Real-time PCR provides improved detection and titer determination of bacteriophage

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The plaque assay is the traditional method for the quantification of bacteriophage, particularly for λ cloning vectors. Unfortunately, this technique is fraught with procedural difficulties, and the quality of the data obtained from this "gold standard" assay may be inaccurate due to the subjective interpretation of the results. The application of quantitative real-time PCR (QPCR) technology can address these issues and be a more accurate platform to evaluate phage growth conditions and quantify viral titers in phage preparations. QPCR, with an improved primer set specific for λ phage and coupled with fluorescent dye detection of PCR products, was used to detect and quantify phages in lysates with no prior DNA purification. Phages were detected below one plaque-forming unit, and at least 89 viral copies were detected from a purified DNA sample. When unknown concentrations of various phage preparations were assessed using QPCR, they were attained more efficiently, with greater sensitivity and precision, and the method produced more accurate quantitative data spanning a wider linear range than those obtained by the plaque assay (six logs vs. one log, respectively). Finally, QPCR for the detection of phage has multiple applications, including conventional cloning and in alternative fields of study such as environmental sciences.

INTRODUCTION

Bacteriophage λ (λ or phage) has been extensively studied for decades and exists as a model system for the lytic and lysogenic pathways in the life cycle of temperate phages. Sanger et al. reported (1) the complete nucleotide sequence of λ , including descriptions of reading frames and genetic functions for many genes relating to these life cycle phases. The λ remains an established genetic tool for many diverse scientific applications. It has been used routinely as a standard cloning vector (2) and, more recently, as a shuttle vector for the detection of mutations in transgenic fish (3).

Research involving viral systems necessitates precise quantification. Currently, the standard quantitative methodology for phage preparations is the traditional plaque assay (2). However, significant limitations of this method include: (i) the requirement for extensive hands-on time (≥ 5 h) for completion of the assay, at least 1.5 days to produce results, and the need for a large quantity of reagents and supplies; (ii) a limited dynamic range

of one log (30–300 plaques/plate); (iii) media that are susceptible to environmental conditions (e.g., drying and aging), resulting in suboptimal bacterial growth, resulting in decreased phage infectivity; (iv) the dependence of accurate titers upon the viability of the host bacteria, with viability less than 100% causing a reduction in the true titer; (v) the inherent subjectivity and potential error when visually counting plaques; (vi) a lack of reproducibility in titer due to procedural errors from multiple dilution and pipetting steps; (vii) the detection of only functional phage rather than total phage DNA content in the preparation; and (viii) an extremely tedious and cumbersome procedure with a low throughput when assessing multiple clones. To address these limitations, we sought an alternative methodology to replace the traditional plaque assay.

Direct quantification by quantitative real-time PCR (QPCR) is relatively simple, cost-effective, and highly accurate over a wide dynamic range. First proposed by Higuchi et al. (4), this kinetic molecular analysis measures the total fluorescence generated during

each PCR cycle as newly synthesized products are produced. The starting copy number of template is then determined when plotted against a standard curve. The potential advantages for using QPCR to supplant the traditional method of plaque assay include: (i) phage titer results within a few hours; (ii) a significant increase in data generation and sample throughput using 96-well microtiter PCR plates with real-time data processing; (iii) cost savings by the reduction of labor and materials; (iv) a more accurate method of phage quantification, with a greater dynamic range and sensitivity; and (v) the possibility of direct detection of phages in preparations requiring no prior or separate sample preparation. These benefits prompted us to investigate the application of the QPCR methodology in the determination of phage titers.

MATERIALS AND METHODS

Traditional Plaque Assay

Recombinant phages (Lambda FIX[®] II) containing human herpes virus 8 se-

quences (5) were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (McKesson HBOC BioServices, Rockville, MD, USA). After the initial plating on NZY agar plates using XL-1 Blue MRA (P2) host cells (Stratagene, La Jolla, CA, USA), individual plaque plugs were picked to establish stock eluates. Phage plaque assays and liquid and plate lysates, along with routinely used NZY media, SM buffer (100 mM NaCl, 8.1 mM MgSO₄, 50 mM Tris-HCl, pH 7.5, 0.01% gelatin), and LB agar/broth, were performed and prepared, respectively, according to established procedures (2).

