Technical Advance

Implementation of a T4 Extraction Control for Molecular Assays of Cerebrospinal Fluid and Stool Specimens

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The use of appropriate extraction and amplification controls for acellular specimens is not standardized in the clinical laboratory community. Extraction controls and checks for inhibitors of amplification in cellular specimens are most often accomplished by amplification of an internal human genomic target. This approach is not feasible for acellular specimens, which may contain little or no amplifiable genomic material. Other specimen types, such as stool, frequently contain amplification inhibitors. Failure to test for these inhibitors can result in the reporting of false-negative results. The goal of this study was to evaluate the use of a T4 bacteriophage as an extraction and amplification control for acellular specimens. The T4 bacteriophage assay was evaluated for use as a control in 290 specimens, including cerebrospinal fluid, serum, and filtered stool. Extraction procedures on two automated instruments were assessed, including the Roche MagNAPure Compact (Roche Diagnostics, Indianapolis, IN) and the QIAGEN BioRobot M48 (QIAGEN, Valencia, CA), along with the manual QIAGEN extraction method. The T4 bacteriophage can be extracted reliably and reproducibly from cerebral spinal fluid, serum, and filtered stool and, therefore, is useful as both an extraction control and inhibitor check for these specimen sources. (*J Mol Diagn* 2008, 10:28–32; DOI: 10.2353/jmoldx.2008.070028)

The use of appropriate extraction and amplification controls for acellular specimens is not standardized in the clinical laboratory community. Inhibitors present in some patient specimens decrease the efficiency of amplification and, if not controlled, can result in the reporting of false-negative results. False-negatives may also arise from undetected extraction failures. Accrediting agen-

cies expect diagnostic laboratories to control for these variables. To meet quality assurance standards recommended by accrediting agencies, a method to detect the presence of inhibitors, while simultaneously providing a control for extraction in acellular specimens, is needed. Extraction controls and amplification inhibitor checks for cellular specimens are most often accomplished by amplification of an internal human genomic target. This approach is not feasible for acellular specimens, which may contain little or no amplifiable genomic material. In our experience, genomic DNA targets are successfully amplified in only approximately 40% of cerebrospinal fluid (CSF) specimens. Other acellular specimen types, such as stool, frequently contain amplification inhibitors.

Several forms of internal controls have been evaluated, including plasmid DNA, ribosomal RNA, armored RNA, and Lambda phage. Plasmid-derived DNA may be degraded before extraction because it is unprotected and subjected to nucleases.¹ To protect the plasmid DNA, further packaging is required in a bacteriophage, which adds more time and expense to the control. Ribosomal 16 or 18S RNA is a convenient control for reverse transcription-polymerase chain reaction (PCR). Armored RNA is a nucleic acid packaged in bacteriophage coat proteins that protect the RNA from ribonucleases. The RNA is released from its protective coat during the extraction procedure and can be used as a control for reverse transcription-PCR.² The disadvantage of this technology is that it is proprietary and expensive, and the reverse transcription process adds unnecessary time and expense to DNA-targeted assays. Additionally, inhibitors of reverse transcription and of PCR cannot be distinguished. Specially engineered Lambda phage DNA fragments have been generated to co-amplify with viral assays, but these fragments are assay-specific, and construction of each synthetic phage is time-consuming.

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These engineered DNA fragments are used to detect the presence of PCR inhibitors during amplification but are not used as extraction controls.³

Unaltered T4 bacteriophage is an attractive alternative, because it is inexpensive to produce, nonpathogenic, and quantifiable. The T4 bacteriophage has a capsid that is structurally complex and composed of more than 1500 subunits that code for approximately 50 different gene products, of which 22 are involved in the tail assembly. The T4 bacteriophage genome is roughly 169-kb long with 289 open reading frames. This assay targets a 163-bp region of the 1.97-kb gp18 tail protein gene, which forms the contractile sheath.^{4,5} The 169-kb T4 bacteriophage genome contains approximately ten times more nucleotide base pairs than a typical plasmid DNA; thus, it should be efficiently extracted. The highly complex T4 bacteriophage capsid effectively protects the encapsulated DNA from nucleases present in biological fluids. Therefore, DNA from phage added directly to a clinical specimen can be efficiently recovered during the extraction process. The T4 bacteriophage can then be used as both an extraction control and a PCR inhibitor control.

