Research in Pharmaceutical Sciences, June 2024; 19(3): 338-346
Received: 22-01-2024
Peer Reviewed: 01-03-2024
Revised: 07-04-2024
Accepted: 14-04-2024
Published: 01-07-2024

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

*Original Article*

# The enhancement of M13 phage titration by optimizing the origin of replication

**Mohammad Hossein Darvishali, Mahmood Fadaie, and Hossein Khanahmad***

*Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.*

## *Abstract*

**Background and purpose:** M13KO7, a modified M13 phage variant, carries the p15A replication origin and Tn903 kanamycin resistance gene. This study aimed to optimize M13KO7's replication by substituting the p15A origin with the higher-copy pMB1 origin (500-700 copy numbers).

**Experimental approach:** A 6431-nucleotide fragment from the M13KO7 plasmid lacking the p15A replication origin and kanamycin resistance gene was amplified using a long polymerase chain reaction (PCR). The modified M13AMB1 plasmid was created by adding adenine to the 3' ends of this fragment and ligating it to the pMB1-containing fragment using T/A cloning. Afterward, to prepare the phage, pM13AMB1 was transformed into *E. coli* TG1 bacteria, and then, using the PEG-NaCl precipitation, the modified phage was propagated. The modified phage titer was determined utilizing the serial dilution and the qPCR methods, compared with the M13KO7 phage.

**Findings/Results:** The results showed that in the serial dilution method, the titers of modified phage and M13KO7 phage were $4.8 \times 10^{14}$ and $7 \times 10^{12}$ pfu/mL, respectively. Besides, the phage titer calculated by the qPCR method for the modified phage was equal to $1.3 \times 10^{9}$ pfu/mL, whereas it was $4.08 \times 10^{8}$ pfu/mL for the M13KO7 phage.

**Conclusion and implications:** This study provides evidence that replication origin replacement led to a significant increase in phage titers. It highlights the importance of replication optimization for molecular biology applications.

**Keywords:** Bacteriophage; M13 Phage; Phage titer; Replication origin.

## INTRODUCTION

Bacteriophages, viruses that exclusively target bacteria, have drawn a lot of attention as invaluable tools in diverse scientific fields. The M13 bacteriophage has long been a cornerstone in molecular biology research, owing to its unique characteristics like excellent safety profile, high stability, manipulability, and non-pathogenic nature. With a filamentous structure and a circular single-stranded DNA (cssDNA) genome, which is approximately 6.4 nucleotides in size, M13 has served as a precious instrument in various endeavors, including phage display, gene cloning, and nanotechnology (1-3). The type n system in phage display offers several advantages over the type n+n system. It is simpler, cost-effective, easier to handle and store, efficient in binder selection, and potentially suitable for polyvalent display. This eliminates the requirement for phagemid and the complications of working with two different genetic components (phage and phagemid), making it a viable tool for many applications in peptide display (4-6).

Phage replication is highly dependent on the replication origin, which also affects the viral load and titration efficiency of the phage.

*Corresponding author: H. Khanahmad
Tel: +98-3137929197, Fax: +98-313668859
Email: h_khanahmad@med.mui.ac.ir

**Access this article online**

**Website:** http://rps.mui.ac.ir

**DOI:** 10.4103/RPS.RPS_14_24

Enhancing the replication kinetics and production process of the M13 phage can be achieved by optimizing its origin of replication. The origin of replication serves as the initiation site for DNA synthesis, allowing for the replication and amplification of the phage genome (7). Traditionally, the p15A ori has been widely used as the replication origin for plasmid vectors due to its compatibility with various host strains. However, its usage in bacteriophage genomes has shown limitations and inefficiencies. It is expected that the introduction of the pMB1 ori, a high (500-700) copy number origin, into the M13 phage genome, will improve the replication efficiency of the phage genome, leading to higher phage titers, which is crucial for the successful development of effective vaccines (8,9). This modification holds great potential for optimizing M13 phage titration, thereby facilitating the development of a reliable and efficient primary vaccination tool (9,10).