Experiments for optimizing phage recovery consisted of varying the quantity of the phage inoculum in relation to a constant amount of host bacteria. All shaking was carried out at 180–300 rpm at 37°C either in a G24 Environmental Shaker (New Brunswick Scientific, Edison, NJ, USA) or on a G10 Gyrotory Shaker (New Brunswick Scientific) located in a Harris Environmental Systems warm room (Andover, MA, USA). Centrifugation of samples was performed at 500–3000× *g* in either a Centra® CL3 (International Equipment Company, Needham Heights, MA, USA) or a CR 412 (Jouan, Winchester, VA, USA) low-speed centrifuge. Incubations of XL-1 Blue bacteria or phage plaque cultures occurred in either the Harris warm room or in a Hybaid Mini-oven MKII (Mid Atlantic Laboratory Equipment Company, Annapolis, MD, USA).

Filter Centrifugation

Due to the large volumes generated from plate lysates, we needed to concentrate the phages before DNA purification. Past experience with polyethylene glycol precipitation (2) found the procedure to be laborious; we therefore attempted a less demanding procedure using filter concentrators. Plate lysates were concentrated in Apollo® high-performance centrifugal concentrators, 70,000 or 100,000 MWCO (Orbital Biosciences®, Topsfield, MA, USA), according to the manufacturer's instructions. Before filtration, the lysates were treated with NaCl (1 M final concentration) to disassociate the phages from the bacterial debris (2).

DNase I Treatment

DNase I endonuclease digestion (6) was used to quantify the contribution of uncoated phage DNA in the lysates that might be adding to the QPCR quantitative signal. The lysates were treated with DNase I (Roche Applied Science, Indianapolis, IN, USA) by combining 25 µL lysate, 20 U DNase I, and LB broth for a final reaction volume of 30 µL. On a Hybaid PCR Express™ thermal cycler (CLP, Franklin, MA, USA), the samples were digested at 25°C for 30 min, and then at 99°C for 15 min to inactivate the enzyme, followed by cooling to room temperature. The digests were stored at -20°C for later evaluation, at which time the samples were diluted 1:200 in SM buffer and analyzed by QPCR.

DNA Extraction

To purify phage DNA, extraction was performed according to the manufacturer's instructions for plate lysates (Lambda DNA Purification Kit; Stratagene). Residual protein contamination was removed using the PUREGENE® DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA), with the following modifications from the manufacturer's protocol. After the addition of two parts Protein Precipitation Solution, the samples were iced for 5 min, and, after the addition of a glycogen carrier (Invitrogen, Carlsbad, CA, USA) and inversion, the samples were stored at -20°C for ≥1 h.

To calculate phage DNA recovery and copy number, spectrophotometric OD_{260/280} measurements of the purified DNA were performed on a DU® 640C spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

QPCR

Primers specific for λ (5' primer, 5'-GATGAGTTCGTGTCCGTACAAGTGG-3'; 3' primer, 5'-GGTTATCGAATCAGCCACAGCGCC-3') have been previously reported (7) and were initially evaluated using Vector NTI® primer design software, version 4.0 (InforMax, Bethesda, MD, USA). However, the 3' primer was modified by the deletion of the two terminal 3' cytosines.

QPCR components consisted of 1×

Platinum® buffer (Invitrogen), 2.5 mM MgCl₂ (Invitrogen), 0.2 µM dNTP (Invitrogen), 0.25 µM each primer (The Biopolymer Laboratory, UMB, Baltimore MD, USA), 50 ng BSA (Sigma, St. Louis, MO, USA), 1.5 U Platinum *Taq* DNA Polymerase (Invitrogen), 10 nM Fluorescein Calibration Dye (Bio-Rad Laboratories, Hercules, CA, USA), and 1:50,000 SYBR® Green I (Molecular Probes, Eugene, OR, USA) in a 50-µL reaction volume. The phage stocks were diluted between 1:50 and 1:1000 in SM buffer, 3-µL portions were analyzed, and dilutions were stored at 4°C. Care was taken to perform the pipetting precisely and, to minimize possible contamination, standard PCR precautions were followed for each step of the procedure.

