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Quantification of M13 and T7 bacteriophages by TaqMan and SYBR Green qPCR

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Abstract

TaqMan and SYBR Green quantitative PCR (qPCR) methods were developed as DNA-based approaches to reproducibly enumerate M13 and T7 phages from phage display selection experiments individually and simultaneously. The genome copies of M13 and T7 phages were quantified by TaqMan or SYBR Green qPCR referenced against M13 and T7 DNA standard curves of known concentrations. TaqMan qPCR was capable of quantifying M13 and T7 phage DNA simultaneously with a detection range of $2.75 \times 10^1 - 2.75 \times 10^8$ genome copies(gc)/ μ L and $2.66 \times 10^1 - 2.66 \times 10^8$ genome copies(gc)/ μ L respectively. TaqMan qPCR demonstrated an efficient amplification efficiency (E_s) of 0.97 and 0.90 for M13 and T7 phage DNA, respectively. SYBR Green qPCR was ten-fold more sensitive than TaqMan qPCR, able to quantify $2.75 - 2.75 \times 10^7$ gc/ μ L and $2.66 \times 10^1 - 2.66 \times 10^7$ gc/ μ L of M13 and T7 phage DNA, with an amplification efficiency E_s of 1.06 and 0.78, respectively. Due to its superior sensitivity, SYBR Green qPCR was used to enumerate M13 and T7 phage display clones selected against a cell line, and quantified titers demonstrated accuracy comparable to titers from traditional double-layer plaque assay. Compared to enzyme linked immunosorbent assay, both qPCR methods exhibited increased detection sensitivity and reproducibility. These qPCR methods are reproducible, sensitive, and time-saving to determine their titers and to quantify a large number of phage samples individually or simultaneously, thus avoiding the need for time-intensive double-layer plaque assay. These findings highlight the attractiveness of qPCR for phage enumeration for applications ranging from selection to next-generation sequencing (NGS).

Keywords

qPCR; TaqMan; SYBR-Green; bacteriophage; M13; T7; plaque assay

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1. Introduction

Bacteriophages (phages) are viruses that infect host bacteria to propagate, and their infection is strain-specific such that each phage only infects a narrow range of strains from the same bacterial species (e.g. *E. coli*) (Penadés et al., 2015). Phages have been exploited for diverse applications such as antibacterial therapeutics, tools in molecular biology, templates for material assembly, and display technologies to identify molecular recognition motifs (Pires et al., 2016). In particular, lysogenic M13 and lytic T7 phage have been developed as genetically modifiable vectors for phage display (Barry, 1996; Pasqualini and Ruoslahti, 1996; Rosenberg et al., 1996). With phage display, exogenous gene sequences encoding proteins, single-chain antibodies, peptides, or a library of peptides, are engineered into the phage genome such that they will be displayed on the surface of the phage (i.e. on their coat protein); for peptide and antibody libraries, each phage will display a different peptide or antibody fragment (Smith and Petrenko, 1997). These resulting phage libraries are screened against target proteins or receptors on tissues of interest; bound phages are eluted, propagated in bacteria to make more copies, and re-screened against the target. This iterative process of biopanning allows selection of a few peptides or antibodies that demonstrate the greatest affinity for the target. During biopanning, it is critical to precisely enumerate M13 and T7 bacteriophages used to determine the amount of phages binding to the target and the enrichment of selected phage-presenting peptides during biopanning (Rodi and Makowski, 1999).