The goal of this project was to implement the T4 bacteriophage as a control to evaluate extraction efficiency and detect inhibitors of PCR in acellular specimens, such as CSF, stool, and serum. To achieve this goal, we first identified compatible sample matrices and extraction protocols. The manufacturer's protocol for the T4 bacteriophage DNA detection assay was then modified to run simultaneously with the set of cycling parameters of in-house infectious disease tests [cytomegalovirus, Epstein-Barr virus, and herpes simplex virus (HSV)] onto the ABI 7500 (Applied Biosystems, Foster City, CA) to streamline workflow and minimize equipment requirements.

Materials and Methods

Sample Collection and Sample Size

This study included 290 clinical samples taken for diagnostic purposes at The Nebraska Medical Center (Omaha, NE). There were 205 CSF, 18 serum, and 67 stool specimens. All stool specimens received were diluted 1:3 in saline and centrifuged for 20 minutes at 4000 rpm. The resulting supernatant was filtered through 0.2-micron filters before extraction.

Bacteriophage

Whole-Enterobacteria T4 bacteriophage (Attostar LLC, Medina, MN), received from the manufacturer already in solution, was diluted in 1× TE to a concentration of 4.2×10^5 pfu/ml. Dilutions were stored at -20°C in 50-μl aliquots.

Positive Control Plasmid

T4 Plasmid DNA (Attostar LLC), containing a 163-bp segment of the T4 bacteriophage gp18 tail protein,

served as a positive amplification control and was used to generate a 4-point standard curve. When used as a positive control, 10 μl of 2 pg/ml plasmid was tested in singlet in a separate tube with each independent assay. To generate a standard curve, three 10-fold serial dilutions in 1× TE of stock plasmid (20 pg/ml) were performed. The plasmid DNA was used to assess the performance of each run of the assay.

Extraction of Nucleic Acids

Nucleic acid was extracted from clinical specimens by either manual or automated methods using input and elution volumes according to the manufacturers' recommended protocol. The manual extractions were performed using the QiaAmp DNA Blood Mini Prep Kit (QIAGEN). In brief, 200 μl of specimen (CSF or filtered stool) with 10 μl of T4 bacteriophage (4.2×10^3 pfu/reaction) was extracted, and the DNA was eluted in 200 μl of AE buffer. Two automated extraction instruments were used in this study. Nucleic acid extractions were performed using the QIAGEN BioRobot M48 with the MagAttract DNA Mini M48 kit (QIAGEN). Two hundred microliters of specimen (CSF) and 10 μl of T4 bacteriophage were extracted, and DNA was eluted in 200 μl of RNase-free water. Total nucleic acid extraction was performed on the Roche MagNAPure Compact using the Total Nucleic Acid Isolation kit (Roche Diagnostics, Indianapolis, IN). Four hundred microliters of specimen (CSF or serum) with 10 μl of T4 bacteriophage were used, and the nucleic acids were eluted in 100 μl of elution buffer.

Real-Time Amplification

A 163-bp region of the gp18 tail protein of the T4 bacteriophage was amplified using primers in conjunction with a molecular beacon probe. Four microliters of nucleic acid template, or 4 μl of water for the negative amplification control, was added to 20 μl of a pre-made master mix (Attostar LLC) containing 0.5 μmol/L primers (T4F: 5'-AAGCGAAAGAAGTCGGTGAA-3'; T4R: 5'-CGCTGTCAT-AGCAGCTTCAG-3'), 0.2 μmol/L Probe (5'-CY5-CCACG-GAAATTTCTTCATCTTCCTCTGGCCGTGG-BHQ2-3'), 10 mmol/L Tris, pH 8.0, 4 mmol/L MgCl₂, 50 mmol/L KCl, and 2.0 U of AmpliTaq Gold (Applied Biosystems). The assay was run on the ABI 7500 instrument with the following cycling conditions: 1 cycle of 55°C for 2 minutes, 1 cycle of 95°C for 10 minutes, and 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. This set of cycling parameters is used for the high-volume infectious disease assays in our laboratory. Amplification of T4 was performed in a separate tube.

Specificity of the T4 Bacteriophage

Nucleic acid from 13 previously extracted (not containing T4 bacteriophage) clinical specimens positive for 13 different pathogens [JC virus, HSV, enterovirus, *Bordetella pertussis*, Epstein-Barr virus, human herpes virus 6, adenovirus, norovirus G2, cytomegalovirus, varicella-zoster

Table 1. Performance of T4 DNA Amplification with Three Extraction Methods

Sample source	Extraction method	Mean C_t	SD	Min	Max	N*
CSF	BioRobot M48	32.30	1.11	30.46	34.85	36
CSF	MagNaPure Compact	32.73	1.97	29.55	43.54	169
Serum	MagNaPure Compact	32.37	1.06	30.73	34.96	18
Stool	Manual Qiagen	32.71	1.17	29.82	36.26	64

SD, standard deviation.