The amplification and detection of λ PCR products were performed using the iCycler® iQ Detection System (Bio-Rad Laboratories). This system includes the iCycler Thermal Cycler, with real-time detection capabilities, and the iCycler iQ Real Time Detection System Software, which allows for the immediate determination of the cycle threshold (C_t), melting curves, and quantification of unknown samples. The PCR cycling parameters were an initial 1.5 min at 96°C and 1.5 min at 95°C, followed by 40 cycles of 15 s at 94°C and 1 min at 68°C. The protocol concluded with a melting curve program using 0.5°C increasing increments (10 s each; 68°–99°C). Standard curves were established from both purified and quantified phage DNA and from crude phage preparations (lysates) serially diluted over a six-log range.

Precision and Reproducibility Studies

To determine the precision of the QPCR assay, we calculated the coefficient of variations (cvs) of the C_s of 10 replicates of two L36 lysate dilutions (1:100 and 1:1,000,000), representing 140,000 and 1.4 plaque-forming units (pfu).

To determine the inter-run reproducibility of the samples when they are stored over an extended period of time, aliquots of five serial dilutions of the L36 plate lysate, diluted in SM buffer and stored at 4°C, were assayed in

replicate six times over a 3.6-month period. The reproducibility of the assay and stability of the samples over time were analyzed by linear regression.

Statistical Analyses

For the analysis of phage preparations amplified by QPCR, initial calculations of PCR efficiencies, correlation coefficients (*r*), means, and standard deviations (SDs) were performed by the iQ Real Time Detection System Software. Raw data were saved to Microsoft® Excel® spreadsheets for further SD and CV calculations. SPSS for Windows®, release 11.0.1, statistical software (Chicago, IL, USA) was used to perform linear regression analysis for the investigation of inter-run reproducibility. This software was also used to perform Student's paired *t* tests in the comparison of the plaque assay titers to those obtained by QPCR.

RESULTS

Modification of the λ Primer Set

A preliminary evaluation of the original, previously reported primer set (7) showed that the 5' primer possessed little potential self-dimer formation, but that the 3' primer had a significant theoretical propensity for self-dimers, one with a free energy close to -5 Kcal/mol. Additionally, the melting temperature (*T_m*) and %GC differences (6.6°C and 4.0%, respectively) between the primers appeared to be excessive. The deletion of the two terminal 3' cytosines on the 3' primer decreased the theoretical chance of self-dimer formation and provided a more efficient primer set by lowering the *T_m* and %GC differences to 0.9°C and 0.2%, respectively. When empirically comparing the performances of the original and the improved primer sets, their respective PCR efficiencies were 88.9% and 98.5%. Additionally, the improved primer set detected an additional replicate of a 10⁻⁸ dilution of the phage standard, which suggested an increase in PCR sensitivity (data not shown).

A homology search in GenBank® using these primers showed that they are 100% homologous to wild-type λ, to the EMBL3 λ cloning vector (Strata-

Table 1. The Effects of DNase I Treatment on Filter Concentrate Fractions

Identifier	Mean C _t	Mean SQ (pfu)	sd SQ	Mean SQ Reduction (%)
L36 neat, no DNase I	14.46	8.61E + 03	6.65E + 02	—
L36 neat, plus DNase I	16.66	2.04E + 03	1.39E + 02	76.3
L36 retentate, no DNase I	17.91	9.03E + 02	2.96E + 01	—
L36 retentate, plus DNase I	19.68	2.84E + 02	5.72E + 00	68.5
L36 eluate, no DNase I	22.11	5.83E + 01	1.09E + 00	—
L36 eluate, plus DNase I	26.22	3.99E + 00	3.65E-01	93.2
L54 DNA, no DNase I	28.18	1.17E + 00	2.60E-01	—
L54 DNA, plus DNase I	N/A	N/A	N/A	100

SQ, starting quantity; L54 DNA, positive DNase I control; and N/A, below threshold and therefore not quantifiable. Reactions were run in duplicate.

gene), to the published sequence of λ TXF97 (8), and, evidently, to the Lambda FIX II vectors used in our study. The position of the 5' primer is located at the E gene, a capsid protein, and the 3' primer is located at the Fi gene involved in DNA packaging; because none of these sites would be disrupted with a modification of the λ cloning vector, it is reasonable to assume the primers will recognize all λ vectors.