Double-layer agar plaque assay (Cornax et al., 1990) is the classical enumeration method to quantify phages based on their infectivity. Here, phages are co-incubated with host bacteria to allow for infection, mixed with nutrient-rich soft agar, and overlaid on a solid agar plate for bacterial growth. Non-infected bacteria will form a lawn of confluent bacteria on the agar substrate; amongst the layer of bacteria, there will be areas of diminished cell growth or lysed cells due to M13 or T7 infection, respectively. These infected areas form visible circular regions, or plaques. The plaques are counted and the concentration of these plaque forming units per milliliter or per microliter (pfu/ml or pfu/μL) are equivalent to the concentration of phages. While plaque assay is the gold standard to quantify the number of phages, the assay depends on the infectivity of phages with bacteria. As a result, there is a potential discrepancy between phages enumerated by plaque assay and the actual number of phages, including non-infective or prematurely degraded phage. Even though T7 and M13 phages are generally stable at a broad range of pH and temperature, their ability to infect host bacteria can deteriorate during amplification, high-speed centrifugation or long term storage (Rakonjac et al., 2011; Steven et al., 1988). Also, the plaque assay is time-consuming (up to ~18 – 24 hours) and can have variable reproducibility. Therefore, more time-efficient and accurate methods are required for quantification of phages.

To enumerate phages, quantitative PCR (qPCR) approaches have been developed to quantify the copies of phage genome, which are equivalent to the number of phages (i.e. one phage encapsulates a single genome). qPCR is a routinely used method for the detection and quantification of gene expression of numerous viruses (Hawkins and Guest, 2017). For example, qPCR methods have been developed to detect and quantify bacteriophages in water samples. A multiplex qPCR method was developed to detect and genotype F⁺ RNA

bacteriophage in water and shellfish (Wolf et al., 2008). qPCR was also used to detect PP7 bacteriophage along with human pathogenic virus enterovirus and adenovirus in California storm water (Rajal et al., 2007). Also, Liu et al used TaqMan Array Card to detect 19 enteropathogens simultaneously in stool samples (Liu et al., 2013). M2 phage and T4 bacteriophage have also been detected and quantified by qPCR (Farkas et al., 2015; Fittipaldi et al., 2010). All these studies demonstrate the feasibility of qPCR as a technique to enumerate M13 and T7 bacteriophages. Building on these studies, the goal of this study was to develop qPCR approaches to detect M13 and T7 phages from biopanning experiments individually or simultaneously and to validate that qPCR is an accurate and efficient method to quantify phages.

2. Materials and Methods

2.1 Materials for qPCR

M13KE DNA (Catalog number: N3541S, Lot number: 0061506) was purchased from New England Biolabs (NEB). T7Select packaging control DNA (Cat. # 69679-1UG, Lot #: D00167945) was purchased from EMD Millipore. MicroAmp™ optical 96-well reaction plate (Cat. # N8010560) and MicroAmp™ optical adhesive film kit (Cat. # 4313663) were obtained from ThermoFisher Scientific.

For biopanning, M13 Ph.D.-C(X)₇C library (Cat. # E8120S), where M13 phage display cysteine constrained random 7-mer peptides on the N-terminus of the p3 coat protein, was purchased from NEB. T7Select 415-1 Cloning Kit (Cat. # 70015, EMD Millipore), was used to engineer random cysteine constrained 7-mer peptides on the C-terminus of the 10B coat protein of T7 phage. To generate the random peptide library displayed on T7 phage, random oligonucleotides (5' TGC (NNK)₇ TGC 3') encoding for C(X)₇C were inserted into T7 phage DNA via EcoRI and HindIII restriction sites following manufacturer's recommendations. DNase I solution (Cat. # 90083) was purchased from ThermoFisher Scientific. UltraPure DNase/RNase-Free Distilled H₂O was purchased from Invitrogen (Ref. # 10977015). PCR reactions were run on ViiA7 Real-Time PCR System (Applied Biosystems), and QuantStudio software was used to analyze qPCR raw data.

2.2 Methods for qPCR

2.2.1 DNA sample preparation—M13KE double stranded DNA (dsDNA) (NEB, 7222 bp, concentration 1 µg/µL) was used as a standard for the calibration curve to quantify M13KE genomic DNA at 10-fold dilutions from 0.01 fg/µL - 10⁶ fg/µL. T7 packaging control dsDNA (Millipore Sigma, 37314 bp, 0.1 µg/µL) was diluted into a serial concentration of 1 fg/µL - 10⁷ fg/µL, which was used to prepare the standard curve for titers of genome copies of T7 phages. To convert phage standard DNA concentration from fg/µL to genome copies per microliter (gc)/µL, the following equation was used: [genome copies(gc)/µL] = [dsDNA g/µL]/[DNA size (bp)*607.4+157.9]*(6.02*10²³). Dilution factor was included in the concentration calculation (Lima et al., 2017; Lock et al., 2014; Tourinho et al., 2015).

