A qPCR Targeted Against the Viral Replication Origin Designed to Quantify Total Amount of Filamentous Phages and Phagemids

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Abstract Filamentous bacteriophages are widely used in phage display technology. The most common quantification method is lysis plaque formation test (PFT). This technique has several disadvantages, and only quantifies infective phages and is not effective when phagemids are used. We developed a qPCR method directed against the M13 replication origin, which detects between 3.3×10^3 and 3.3×10^8 viral genome copies with a linearity of $R^2 = 0.9998$. Using this method we were able to observe a difference of approximately ten more phages than with the PFT. This difference was not due to the presence of a free genome, which suggests the presence of non-infective particles. Using a DNaseI treatment, we observed the presence of 30% to 40% of unpackaged genome in recombinant phage modified in PIII or PVIII. The qPCR method with a DNase I treatment is an efficient method to quantify the total amount of filamentous phages.

Keywords Phage quantitation · Phage display · qPCR · Phagemids

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Introduction

Filamentous phages (FPs), and in particular the M13 phage are a common tool in biotechnology because they are amenable to genetic engineering by phage display technology, which consists in displaying foreign proteins or peptides on the virion surface by inserting foreign DNA fragments into their genome [1]. Using this technology it is possible to produce libraries that expose antibodies or peptides on the phage surface, and used them to isolate proteins that bind specific targets [2, 3]. Phage display technology has become increasingly important in the last years and now is no longer restricted to the construction of peptides or antibody libraries; it uses extend to nanotechnology, since FPs were used as nanometric building blocks in material science [2]. FPs have recently been used as epitope carriers in vaccine development [4, 5].

FPs quantification represents a critical aspect for its use in phage display [6]. The current methods of choice are PFT and the antibiotic resistant colony formation test. Since both methods relays on infectivity of the phage, they can be affected for several factors including those affecting the function of the F-pilus or the solvation of coat proteins [7]. It has been shown that high centrifugation speeds and long term storage can also affect infectivity [8, 9]. Aside from infectivity several other factors such as ion content, thickness of agar layer, the concentration of agar, and the health of host bacteria can affect quantification by these methods [9–11].

As an alternative, qPCR has been used to quantify phages [12, 13]. This technology offers several advantages over PFT since it quantifies the total phages, not only infective phages. Currently, qPCR methods have been developed to quantify FP [14–18]. Some groups developed qPCR methods against viral genomes, however these

methods did not allow the quantification of total phages when phagemids were used, because phagemids do not possess most viral genes [14, 17]. Other groups developed methods directed against foreign sequences present in phagemid genomes [15, 18]. However, these methods do not measure the phage with a helper genome that carries the recombinant peptide or protein in their capsids. For that reason, these methods do not allow the quantification of the total phage production when phagemids are used.

Materials and Methods

Primer Design and qPCR

To obtain primers against the viral replication origin we used the Primer 3 plus program [19], setting the product size from 150 to 250 bp and an optimal annealing temperature of 60 °C. Later primers OriM13 Fw-TTCCCTTCCTTTCTCGCCAC and M13Ori Rv2 AGAACGTGGACTCCAACGTC were synthesized by Macrogen (Korea). The primers were tested by PCR using pcDNA3.1 plasmid (data not shown), showing a single amplification product.

All qPCR reactions were performed by dye-based quantification, for which 10 μL reaction mix was prepared with 5 μL SsoAdvanced Universal SYBR Green Supermix (BIORAD), 0.5 μL of each 10 mM primer and 2 μL of each template. Subsequently they were amplified in a LightCycler Nano (Roche) real-time thermal cycler, using the following program: initial denaturation of 95 °C for 5 min, 35 cycles of two-stage amplification: 95 °C for 15 s, 60 °C for 30 s. Finally, a denaturing curve of the products was made starting at 60 °C for 20 s, then increasing by 0.1 °C/s up to 95 °C, and then finishing at 95 °C for 20 s. Reactions without template were performed as a negative control.