The Sensitivity, PCR Efficiency, and Linearity of the Phage QPCR Method

Serial dilutions of a plaque assay titrated, plate lysate stock (clone L36) were amplified by QPCR with no prior DNA extraction. The exponential amplification curves showed the expected three cycle increase per log reduction of starting phage (data not shown). The sensitivity of the detection of λ in the crude lysate was at least 0.014 pfu (Figure 1A). Conversely, the traditional plaque assay can only detect the presence of λ at one pfu per plate. The standard curve generated from the L36 dilutions exhibited a PCR efficiency of 94% and an *r* of 0.998 over a six-log range extending from 14,000 to 0.014 pfu (Figure 1A). This is in contrast to the single-log range (30–300 pfu) of the traditional plaque assay. Melting curve analysis of the serially diluted samples displayed one melting peak specific to the amplified λ PCR product (93.5°C), with the absence of primer dimer formation, even at the highest dilution (Figure 1B).

Using serial dilutions of purified λ DNA from clone L54 diluted in SM buffer, a QPCR standard curve was generated from reactions run in duplicate. The molecular assay was able to detect at least 89 λ copies, exemplifying the excellent sensitivity of the assay. The standard curve displayed a PCR efficiency of 99.6% and an *r* of 1.000 over a six-log range (89–89,000,000 copies).

The Precision and Inter-Run Reproducibility of the Phage QPCR Method

The low CVs of the mean sample C_s exemplified the precision of the QPCR method. For example, the 1:100 sample of a L36 phage stock had a mean C_t of 16.88 (16.25–17.73) with a CV of 2.64%. In addition, the mean of the 1:1,000,000 standard was 30.61 (30.00–31.17) with a CV of 1.31%. In contrast, for the traditional plaque assay, the precision was less. In an experiment using six or seven replicate plates for each of three different expectant titers (155, 78, and 39 pfu/plate), the mean observed titers were 192, 76, and 39, with CVs of 7.6%, 14.9%, and 7.9%, respectively.

The inter-run reproducibility of the QPCR assay was determined by analyzing aliquots of five serial dilutions of a L36 standard over a period of almost four months (data not shown). Linear regression analysis showed that six separate experiments over the 3.6 months of storage did not significantly impact C_ts in any of the five different dilutional groups (*P* = 0.108).

The Contribution of Uncoated λ DNA to the QPCR Signal

We determined the extent of the quantitative signal associated with uncoated phage DNA that does not represent complete phage particles or a pfu by comparing DNase I-treated phage samples to untreated phage samples via QPCR. Treatment with DNase I shifted the C_t s approximately 2.5 cycles to the right as compared to untreated phage samples, indicating the presence of approximately one additional log of uncoated phage DNA in the lysate (Figure 2). However, the ability to amplify and quantitate the phages in the sample after DNase I treatment indicated the presence of DNA resistant to the endonuclease, presumably derived from functional phages.

These findings are illustrated by fractions obtained from the filter concentration of plate lysates. Table 1 shows that 76.3% of the target DNA in the neat lysate was uncoated. However, 7.8% less DNA in the filter retentate

(68.5%) was uncoated, with a greater amount of uncoated DNA in the eluate (93.2%), which indicated a strong preferential retention effect by the filter membrane for whole virion as opposed to uncoated DNA.