For samples, their DNA was prepared for qPCR from purified phages. M13KE and T7 phages were amplified following manufacturers' protocols. After amplification, M13KE and T7 phages were precipitated overnight with 1/5 volume PEG 8000/20% 2.5 M sodium chloride and then centrifuged at 11627g for 15 minutes. The resulting phage pellets were resuspended in phosphate buffer saline (PBS, Corning) and diluted into eight-serial ten-fold concentrations. Before isolating DNA from phages for qPCR, it was necessary to remove residual, free DNA that is not from intact phage particles (i.e. DNA floating from degraded or ruptured phage particles). This pre-treatment minimizes the discrepancy between quantification of phage genome copies by qPCR and phage infectivity by plaque assay. To remove residual DNA, phage samples were pre-treated with DNase I. Five units of DNase I (2500 units/mL) were added to 200µL of each diluted concentration of T7 and M13KE phage samples, and then incubated at 37°C for 10 minutes. To isolate DNA from phage particles without the need for additional purification steps (e.g. spin column purification kit), DNase I pre-treated and non-treated M13KE or T7 phage samples were heat-denatured at 100°C for 15 minutes (Famm et al., 2008; Fittipaldi et al., 2010; Lock et al., 2014). After, each concentration of denatured M13KE and T7 phage samples were prepared for qPCR. The workflow of the qPCR sample preparation and double layer plaque assay is shown in Fig. 1.

2.2.2 TaqMan qPCR—Probe-based qPCR Master Mix, PrimeTime Gene Expression

Master Mix (Cat. # 1055770) was purchased from IDT. Primers and probe (IDT) for M13KE bacteriophage DNA were forward 5'-ATG GTA ATG GTG CTA CTG GTG-3', reverse 5'-GAC AAA AGG GCG ACA TTC AAC-3' and probe 5'-6-FAM/AAT GGC TCA/ZEN/AGT CGG TGA CGG T/3'IABkFQ. The probe was the complement sequence to the M13KE DNA template with 5' reporter dye FAM and dual quenchers (internal ZEN and 3' IBFQ). The primers and probe for T7 bacteriophage DNA were forward 5'-TGG ATG GGA TAA CTG GTA AGC-3', reverse 5'-TGG TTC TTA GTG TGG ATG TCG-3' and probe 5'-HEX/TGG CTC ACT/ZEN/TCA TGG CTC GCT TT/3'IABkFQ (reporter dye 5'HEX). The 20 µL PCR reaction mixture was prepared with 10 µL PrimeTime gene expression master mix, 1 µL mix of 250 nM probe and 500 nM phage sequence-specific primers for M13KE phage DNA, 1 µL mix of 250 nM probe and 500 nM primers for T7 phage DNA, 2 µL denatured M13KE phages, 2 µL denatured T7 phages and 4 µL UltraPure dH₂O. PCR cycling conditions were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min.

2.2.3 SYBR Green qPCR—PowerUp SYBR Green Master Mix (Cat. # A25742) was

bought from ThermoFisher Scientific. Primers (IDT) for M13KE phage DNA were forward 5'-CAC CGT TCA TCT GTC CTC TTT-3' and reverse 5'-CGA CCT GCT CCA TGT TAC TTA G-3'. Primers for T7 phage DNA were forward 5'-CCT CTT GGG AGG AAG AGA TTT G-3' and reverse 5'-TAC GGG TCT CGT AGG ACT TAA T-3'. A 10 µL PCR reaction mixture was prepared with 5 µL 2x SYBR Green Master Mix, 1 µL 500 nM primers, 2 µL UltraPure dH₂O and 2 µL denatured M13KE phages or T7 phages. PCR cycling conditions were 50°C for 2 min, 95°C 2 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by the melt curve setting of 1 cycle of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s.