In this study, we reported a modified M13KO7-derived phage with an optimized origin of replication, which has a higher titer compared to the wild-type phage. To this end, our research focused on genetically engineering the M13KO7 helper phage to replace its replication origin with a high copy number, pMB1, origin. With this change, our goal was to construct an n-type system that could be used in various scientific applications. This approach combines the well-established features of M13 as a bacterial targeting vehicle with the advantages offered by pMB1 origin-driven replication. By doing so, we anticipate that the modified M13 phage will exhibit increased replication rates, higher production yields, and improved overall efficacy. By optimizing the origin of replication, we aim to unlock the full potential of the M13 bacteriophage, enabling its use as a more efficient and cost-effective tool in various scientific domains.

## MATERIALS AND METHODS

### *Bacterial and bacteriophage strains*

In the current study, two widely used strains of *Escherichia coli* bacteria, i.e. Top10 (DH10b) and TG1 strains, were purchased from the Pasteur Institute of Iran. *E. coli* Top10 bacteria, which has a tetracycline resistance gene in its genome, was used for cloning and plasmid storage. Also, *E. coli* K12 TG1 bacteria, which has abundant F-pili, was used in the phage extraction process. The two phage strains used in this research were the wild-type M13KO7 phage carrying the p15A ori, which was bought from the Pasteur Institute of Iran, and the modified M13 phage carrying the pMB1 ori (M13AMB1).

### *Media and culture preparation*

For growing bacteria in a standard manner, cells were cultured in Luria-Bertani (LB). LB Agar plates, which contain 1.5% added agar, were utilized for the solid culture of bacteria. The tetracycline, ampicillin, and kanamycin antibiotics were used at concentrations of 12.5, 100, and 50 μg/mL, respectively, to select antibiotic-resistant genetic markers. The phage extraction process was carried out in a super broth (SB) medium, which contains 20 g/L yeast extract, 35 g/L peptone, and 5 g/L NaCl. Indeed, the extracted phages were diluted in 1× phosphate-buffered saline (PBS) solution containing NaCl, KCl, $Na_2HPO_4$, and $KH_2PO_4$. In addition, TBE buffer containing tris, boric acid, and ethylenediaminetetraacetic acid (EDTA) was used to perform gel electrophoresis.

### *Construction of modified plasmid pM13AMB1*

To construct the modified pM13AMB1 plasmid, the helper phage M13KO7 genome was genetically manipulated to amplify a 6431-bp fragment and remove the p15A ori coding sequence and kanamycin antibiotic resistance gene. The 6431-bp fragment was amplified by polymerase chain reaction (PCR) based on the pM13KO7 vector, using Super PCR Master Mix 2X (Yekta Tajhiz, Iran) as well as PFHindIIIM13 and PREcoRIM13 primers (Table 1, Fig. 1A and B). For this purpose, a temperature program of 4 min at 94 ℃, 30 cycles of 30 s at 94 ℃, 30 s at 60 ℃, and 7 min at 72 ℃, followed by 7 min at 72 ℃, was applied to the Thermocycler (Eppendorf, Germany). Subsequently, adenine was added to the 3′ ends of the amplified fragment through the Taq treatment method and then, it was

ligated to the pMB1-containing fragment (the pGetII vector) by the T/A cloning approach. Transformation of the modified M13AMB1 plasmid into competent *E. coli* Top10 cells was carried out using the calcium chloride protocol, as previously described (11,12). The construction of the M13AMB1 plasmid was confirmed using colony PCR on positive clones with PFHindIIIM13 and PREcoRIM13 primers (Table 1) and plasmid digestion with *PstI* restriction endonuclease (Thermo Fisher Scientific, USA), as well as Sanger sequencing.

### *Preparation of wild-type and modified M13 phages*

Plasmids pM13KO7 and pM13AMB1 were separately transformed into *E. coli* K12 TG1 bacteria using calcium chloride protocol. The colony PCR using FRealM13 and REcoRIM13 primers (Table 1) was used to confirm the presence of plasmids in the positive colonies resulting from the transformation, and these clones were then used for phage propagation. TG1 cells containing pM13KO7 were grown in a 1-L Erlenmeyer flask (25% working volume) using SB medium containing 50 µg/mL kanamycin, and in the same way, TG1 cells containing pM13AMB1 were cultured in SB medium containing 100 µg/mL ampicillin. Then, TG1 cells were incubated overnight in a shaker incubator (37 ℃ and 200 rpm). Next, the culture medium containing bacteria and phage was centrifuged for several steps at 5500 rpm and 4 ℃ to precipitate the bacteria. Then, the supernatant was passed through a sterile 0.22 µm syringe filter to obtain pure phage. Finally, by appending PEG-NaCl solution (20% polyethylene glycol 6000 with 2.5 M NaCl) and spinning at 12000 rpm, the modified and wild-type phages were precipitated, and then the pellet was dissolved in sterile PBS.