Comparison of Phage Titters by QPCR Versus the Traditional Plaque Assay

The phage titer in three liquid lysates was determined using the traditional plaque assay (range = 5.64×10^4 – 7.27×10^4 pfu/3 μ L; avg = $6.31 \times 10^4 \pm 8545$) and then compared to the titer, as determined by QPCR using two different lysate standards (I and II) previously titered by plaque assay; all with and without DNase I treatment, each assayed in duplicate. When no DNase I treatment was used for standards I and II, QPCR titers (pfu/3 μ L) were on average $5.96 \times 10^4 \pm 7750$ and $5.86 \times 10^4 \pm 12,619$ for untreated samples but $1.40 \times 10^4 \pm 2887$ and $1.08 \times 10^4 \pm 2481$ for DNase I-treated samples. Alternatively,

whereas standards I and II were DNase I-treated, QPCR titers were on average $3.39 \times 10^5 \pm 70,002$ and $3.29 \times 10^5 \pm 43,000$ for the untreated samples but $6.38 \times 10^4 \pm 14424$ and $7.54 \times 10^4 \pm 15,647$ for DNase I-treated samples. A Student's two-tailed t test was used to calculate any significant differences in pfus from the expected to the observed titers; all titers were normalized to 3 μ L of diluted sample. The highest correlations of titers between the two assays were between untreated samples plotted against untreated standards and between DNase I-treated samples plotted against DNase I-treated standards; each comparison generated no significant differences in titer ($P = 0.106$ – 0.862). However, the titer disparities were significantly different when comparing DNase I-treated samples to untreated standards and vice versa ($P = 0.005$ – 0.017). Both standards produced equivalent data.

Applications of the QPCR Assay

To determine optimal phage yield from a liquid lysate, XL-1 Blue host bacteria (100 μ L) were inoculated with four different amounts of phage plug eluate (30, 100, 200, and 300 μ L). Each condition was assessed by QPCR, and the starting quantity of each preparation was determined using a standard curve (Figure 1A). Replicates of the 100- μ L inoculum produced the highest titer with a mean of 8.17×10^3 pfu/3 μ L diluted sample, whereas the 30, 200, and 300 μ L inocula had titers of 6.39×10^3 , 4.66×10^3 , and 2.90×10^3 , respectively. The SD and CV of the 100- μ L inoculum were 1.39×10^2 and 1.7%, respectively.

The quantity of phage DNA in neat, retentate, and eluate fractions generated via filter concentration from a liquid lysate was determined using a purified phage DNA standard curve. The results indicated that approximately 10% of the DNA (84.5 μ g) was found in the retentate. However, approximately 90% (736 μ g) of the phage DNA present in the 3 mL original lysate was lost during the procedure, with only a minute portion (2 μ g) found in the eluate.

DISCUSSION

Our data indicate that QPCR per-

Figure 1. Sensitivity, PCR efficiency, and linearity in the determination by QPCR of phage titers in a liquid lysate. (A) The standard curve, derived from serial dilutions of a previously titered lysate (by plaque assay), is linear over six logs. Replicate unknown samples are of L36 lysates generated from different amounts of inocula (30, 100, 200, and 300 μ L) from phage plug eluates. (B) The post-amplification melting curve analysis of PCR products. The graph represents L36 lysate reactions plotted as the negative first derivative of the temperature versus fluorescence ($-dRFU/dT$) against temperature, representing a function of the decrease in SYBR Green I fluorescence due to increasing temperature.

formed directly on phage preparations is superior to the traditional plaque assay for the quantification and detection of λ phage. The advantages, which are numerous, include a significant increase in sensitivity, efficiency, accuracy, and linear quantitative dynamic range. In addition, the precision of the assay is excellent, having cvs below 2%, which are much lower than those of the traditional plaque assay (7%–14%). Thus, we have shown that QPCR is a more accurate method for the quantification of phage titers. Excluding human error, samples can be assayed with confidence without resorting to duplicate reactions, thereby saving considerable cost and labor. Additionally, reproducibility of the test and long-term stability (≥ 3.6 months) of the phage target were established, demonstrating the high quality of the method.

Several authors have amplified λ with a commonly used set of primers (3,7,9), but these primer sequences do not possess the necessary measure of proficiency that is essential for reliable and accurate results. We showed that by truncating the 3' primer, the modified primer set could improve the efficiency of the QPCR by approximately 10%, with the complete absence of primer dimers. Although specific λ clones were used in this study, they were only a convenience sample, and this method should work with any phages possessing the same primer sites. For those phages that do not, we are exploring the development of specific or consensus primers to other species, perhaps in a multiplex QPCR format for use in such diverse applications as detecting phages in wastewater, recycling, and environmental samples. Phages have been reported as surrogate markers for the presence of human pathogens in these environmental sites (10–12).