2.3 Double layer plaque assay of M13KE and T7 phage

Double layer plaque assay of M13KE and T7 phage was performed following the NEB protocol (<https://www.neb.com/protocols/2014/05/08/m13-titer-protocol>) and Novagen T7Select System Manual (http://www.emdmillipore.com/US/en/product/T7Select-415-1-Cloning-Kit,EMD_BIO-70015#anchor_USP), respectively.

3. Statistical analysis

Statistical analysis was performed using two-way ANOVA to compare enumeration of M13 and T7 phages from qPCR to double layer plaque assay. A value of $P < 0.05$ was considered statistically significant.

4. Results

4.1 TaqMan qPCR

In a single PCR reaction, TaqMan qPCR can quantify genome copies of M13KE and T7 phages simultaneously using two fluorescent probes (FAM and HEX) chosen for M13KE and T7 phage DNA. Denatured M13KE and T7 phages were mixed together and along with M13KE and T7 DNA standards, were prepared in separate PCR reactions, and the cycle threshold (Ct) values were acquired from each PCR reaction. Ct is the cycle number at which the fluorescence signal from amplification exceeds the fluorescence threshold, which is indicative of amplification of the nucleic acids (i.e. DNA of phage genomes). The amount of M13KE and T7 phage DNA can be interpolated from the standard curves constructed from eight serial concentrations for M13KE and T7 standard DNA samples, respectively; the amount of genome copies of phages from samples can approximate the number of phages.

We determined the sensitivity of qPCR to quantify M13KE DNA. Converting concentration from fg/ μ L to genome copies (gc)/ μ L, M13KE DNA standard with concentrations of 0.1 fg/ μ L - 10⁶ fg/ μ L was 2.75*10¹ – 2.75*10⁸ genome copies(gc)/ μ L. Logarithm transformation of DNA concentration (log gc/ μ L)) for M13KE was 1.4 – 8.4. At the given concentrations, Ct values were 33.1 – 9.6 for M13KE phage DNA. Linear regression of Ct against log (gc/ μ L) for M13KE phage DNA was $Y = -3.386 \cdot X + 38.20$ (Fig. 2A, $R^2 = 0.9996$). To determine the amplification efficiency (i.e. the efficiency of PCR to make 2ⁿ copies per n cycles), the following equation was used:

$$E_s = 10^{-(1/\text{slope})} - 1 \quad (\text{Rutledge and Côté, 2003}), \quad (1)$$

where E_s is the amplification efficiency (e.g. 100% amplification efficiency has $E_s = 1$). Since the slope was 3.386, the calculated E_s was 0.97. The standard deviation (SD) of Ct of the triplicate samples of M13KE DNA at all eight concentrations ranged from 0.013–0.362 cycles, with an average of 0.153 cycles. Since each phage genome DNA can be treated as a single molecule, the DNA molecule deviation (i.e. deviation of phage genomes) was calculated per following equation:

$$\pm\% \text{ molecules} = [(E_s + 1)^{SD} - 1] * 100\% \text{ (Rutledge and Côté, 2003)} \quad (2)$$

With $E_s = 0.97$ from previous calculation, the DNA molecules deviation was ± 0.88 to $\pm 27.9\%$, with average $\pm 11.0\%$.