**Table 1.** The sequences of used polymerase chain reaction primers.

| Primer | Primer sequence |
|---|---|
| PFHindIIIM13 | 5'TTTAAGCTTGTCTCGGGCTATTCTTTTGAT3' |
| PREcoRIM13 | 5'AAAGAATTCCGAGATAGGGTTGAGTGTTGTT3' |
| FRealM13 | 5'GTTCCGATTTAGTGCTTTACG3' |

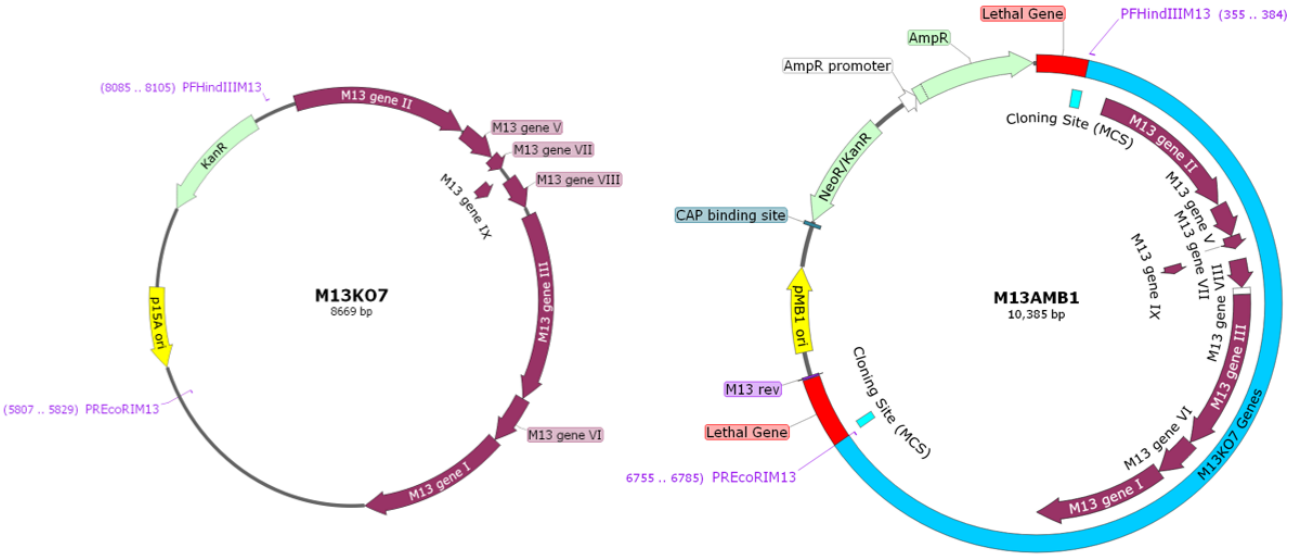


**Fig. 1.** The M13AMB1 plasmid construction. (A) The M13KO7 vector map; (B) the M13AMB1 vector map. The pM13KO7 derivative, pM13AMB1, was produced through genetic engineering by substituting the origin of replication, p15A, with pMB1.

