). For *B. anthracis* Target 3, the only inconsistent sample set was the infective/lethal dose, with two inconclusive and two negative results. The infective dose of *B. anthracis* was below the system LOD for the R.A.P.I.D.® System for this bacteria.

For *Brucella suis*, 90% (19 out of 21) of the sample sets showed consistent results. For this bacteria, two PT sample sets showed inconsistent results (two positive and two inconclusive replicates at 2×10^3 cfu/mL and one inconclusive and three negative replicates at the infective/lethal dose). The infective dose of *Brucella suis* was below the system LOD for the R.A.P.I.D.® System for this bacteria. For *E. coli*, 100% of the sample sets showed consistent results among the replicates.

Other performance factors: A Battelle technician with prior PCR experience who was trained by the vendor operated the R.A.P.I.D.® System at all times. All three components of the R.A.P.I.D.® System (the ITI 1-2-3 Flow Kit, the freeze-dried reagents, and the R.A.P.I.D.® 7200 instrument) were straightforward and easy to use. Three separate work areas were needed for testing to minimize cross-contamination. The freeze-dried reagents were color coded, contained all of the necessary components for PCR in one vial, and were reconstituted in the same vial, making PCR setup easy. Reagents for the DNA purification and PCR setup had room temperature storage requirements. The glass capillaries used on the R.A.P.I.D.® 7200 instrument were problematic when the rotors on the carousel were dirty, but posed no problems once the rotors were properly cleaned. The sample throughput for this verification test was 36 to 72 samples per day. Approximate operational times were two hours for DNA extraction/purification, 30 minutes for reconstituting the reagents and loading the capillaries for one carousel batch, and 30 minutes/carousel batch for PCR. The R.A.P.I.D.® 7200 instrument can hold up to 32 capillaries (14 split samples, plus controls). The R.A.P.I.D.® software was easy to use, and additional software analysis tools other than those used in this test are available.

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