The standard curve of M13KE phage DNA was used to quantify the unknown concentration of DNA isolated from heat denatured M13KE phages (see **Materials and Methods**). To improve the accuracy of quantification of genomic DNA from intact phages, samples were pre-treated with DNase I to remove non-encapsulated DNA or DNA from phages that degraded during amplification, purification, and storage. In parallel, double-layer plaque assay was run to compare the number of infective phages (pfu/ μ L) with the number of phages quantified by qPCR (gc/ μ L). M13KE WT phages were prepared in serial ten-fold dilutions from $\sim 10^1$ – 10^8 pfu/ μ L in PBS from the same stock of M13KE WT phages to determine the detection sensitivity of TaqMan qPCR. The comparison between quantified phage genome copies and plaque assay is shown in Fig. 3A. As stated earlier, since one M13KE phage particle encapsulates one copy of genomic DNA, genome copies (gc/ μ L) are equivalent to plaque forming units (pfu/ μ L). From two-way ANOVA between qPCR with and without DNase I pre-treatment and plaque assay (Fig. 3A), there were significant differences between M13KE phage titers enumerated by qPCR and plaque assays. At the highest concentrations of $\sim 10^8$ / μ L, qPCR titers of M13KE phages without DNase I pretreatment (G1) was 6.9-fold higher than qPCR titers with DNase I pretreatment (G2) and 3.6-fold higher than plaque assay (G3) (Fig. 3A). G3 had two-fold higher titers than G3. These findings suggest that there was a significant effect of DNase I pre-treatment on qPCR quantitation.

Next, we quantified the sensitivity of TaqMan qPCR for T7 phage DNA. Standard T7 DNA concentrations ranged from 1 fg/ μ L - 10^7 fg/ μ L, which are equivalent to $2.66 * 10^1$ – $2.66 * 10^8$ gc/ μ L (1.42 – 8.42 log (gc/ μ L)). At given concentrations, Ct of T7 phage DNA was 33.9 – 8.9 . The linear regression of Ct against log (gc/ μ L) for T7 phage DNA (Fig. 2B) was $Y = -3.588X + 38.86$; $R^2 = 0.9992$. E_s was 0.90 . The SD of Ct ranged from 0.032 to 0.164 cycles for all eight concentrations, with an average of 0.089 cycles. The respective DNA molecule deviation ($\pm\%$ molecules) was $\pm 2.07\%$ – $\pm 11.1\%$, with a mean value 5.88% .

The standard curve in Fig. 2B was used to quantify genome copies of T7 phages at unknown concentrations. Using the same T7 WT phage stock solution, all eight concentrations ($\sim 10^1$ – $\sim 10^8$ pfu/ μ L, denoted as E1–E8) were diluted with PBS buffer. At E7 and E8, there were significant differences between the enumeration of T7 phages by qPCR with and without DNase I pre-treatment (G1, G2) and plaque assay (G3) (Fig. 3B). Samples without DNase I pre-treatment (G1) had significantly higher titers than qPCR with DNase I pre-treatment (G2) and titers from plaque assay (G3), which suggests that DNase I pre-treatment improves the accuracy of quantification of qPCR for T7 phage. Analyses of TaqMan qPCR raw data from amplification plot and multicomponent plot (Fig. S1) indicates highly efficient and specific amplification of fluorescent signal of M13KE and T7 phage DNA from TaqMan qPCR.

4.2 SYBR Green qPCR

SYBR Green qPCR can quantify any double stranded DNA. However, due to the lack of specificity of the SYBR Green fluorophore for double stranded DNA, M13 and T7 phage qPCR were run in separate reactions. M13KE standard DNA was diluted to the range of $10^{-2} - 10^5$ fg/ μ L, which was equivalent to $2.75 - 2.75 \times 10^7$ gc/ μ L ($0.4 - 7.4$ log (gc/ μ L)). Ct was $32.4 - 9.9$. The linear relationship of Ct against log (gc/ μ L) was $Y = -3.188X + 34.52$, $R^2 = 0.9938$ (Fig. 4A). E_s was 1.06. SD of Ct ranged from $0.05 - 0.34$ cycles with an average SD of 0.21 cycles, and the respective DNA molecules deviation was $\pm 3.7\% - \pm 27.8\%$, with an average $\pm 16.4\%$.