***Enumeration of wild-type and modified phages by serial dilution***

The fifteen 1.5-mL vials were filled with 90 µL of sterile PBS to create a dilution series, necessary to calculate the titer of modified and helper phages. A dilution rate of 1:10 was employed, starting with 10 µL of acquired phage added to the first vial to achieve a titer of 0.1. Subsequently, 10 µL from the first vial were put into the second vial for a titer of 0.01, and so forth until the fifteenth vial reached a titer of $10^{15}$. Finally, 10 µL of solution from the fifteenth vial was taken out to ensure uniformity across all vials. The resulting phage solutions, with a dilution ratio ranging from $10^{10}$ to $10^{15}$, were then added to bacterial tubes (containing 90 µL of bacteria with an optical density of 0.4-0.6) for transduction. It was placed in an incubator set to 37 ℃ and left for 30 min. Subsequently, each vial's content was cultivated on agar plates that had 100 µg/L of the antibiotic ampicillin on them. The plates were then left to incubate for a whole night at 37 °C. Finally, the grown colonies on each plate were counted and then the titer of phages per milliliter was calculated using the following equation (13,14):

Phages concentration = Colony number × dilution coefficient × volume of bacteria culture per milliliter

***Quantification of wild-type and modified phages titer by qPCR***

Phages can be enumerated utilizing the quantitative PCR (qPCR) method to calculate the copies of phage genomes, which are equal to the number of phages (*i.e.* a single phage contains a single genome). The 181-bp fragment amplified from the M13KO7 plasmid was used as a standard to prepare serial dilutions from $10^6$ to $10^{11}$ (15). For the samples, ssDNA was prepared for qPCR from the modified and wild-type phages. Primers FRealM13 and REcoRIM13 were designed using GeneRunner software version 6.5.47 (Table 1). A 12 µL PCR reaction mixture was prepared with 6 µL 2× SYBR Green Master Mix, 0.5 µL of each 10 µM primer, 4 µL sterile $ddH_2O$, and 1 µL ssDNA of phage as a template. PCR cycling conditions were 94 ℃ for 10 min, 40 cycles of 94 ℃ for 20 s, 63 ℃ for 30 s, and 72 ℃ for 20 s. To calculate the phage titer in pfu/mL, the quantity of modified and helper phages in 1 mL had to be ascertained first. The mean counts for both modified and helper phages were then determined by calculating the three qPCR experiment replicates. Considering that the volume of 500 µL of phages was used to extract the genome, and these were then dissolved in 75 µL of water, to convert the number of phages present in 1 µL to 1 mL, the average number of each phage was multiplied by 75 (to obtain the number for the total retrieved genome) and then by 2 (to calculate the number in 1 mL).

***Statistical analyses***

The statistical analysis was performed using GraphPad Prism version 10 (GraphPad Software, USA). The data are presented as mean ± SD. Statistical significance was determined using an unpaired t-test, and a *P*-value $< 0.05$ was considered statistically significant.

## RESULTS

***Construction and confirmation of modified pM13AMB1***

In this work, we attempted to use genetic engineering to produce a modified M13 phage harboring the pMB1 ori and investigate the relationship between the titration of modified bacteriophages and the presence of high copy number origin of replication (Fig. 2). To achieve this, a 6431-bp fragment containing all the genes of the M13KO7 phage except the kanamycin resistance gene and the p15A ori coding sequence was amplified by PCR from the M13KO7 plasmid (Fig. 3A). In the following, adenine base was added to 3′ ends of this fragment, and it was then ligated to the pGetII vector using the T/A cloning method, generating the modified M13AMB1 plasmid. This modified construct was transformed into competent *E. coli* Top10 cells, and the successful construction of the pM13AMB1 plasmid was confirmed using colony PCR (Fig. 3B), which amplified the 6431-bp fragment, and plasmid digestion with *PstI* restriction endonuclease (Fig. 3C), which indicated a linearized plasmid size of ~10 kb.

Also, the presence of the insert (6431-bp) fragment in the pM13AMB1 was confirmed through amplification by PCR (Fig. 3D). Indeed, the Sanger sequencing result confirmed the presence of the M13KO7 phage genome within the modified construct. Ultimately, the existence of pM13AMB1 in *E. coli* TG1 was verified using colony PCR before the phage preparation (Fig. 3E).