The phage genome was obtained using a phenol-chloroform extraction as previously described [20]. Briefly, a volume of phenol was added to a volume of purified M13KO7, vortexed for 30 s and centrifuged at maximum rpm for 5 min at RT, twice. Then a volume of chloroform was added, vortexed and centrifuged again, adding a tenth of the volume of 3 M Sodium Acetate pH 6, 2.5 volumes of cold ethanol and incubating overnight at − 20 °CC. The ssDNA was recovered by centrifuging at maximum speed for 30 min at 4 °C, removing the supernatant and briefly centrifuging again in order to remove supernatant remnants. The pellet was washed with cold 70% ethanol, and centrifuged for 5 min at 4 °CC. The pellet was dried at RT and finally resuspended in Tris 50 mM pH 8. The purified genome was measured by UV absorbance quantification in a ThermoScientific[TM] NanoDrop[TM] 8000

Spectrophotometer (Applied Biosystems). Then, tenfold serial dilutions were prepared ranging from 1.66×10^3 to 1.66×10^8 VGC/μL. Each dilution was performed in duplicate and analyzed by qPCR independently. For the determination of the PCR efficiency the following mathematical equation was used:

$$E_s = 10^{(-1/m)} - 1$$

where "m" is the slope of the line of the graph of the number of DNA copies according to the averages of Cq [21].

The plasmid pcDNA 3.1 was purified by miniprep using the GeneJet kit (Thermo Scientific) and quantified by UV absorbance method in a ThermoScientific[TM] NanoDrop[TM] 8000 Spectrophotometer. Then, tenfold serial dilutions were prepared ranging from 1.66×10^3 to 1.66×10^8 copies per microliter. Each dilution was performed in duplicate and analyzed by qPCR independently.

To perform the calibration curve of PFU as a function of Cq we used a commercial M13KE available from New England Biolabs (NEB), this was used to avoid purification bias in the quantitation. Serial dilutions of stock M13KE phage ranging from 10^3 to 10^8 PFU/μL were made. These dilutions were used as template for the qPCR, as described previously.

DNase I Treatment

Ten microliters of 1×10^8 VGC/μL were diluted in 190 μL DNase buffer (2.5 mM $MgCl_2$, 10 mM Tris-HCl and 0.5 mM $CaCl_2$, pH 7.6) and incubated with 2 μL of DNase I (PROMEGA) at 37 °C for 30 min. In order to inactivate the DNase the sample was incubated for 15 min at 100 °C and the qPCR was performed as described above. The assay was performed in triplicate for each phage; pcDNA was used as control. The data was analyzed with Microsoft Excel and GraphPad Prism software, finding the average and the standard deviations of the Cq values of each phage. The VGC/μL was calculated, using the calibration curve of Log_{10} VGC versus Cq. t-test was performed to analyze different conditions.