Since SYBR Green qPCR demonstrated increased sensitivity compared to TaqMan PCR, we next used SYBR Green qPCR to enumerate phages from biopanning. A C(X)₇C phage-presenting peptide library displayed on the N-termini of p3 coat protein (pIII) of M13KE phage was panned against a human brain microvascular endothelial cell line, hCMEC/D3 (Weksler et al., 2013), and individual plaques were isolated and amplified. A M13KE C(X)₇C clone was selected and amplified, serially diluted with PBS, and prepared for plaque assay and qPCR. SYBR Green qPCR for the M13KE C(X)₇C clone was compared with the plaque assay (Fig. 5A). At the highest concentration E7, qPCR titers without DNase I treatment were 3.3-fold and 2.5-fold higher than plaque assay and qPCR with DNase I pre-treatment (G1 versus G3 and G2), respectively (Fig. 5A). There was a significant effect of DNase I pre-treatment prior to heat treatment of M13KE phages for qPCR and significant differences between the enumeration of M13 phages by qPCR and plaque assay.

Next, we wanted to compare the titers of T7 phage identified from biopanning. T7 standard DNA was serially diluted from 1 fg/ μ L - 10^6 fg/ μ L, or $2.66 \times 10^1 - 2.66 \times 10^7$ gc/ μ L (log (gc/ μ L): $1.4 - 7.4$). The Ct of T7 phage DNA at the given concentrations ranged from $33.04 - 8.70$. The linear relationship between Ct versus log (genomes/ μ L) was $Y = -3.984X + 38.32$ ($R^2 = 0.9983$, Fig. 4B). E_s was 0.78. SD of Ct ranged from 0.079 to 0.41, with mean 0.26 cycles; the DNA molecule deviation was $\pm 4.7\%$ to $\pm 26.7\%$, with average $\pm 16.2\%$.

The T7 C(X)₇C library was panned against hCMEC/D3 cell line, and from the eluate, individual plaques were isolated and amplified. Serial dilutions of a selected T7 clone were prepared for qPCR. The titers were interpolated from the standard curve and compared with titers from plaque assay (Fig. 5B). At E4, there was a significant 1.5-fold difference between qPCR titers with and without DNase I treatment. Additionally, there was a significant difference between DNase I pre-treated T7 phage enumerated by qPCR compared to plaque assay. Analyses of SYBR Green qPCR raw data for M13 and T7 phage DNA, including amplification, multicomponent, and melt plots (Fig. S2), indicate efficient and specific SYBR Green PCR amplification for T7 and M13KE phage DNA.

5. Discussion

TaqMan and SYBR Green qPCR were developed to quantify genome copies of M13 and T7 phages as methods to enumerate M13 and T7 phage particles. qPCR is based on the increase in the fluorescent signal that accompanies with each round of amplification of nucleic acids; from a standard curve, titers of phages at unknown concentrations can be determined from

the number of phage genome copies. SYBR Green and TaqMan qPCR offer the ability to detect phage genome copies accurately and with excellent sensitivity, as observed with qPCR-based detection of bacteria or parasites in patients' specimens (Gomes et al., 2017; Maeda et al., 2003). In addition, qPCR methods have been developed to quantify a variety of dsDNA, ssDNA and RNA-enveloped viruses, such as human Herpes virus, Hepatitis, AIDS, Filoviruses, and Arenaviruses (Casabianca et al., 2003; Dhar et al., 2001; Gut et al., 1999; Lima et al., 2017). Here, each qPCR method can be used to enumerate phage particles, depending on the application and requirements for detection sensitivity. TaqMan offers the advantage of quantifying multiple phages in a single reaction since multiple TaqMan fluorescent probes can be used with equivalent types of phages. Alternatively, SYBR Green qPCR uses a single green dye that ubiquitously binds to double-stranded DNA, and therefore, SYBR Green dye cannot differentiate between different genomic DNA templates. As a result, SYBR Green qPCR was run to quantify genome copies of M13KE or T7 phages in separate reactions. The limit of detection of SYBR qPCR is 2.75 gc/ μ L for M13KE, which is 10-fold more sensitive than TaqMan qPCR (2.75 * 10¹ gc/ μ L). For T7 phage DNA, the Ct value from SYBR Green qPCR was lower than TaqMan (at 2.66*10¹ gc/ μ L, Ct from TaqMan and SYBR Green qPCR were 33.9 and 33.0 respectively), which indicates that SYBR Green is more sensitive than TaqMan qPCR. Due to its superior sensitivity, SYBR Green qPCR was selected to quantify M13 and T7 C(X)₇C clones isolated from selection experiments. In Fig. 6, there is a significant difference in titers between qPCR and double layer plaque assay from clones identified from biopanning. This difference detected using qPCR is critical because it is important to accurately quantify titers from biopanning. With each subsequent round of panning, there should be enrichment for selected phage (i.e. increased output of eluted, targeted phage over input phage); as a result, it is important to have reliable enumeration of phage binding to the target during biopanning (Dennis, 2002; Rodi and Makowski, 1999). Generally, SYBR Green qPCR is preferred since the fluorescent dye binds double-stranded DNA molecules by intercalating between the DNA bases and does not require additional conjugation of fluorophores onto the probes, which is necessary for multiplex TaqMan qPCR.