*These data include results for the 287/290 specimens with detected C_t values. Three stool specimens found to contain significant levels of PCR inhibition are not included in this table.

virus, parvovirus B19, and BK virus] were tested by the T4 bacteriophage assay. This was to verify that the nucleic acids of the pathogens encountered in clinical samples did not amplify with the T4 bacteriophage primers and probe.

In addition, pathogen-negative specimens were extracted with the addition of T4 bacteriophage and tested with nine viral target assays (HSV, enterovirus, adenovirus, human herpes virus 6, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus, West Nile, and parvovirus B19). The goal of these tests was to show that the T4 bacteriophage did not cause false-positive results on any of these nine viral assays.

Parallel Testing

Forty-six specimens were extracted both with and without the T4 phage to show that T4 phage did not interfere with infectious disease testing. Seventeen specimens were tested for HSV, six specimens for adenovirus, and 23 specimens for enterovirus.

Data Analysis

Analysis was performed using ABI Prism Sequence Detection Software version 1.2.3. (Applied Biosystems). Measurements of fluorescence were taken in real time, with the cycle threshold (C_t) value for each specimen designated as the point at which fluorescence was detected above the background. The baseline was set as 6 to 15, and the threshold was set at 5100. All specimens were tested in singlet.

Statistical Analysis

The C_t from each PCR was recorded. The mean and SD for samples within experiments and the mean and SD for all experiments for the same extraction procedure were calculated using Excel (Microsoft Inc., Redmond, WA). Interassay reproducibility was recorded as the coefficient

of variation (CV), based on the C_t values for all of the sample replicates tested in several study experiments.

Results

Real-Time Amplification of T4 Bacteriophage Targets

The average C_t values obtained for each specimen source and extraction method are presented in Table 1. In summary, the average C_t value for T4 bacteriophage in CSF specimens extracted on the BioRobot M48 was 32.3 (± 1.11) and on the MagNaPure Compact was 32.73 (± 1.97). The average C_t value for stools extracted manually was 32.71 (± 1.17). The average C_t value for serum specimens extracted on the MagNaPure Compact was 32.37 (± 1.06). The most variability in C_t values was seen with CSF specimens extracted on the MagNaPure Compact, with a range of 29.55 to 43.54. The T4 plasmid-positive control (average was 27.92 ± 0.97) had a range of 25.88 to 29.94. The standard curve data are presented in Table 2.

The data in this study show that failure to adequately amplify ($>38 C_t$ to undetected) T4 DNA occurred in 2.8% (8 of 290) of all of the specimens tested. However, analysis by specimen type reveals a failure rate of 4.4% (3 of 67) for stool and 2.4% (5 of 205) for CSF (Table 3). One of the five CSF specimens, which had sufficient volume for re-extraction and an initial C_t value of 40.64, was successfully re-extracted with a final C_t value of 35.28. This result suggests an initial unsuccessful extraction. T4 bacteriophage was initially undetected in three stool specimens. However, on repeat using the same DNA at a 10-fold dilution, the T4 bacteriophage was amplified in all three samples with an average C_t value of 35.2. These

Table 2. Average C_t Values for the Plasmid Standard Curve

	20 pg/ml	2 pg/ml	0.2 pg/ml	0.02 pg/ml
Average C_t	23.34	27.15	30.63	33.95
Standard deviation	0.89	0.46	1.07	0.97
CV (%)	3.82	1.68	3.49	2.85

All data are from seven independent runs.

Table 3. Specimen Failure Rates

Sample source	Extraction method	T4 failure
CSF	MagNaPure Compact	5* of 169 (<3%)
CSF	BioRobot M48	0 of 36
Serum	MagNaPure Compact	0 of 18
Stool	Manual QIAGEN	3† of 67 (4.4%)

*Repeated successful extraction and testing of one specimen revealed the initial T4 failure was due to extraction failure.

†Nucleic acids were diluted 1:10 and retested; T4 bacteriophage result was positive, demonstrating that failure was due to the presence of PCR inhibitors.

Table 4. Quantitative Comparison of Effect of T4 on Viral Detection

Case	HSV C_t value without T4	HSV C_t value with T4	Specimen source
1	33.59	34.34	CSF
2	32.44	32.25	CSF
3	36.80	34.34	CSF
4	35.87	34.47	CSF
5	39.63	36.34	CSF

results suggest inhibition of PCR in these undiluted stool specimens.

