Journal of Virological Methods 252 (2018) 100–107

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

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

Xiujuan Peng[a], Alex Nguyen[b], Debadyuti Ghosh[a,*]

[a] Division of Molecular Pharmaceutics and Drug Delivery, College of Pharmacy
[b] College of Natural Science, University of Texas, Austin, United States

ARTICLE INFO

Keywords:
qPCR
TaqMan
SYBR-green
Bacteriophage
M13
T7
Plaque assay

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 $2.75*10^1$–$2.75*10^8$ genome copies (gc)/μL and $2.66*10^1$–$2.66*10^8$ genome copies(gc)/μL 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–$2.75*10^7$ gc/μL and $2.66*10^1$–$2.66*10^7$ gc/μL 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).

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 et al., 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

* Corresponding author at: 2409 University Ave, PHR 5.218B, Austin, TX, 78712, United States.
E-mail address: dghosh@austin.utexas.edu (D. Ghosh).

https://doi.org/10.1016/j.jviromet.2017.11.012
Received 29 June 2017; Received in revised form 23 October 2017; Accepted 27 November 2017
Available online 02 December 2017

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 h) 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 10 B 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 H_2O 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–10^6 fg/μL. T7 packaging control dsDNA (Millipore Sigma, 37314 bp, 0.1 μg/μL) was diluted into a serial concentration of 1 fg/μL–10^7 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]*($6.02*10^{23}$). 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 vol PEG 8000/20% 2.5 M sodium chloride and then centrifuged at 11627*g* for 15 min. 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 200 μL of each diluted concentration of T7 and M13KE phage samples, and then incubated at 37 °C for 10 min. 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 min (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

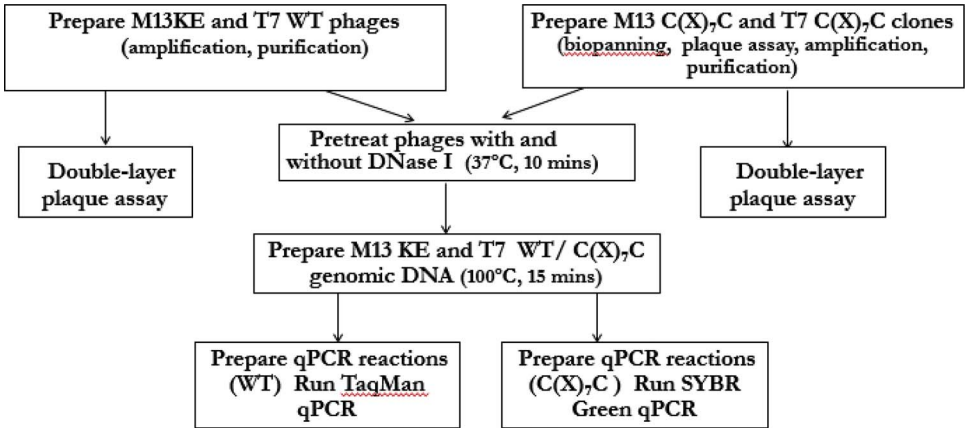

Fig. 1. The workflow of TaqMan and SYBR Green qPCR of genomic DNA from M13KE and T7 wild type (WT) phage and $C(X)_7C$ clones, as compared to double-layer agar plaque assay.