While double-layer agar plaque assay is the gold standard to quantify viable, infective phages from biopanning, there are challenges that limit its accuracy to enumerate phages. Even though plaque assay does not require extensive training or special equipment, variables including the thickness of agar layer, the concentration of agar and the other ingredients (e.g. CaCl₂), and the health of host bacteria, can impact the accuracy of phage titers. In the traditional "pour plate" approach (i.e. phages and host bacteria are pre-mixed with agar and poured on solid agar plate), phage particles need to diffuse through agar gel to infect the bacteria for plaque formation; however, it has been reported that this approach underestimated the titers of MS2 phages compared to "spread plate" (i.e. bacteria and phage directed were directly spread on the surface of agar plate (Cormier and Janes, 2014). Additionally, phage particles are susceptible to organic solvents (e.g. ethanol) due to their extensive use for antimicrobial sterilization. As a result, phage particles can lose infectivity due to compromised function of the F-pilus or solvation of coat proteins (Olofsson et al., 2001). Also, phages degrade under harsh conditions including high temperature (>80°C), pH less than 3 or greater than 11, high centrifugation speeds, or long term storage (Irie et al.,

2001; Tsuchiya et al., 2005). Plaque assay cannot enumerate these degraded phages that are present in the phage stock over time. Therefore, compared to qPCR, plaque assay may underreport the number of phage particles in samples. Moreover, the plaque assay is more labor intensive and time-consuming compared to qPCR, especially for large number of samples. The plaque assay can require up to an overnight incubation for plaque formation and is only efficient in terms of hands-on preparation for less than 10 samples. For example, in this study, enumeration of 30 M13KE phage samples using plaque assay required ~12 – 18 h, whereas qPCR needed only ~4 – 6 h. qPCR allows us to account for all intact and degraded phage particles and the impact of DNase I pre-treatment to accurately quantify phage particles.

For qPCR various DNA extraction methods are used to isolate phage DNA but most depend on DNA purification kits (Liu et al., 2016, Dong et al., 2010). To isolate genomic DNA directly from phage particles for qPCR and avoid the additional steps and reduced yield from kit-based DNA purification, we isolated phage DNA from phage particles by heat denaturation at 100°C for 15 min (Lock et al., 2014). This approach was time-saving and minimized the use of reagents for DNA preparation prior to preparation of qPCR. After heat denaturation, the coat proteins of phages disassemble, and the genomic DNA is released and available to bind primers for qPCR (Famm et al., 2008). Testing different temperature and timing conditions, 100°C for 15 min was found to optimally denature both M13 and T7 phages for DNA isolation.

In addition to enumerating phages from biopanning, qPCR is potentially beneficial to quantify phage DNA and enumerate phages for applications such as next generation sequencing (NGS), where it is critical to accurately quantify multiple library DNA samples before pooling library DNA for NGS analysis. Specifically, if pooling biopanning samples from different experiments or using different kinds of phage libraries (e.g. M13 and T7 libraries) for NGS, TaqMan qPCR is more time-efficient to quantify DNA concentration compared to SYBR Green qPCR. While the focus of this work was to demonstrate the feasibility of qPCR to reproducibly quantify genome copies of phages from phage display, this method can be applied towards multiple applications involving bacteriophages.