Specificity testing showed that all 13 pathogen-positive specimens showed an undetected C_t value on the T4 assay, which confirmed that the phage primers did not amplify these infectious disease agents. This also demonstrated that the T4 bacteriophage was not naturally occurring in these specimens. In addition, none of the nine viral primer sets tested co-amplified T4 bacteriophage. Therefore, false-positive results should not occur with specimens extracted with T4 bacteriophage on these nine assays.

Results from the parallel testing showed that the T4 phage did not interfere with infectious disease testing. The results for all 46 specimens tested were concordant (Tables 4 and 5). Five HSV-positive specimens quantitated by real-time PCR had an average difference of 1.32 C_t and a SD of 1.64 (Table 4). No change in qualitative infectious disease result was seen in any of the 41 specimens tested in parallel (Table 5).

Discussion

A quality control material and a laboratory procedure for evaluation of extraction and PCR efficiency in acellular patient specimens was evaluated and implemented. For cost efficiency and convenience, a naturally occurring T4 bacteriophage was selected as the control material. The phage was shown to function as an effective control in all three of the specimen matrices tested and on all three of the extraction platforms tested. The presence of T4 DNA in the extracted material did not interfere with concurrent infectious disease testing.

The demonstrated consistent recovery and amplification of T4 bacteriophage DNA across all extraction platforms and specimen types enables the laboratory to begin a laboratory-wide process to define acceptable limits for extraction and amplification efficiency. These limits must be independently established for each extraction platform, as extraction efficiencies are reported to vary across platforms.⁶

Extraction failures are distinguished from the presence of inhibitors by repeat testing following 1:10 dilution of the extracted nucleic acid with water. In our experience, this 10-fold dilution decreases the concentration of inhibitors to levels sufficient to allow amplification of the T4 DNA. In contrast, failure to detect T4 DNA after a 1:10 dilution indicates an extraction failure and is verified by re-extraction of the original specimen. Dilution studies confirmed

Table 5. Qualitative Comparison of Effect of T4 on Viral Detection

	With T4	Without T4
HSV		
Positive	5	5
Negative	7	7
Enterovirus		
Positive	20	20
Negative	3	3
Adenovirus		
Positive	2	2
Negative	4	4

that the failures in stool resulted from the presence of inhibitors in stool.

More stringent criteria than “detected” versus “not detected” are desired for evaluation of amplification efficiency. In this study, when a CSF with a T4 C_t value of 40.64 was re-extracted and re-tested, the T4 C_t improved to 35.28, suggesting that efficiency of the initial extraction was poor. In the current studies, the average observed T4 C_t value for all of the specimens tested was approximately 32.75, with a SD of approximately 2 C_t . Based on this information, C_t values for T4 amplification that are unaffected by inhibitors would be expected to range from 30.75 to 34.75. Assuming equivalent amplification efficiency for the T4 assay and all concurrent testing, a delay of 3.3 C_t corresponds to a 10-fold reduction in amplification efficiency. Therefore, our standard laboratory practice is that any specimen, with a T4 bacteriophage amplification curve crossing the threshold after 38.05 C_t , is retested at a 1:10 dilution to determine whether the result is due to inefficient extraction or the presence of an inhibitor.

Uncertainties regarding the final direction of quality control in the molecular laboratory make laboratories hesitant to commit time and resources to develop a control strategy that may not become widely adopted over time. The T4 bacteriophage studied offers an inexpensive external control, at an estimated cost of 3.50 dollars per reaction, for two critical steps in PCR-based testing.

Our results show that the use of the T4 bacteriophage as an extraction control as well as an inhibitor control is very useful in clinical assays. The presence of the T4 bacteriophage does not obviously decrease efficiency of extraction or amplification of infectious disease targets. This is proven by equivalent qualitative results and C_t values for target assays during parallel studies when dual extraction of a specimen was performed with and without spiking in T4 bacteriophage. The use of the T4 bacteriophage facilitates the reporting of truly negative results, instead of false-negatives masked by inhibitors or caused by extraction failures. We found no evidence of false-positive amplification due to naturally occurring T4 bacteriophage in the three specimen sources tested in our laboratory.

In this report, we have shown a protocol for the extraction and amplification of the T4 phage that is adaptable to different extraction platforms and has a set of cycling parameters that can be run simultaneously with multiple infec-

tious disease assays. This can help to streamline the workflow and provide more timely results for busy laboratories.

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