The QPCR assay was able to detect λ phage as low as 0.014 pfu. Using a nested PCR procedure, Puig et al. (11) also showed that a molecular method could detect between 0.1 and 0.01 pfu purified HSP40 phages in sewage and seawater. These data suggest that traditional plaque assays are orders of magnitude less sensitive than PCR. We postulate that either PCR is detecting uncoated phage DNA, which our data support, and/or plaque assays are quite

inefficient at providing the optimal growth conditions for the detection of all functional phages being assayed.

We were initially unaware whether phages would amplify directly from lysates with no prior DNA purification because the phage capsids would need to be ruptured, and the components of the SM storage buffer and lysate (e.g., cellular debris) might inhibit the QPCR assay. We observed excellent amplification and no indication of inhibition when the phage samples were simply heat-treated as part of the initial denaturation step in the QPCR amplification. In support of our findings, two reports documented that heat treatment is efficacious with the PCR screening of phage libraries (13,14). However, unlike the QPCR method in which heating is incorporated into the cycling program itself, both previous reports performed the specimen preparation as a two-step procedure. Additionally, Towers et al. (15) described a direct

quantification of recombinant retrovirus via QPCR from culture supernatant with no extensive pretreatment.

We assessed whether the quantitative signal of the phage DNA was due solely to DNA with a capsid coat or, perhaps, uncoated DNA present in the media contributed to this signal. The data showed that a large proportion of the signal (>68%) was from uncoated DNA, and we theorized that accurate determinations of functional phage pfu titers via QPCR required the prior DNase I digestion of these uncoated DNA. However, in contrast to our expectations, the data indicated that a plaque assay titer shows excellent correlation with lysate quantified by QPCR if the standard and sample are treated similarly (i.e., DNase I digestion is not necessarily required for accurate titers of functional phages). This was surprising, and further investigation is currently under way in an attempt to explain this finding, although

it might be due to the relative shift of sample C_s in relation to the shift of the standard curve after DNase I treatment.

It must be noted that the lysate tested in the DNase I experiment described earlier had been stored at 4°C for several weeks with unknown subsequent quantitative effects. It is common knowledge that plaque assay titers decrease with time when stored at 4°C, but possibly the phage DNA remains intact outside of the capsid. This hypothesis is supported by our findings of the good stability of the plate lysate standard over time. Further experiments will be required to determine the extent of the uncoated DNA, and whether growth and storage conditions affect the amount of the signal that is derived from the specific DNA. However, if phage are being cultivated only for their DNA content, then DNase I treatment is unnecessary and even undesirable. We speculate that it is unlikely that uncoated phage DNA will be defective in the cloned gene of interest because the defect would most likely reside in a normal biological function of the phages themselves.

One practical application of this technology is for the optimization of λ growth conditions, such as in liquid lysates, to harvest the largest number of phages. Of four inocula from a phage plug eluate that we investigated, the 100- μ L inoculum produced maximal titers; most likely because it presented the best ratio of host to infecting phage. The two other higher volume inocula caused the culture to lyse too rapidly, thereby not allowing sufficient replication of phages to occur before the total lysis of their bacterial hosts, which resulted in lower titers.

There were many variables to consider when optimizing phage yield from lysates. For example, to determine the best growth conditions using the plaque assay for a set of seven viral genomic λ clones with four different phage inocula each, and using two different dilutions of lysate in duplicate, it would require 112 agar plates. The amount of time and reagents necessary would be considerable, requiring up to three separate plaque assays and a week of associated labor. QPCR, however, provided a quantitative determination of the optimal lysate conditions in the same day

with only 56 PCRs (including standard/control wells) in about 2.5 h of hands-on time. The use of QPCR resulted in saving hundreds of dollars in labor costs for this experiment alone. Furthermore, a shortened assay time will allow researchers to perform multiple experiments in one day, an achievement that is almost impossible with traditional plaque assays. Many laboratories with high-throughput testing would benefit from this method.