6. Conclusion

Both SYBR Green and TaqMan-based qPCR methods have been developed and validated to enumerate the M13 and T7 bacteriophages individually or simultaneously. qPCR is a sensitive method, able to quantify less than ten M13 or T7 phage particles per microliter. Compared with traditional plaque assay, qPCR was more time-efficient and accurate. Moreover, both qPCR methods were approximately 100–1000 times more sensitive than standard direct and sandwich ELISAs to detect M13 (Fig. S3), which further highlight their ability to quantify low phage titers compared with gold standard ELISA and plaque assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- SYBR Green and TaqMan qPCR were developed to quantify T7 and M13 phage
- SYBR Green qPCR sensitively detects selected phage from biopanning
- qPCR methods have improved detection sensitivity than ELISAs
- Enzymatic DNase I pre-treatment impacts values of phage titers from qPCR
- qPCR is time efficient and less intensive for large samples (>30) than plaque assay

Figure 1.

The workflow of TaqMan and SYBR Green qPCR of genomic DNA from M13KE and T7 wild type (WT) phage and C(X)₇C clones, as compared to double-layer agar plaque assay.

A

B

Figure 2. Standard curves of M13KE (Panel A) and T7 (Panel B) phage DNA from TaqMan qPCR

Log (gc/μL) was calculated from standard M13KE DNA from 0.1 fg/μL - 10⁶ fg/μL and T7 packaging control DNA at concentrations ranging from 1 fg/μL - 10⁷ fg/μL. At each

concentration, every sample had a Ct value from TaqMan qPCR. Linear regression was plotted with Ct versus log (genome copies (gc)/ μ L). Linear equation and correlation coefficient are in the inset.

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* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$

Figure 3. Titers of different unknown concentrations of M13KE and T7 wild type (WT) phage using TaqMan qPCR and plaque assay. Panel

A is the enumeration of M13KE WT phage. **Panel B** is quantification of T7 WT phage. Serial dilutions of M13KE (**panel A**) and T7 (**panel B**) WT phage were prepared from phages purified from amplification. E1 to E8 represent the quantification scale $\sim 10^1$ – 10^8 pfu/ μ L. Left Y-axis indicates qPCR enumeration results in gc/ μ L for M13KE (**A**) and T7 (**B**) WT phages with and without DNase I pre-treatment at their respective concentration. Titers for double layer plaque assay is shown in right Y-axis in both panels; units were in plaque forming units/ μ L (pfu/ μ L) plotted against the concentration scale (X-axis). P values are indicated in the graph.

Figure 4. Standard curves of M13KE (Panel A) and T7 (Panel B) phage DNA from SYBR Green qPCR

Log (gc/ μ L) was calculated from serial concentrations of M13KE DNA from $10^{-2} - 10^5$ fg/ μ L and of T7 DNA from $10^0 - 10^6$ fg/ μ L. Ct (threshold cycle) values were the SYBR Green qPCR results at the respective concentration. Linear regression was plotted with Ct versus log (gc/ μ L). Linear equation and correlation coefficient are in the inset.

Figure 5. Titers of selected M13 phage clone (Panel A) and T7 phage clone (Panel B) from biopanning using SYBR Green qPCR and plaque assay
M13KE PIII C(X)₇C and T7-select-415 C(X)₇C phage display library was selected against hCMEC/D3 cells for three rounds of biopanning, the plaque (clone) was chosen and amplified at 3rd round of selection. A serial dilution was conducted to prepare the unknown concentrations of the M13 clone. For the slow grower T7 clone, only 10-fold dilution was used to prepare the samples. The qPCR titers of M13KE clone (**Panel A**) and T7 clone

(Panel B) by SYBR Green qPCR were shown in the left Y-axis with and without DNase I pre-treatment before qPCR sample preparation (units in gc/ μ L), and plaque assay (right axis, units in pfu/ μ L) were plotted against the quantification scales.