Results and Discussion

Due to the difficulties mentioned in the previously described methods, we developed a qPCR method that allows the quantification of phages carrying whole genome or phagemids. To that end, we designed the primers OriM13-Fw and OriM13-Rv2, directed against the M13 replication origin. Since this sequence is the packaging signal, it is present in all F′-pilus filamentous phage genomes and phagemids. It is noteworthy that these primers also were
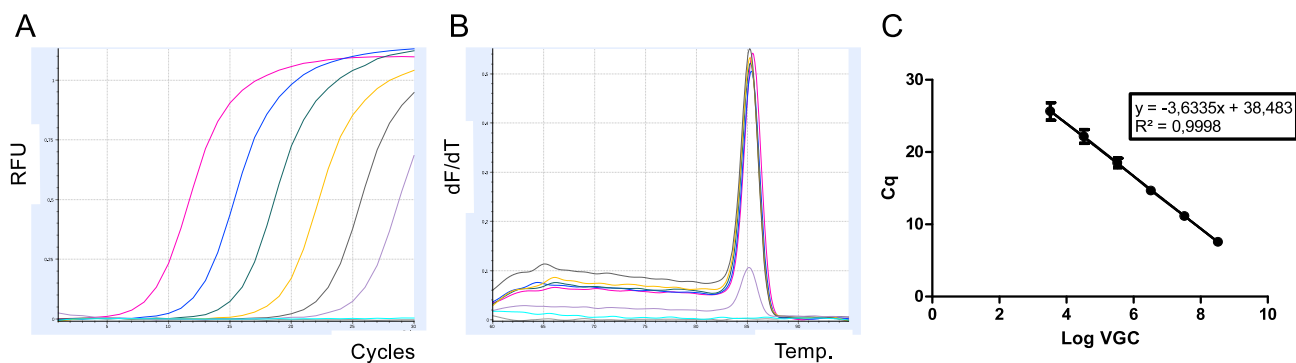
Fig. 1 Calibration curve according to the number of VGC. **a** Amplification curve. Dilutions of viral genome were made and amplified by qPCR. **b** Melting curve, where the presence of a single amplicon is observed. **c** Linear regression analysis of the data
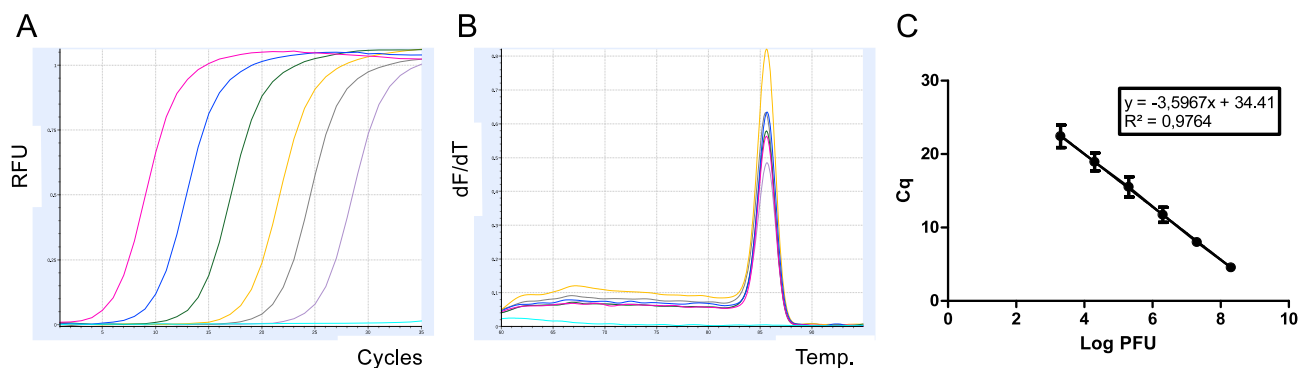

Fig. 2 Calibration curve according to PFU. **a** Amplification curve. Dilutions of M13KE virus were made and amplified by qPCR. **b** Melting curve, where the presence of a single amplicon is observed. **c** Linear regression analysis of the data

designed to allow the amplification of the truncate replication origin present in most common commercial helper phages like M13KO7 or M13KE.

Using the genome of M13KO7 we made viral genome standard curves with concentration ranging from 3.3×10^3 to 3.3×10^8 VGC. We observed a Cq of 7.4 to 26.8 (Fig. 1a, b), with a linear regression of $y = -3.6335x + 38.48$ ($R^2 = 0.9998$) where y is the Cq values and x is the $\log_{10}$ of VGC (Fig. 1c). Similar results were obtained using standard curves made with a plasmid carrying the M13 replication origin (Fig S1) with a linear regression curve of $y = -3.556x + 34.36$. We also observed a qPCR efficiency of 88.5% in the case of VGC and 91.1% with plasmid copies.

Next, we performed plaque formation units (PFU) against Cq standard curves with M13KE ranging 2×10^3 to 2×10^8 PFU with log PFU between 3.3 and 8.3. We observed a range of Cq from 4.1 to 24.6, and a linear relationship of the Cq against $\log_{10}$ PFU of $y = -3.5967x + 34.41$ ($R^2 = 0.9764$) (Fig. 2), indicating a qPCR efficiency of 89.7%.