There are other unique advantages to phage quantification by QPCR. Often phages are grown for isolation of their cloned DNA insert and, because plaque assays only detect functional phages, they are not the correct method for estimating the total amount of phage DNA in a preparation before DNA purification. Our data showed that the QPCR could quantify the amount of phage DNA in crude lysates and in filter concentrate fractions and was useful for determining the loss and recovery of phage DNA from filtered stock preparations. Although filter concentration was a relatively quick method to concentrate the phages, it was not particularly efficient, most likely due to excessive binding of the virus to the filter membrane. Preblocking of the filter membrane might prove to be efficacious for this application.

Other authors have reported supportive data that remain significantly different from our methodology. Lunde et al.

(16) used purified λ DNA as the analyte in competitive PCR but targeted specific cut-and-join sites in the phage genome. These sites and not the number of phages were quantified using a fluorescently tagged primer. Unlike QPCR, the authors detected PCR products only after analysis on an ABI PRISM® 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Winn et al. (3) used λ DNA as the starting template to detect via qualitative PCR the presence of λ in transgenic fish tissues. Utilizing a second primer set along with a fluorescent probe against a transgene target, *lacI*, they performed QPCR to quantify the number of integrated λ copies. Although their work is similar to the method presented here, the differences are significant. We contend that no DNA extraction is necessary for analysis by QPCR, well-characterized phage lysate preparations can act as standards, and a fluorescent probe is unnecessary. Most importantly, the *lacI* transgene is not found on all λ vectors, which makes those primer and probe sequences inappropriate for general λ QPCR. Finally, in 1994, Kuehnelt et al. (7) published one of the first reports on the quantification of λ DNA by PCR. Because this predated the advent of QPCR, our proposed method adds a technical advancement to our ability to quantify and detect phages efficiently and rapidly. Thus, the QPCR method presented here is significantly different from previous studies, requires

Figure 2. PCR amplification plot of untreated versus DNase I treated phage stock. Note the right shift of the curves due to the diminished number of targets after the DNase I treatment of the phage stock. The signal from the purified λ DNA positive control was completely abolished by treatment with DNase I.

no special specimen preparation, and exists as the simplest and most direct state-of-the-art methodology for the quantification and detection of phages at a lower cost and time requirement.

Companies that create and market expression, cDNA, genomic libraries, and phage display kits could successfully utilize this QPCR method. National repositories may find this technology useful for their upkeep and replenishment of bacteriophage stocks, as would biotechnology companies that develop and market λ vectors and kits. The assay could also be applied to diagnostics in the dairy industry, where there is interest in protecting milk from phage contamination before fermentation or curdling procedures (17). Financial losses occur to dairy farmers when the bacterial starter cultures do not grow due to phage infection (18). Field-deployable QPCR units are already in existence (19) and would complement our method for the survey of phages at field sites. However, in this application, the quantification of phages might not be as critical as the sensitivity required for the detection of low numbers of phages. Our preliminary experiments have shown that QPCR is able to detect at least 89 λ viral copies in a 3- μ L sample.

Another novel application of this effective method could be in the in vivo quantification of phages used in bacteriophage therapy. This distinctive therapy, long used in the Eastern European medical field, is now gaining interest in the Western Hemisphere as a solution to the increasing antibiotic resistance exhibited by infectious bacteria (20,21). The use of a QPCR platform to accurately determine the amount of phage in a therapeutic dose and to monitor pharmacometrics would be a valuable test in patient care in this new medical arena.

For researchers and institutions that utilize phages in their biotechnological advancement, QPCR technology promises an economical ease of use and accelerated turnaround time previously unattained by the traditional plaque assay. These advantages are especially applicable for investigators familiar with the technology of QPCR and who have the hardware and expertise in place. Furthermore, the assay excels in the generation of accurate quantitative data more efficiently and sensitively

than previous methods used for the determination of phage titers. QPCR has multiple applications to other industries, for which it can provide a highly sensitive and extremely rapid detection platform for phages from many diverse sample sources.

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