As shown in Fig. 3, we observed a strong correlation between VGC and PFU standard curves with $y = 0.9898x + 1.12$ ($R^2 = 0.9764$). However, we observed a 13-fold increase in the amount of phages quantified with VGC compared with PFU. Since, some previous reports indicate that this difference could be caused by the presence of unpackaged genome [12, 14], we quantified the VGC by qPCR with or without previous DNase I treatment. As shown in Fig. 4, 92% of the M13KE viral genome was resistant to DNase treatment. These results, indicate that of the most viral genome was packaged, and the difference between VGC and PFU quantification was not caused by free unpackaged genome. Reitinger et al. [22] also observed tenfold difference between VGC (resistant to DNase I treatment) and PFU using absolute quantification by digital PCR. Similar results were also observed in MS2 phage quantification [23, 24].

To evaluate this method with different phages, we quantified the helper phage M13KO7 and recombinant phages modified in PIII and PVIII proteins by qPCR. The recombinant phages showed around 40% of digested genome, suggesting that the presence of this modification could alter the phage assembly allowing the presence of unpackaged genome (Fig. 4). Similar results, were observed in recombinant M13 phages [14].
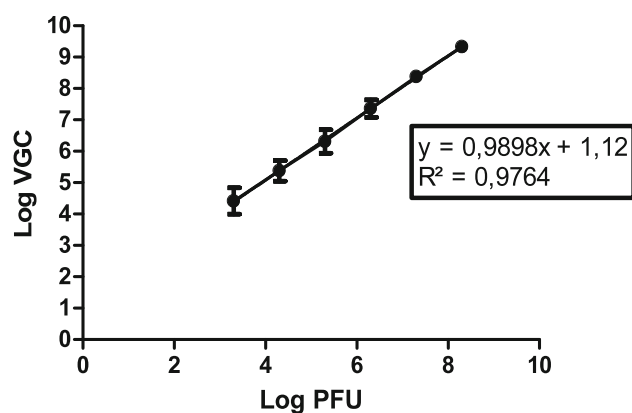
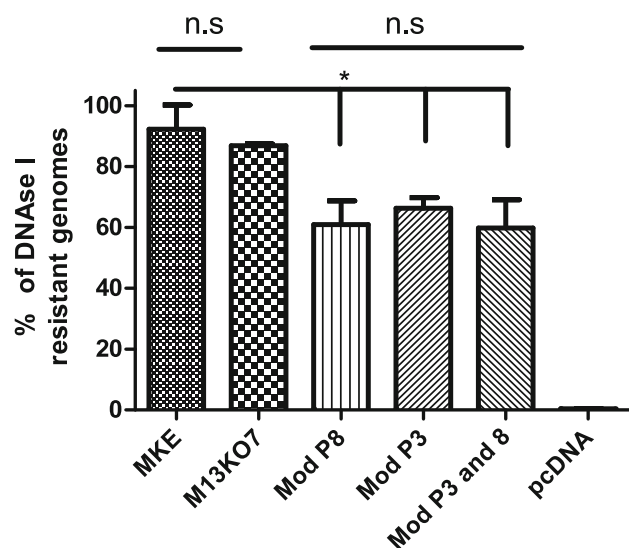**Fig. 3** Correlation between the results obtained using VGC and PFU

Fig. 4 Evaluation of unpackaged genomes present in the phage preparation. The purified phages were treated with DNase I and inactivated by temperature. The amount of VGC was analyzed by qPCR and normalized by undigested samples. As a control of DNase I treatment pcDNA 3.1 plasmid was used. KO7: M13KO7, Mod P3: M13 phage expressing an antibody coupled to PIII, Mod P8: M13 phage expressing an octapeptide coupled to P8, Mod P3 P8: M13 phage expressing a peptides coupled to PIII and PVIII. *$p > 0.05$. n.s.: non significant

Considering our results, the qPCR method targeting the M13 replication origin is efficient for the quantification of the total amount of phages and could be a simple alternative to infectivity-dependent methods, especially when phagemids are used.

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