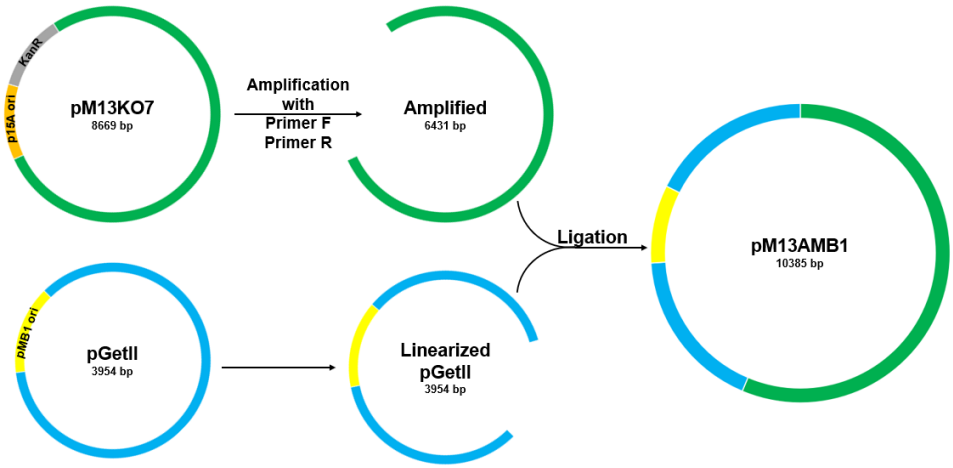


**Fig. 2.** Construction of M13AMB1 plasmid. Using the T/A cloning technique, a 6431-bp fragment amplified from the M13KO7 plasmid and then ligated to the pGetII vector to create a modified variant of phage M13, known as M13AMB1. The 6431-bp fragment amplified from the M13KO7 plasmid lacks the sequence related to the p15A ori and kanamycin resistance gene (KanR).

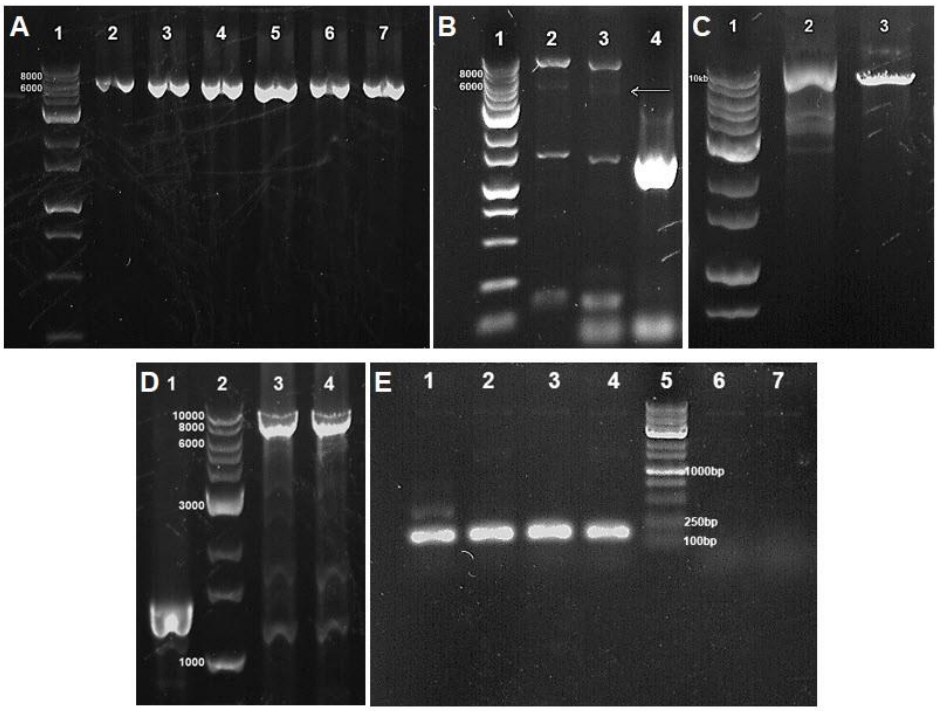


**Fig. 3.** Gel electrophoresis figures. (A) Using PCR, a 6431-bp fragment was amplified from the M13KO7 plasmid. In this figure, lane 1 represents the 1-kb ladder, and lanes 2 to 7 correspond to the 6431-bp fragment; (B) to confirm the presence of the 6431-bp fragment, a colony PCR reaction was conducted on the positive colonies grown on the plate, the result of which is displayed in lanes 2 to 4 next to the 1-kb ladder in lane 1; (C) the modified M13AMB1 plasmid underwent an enzymatic digestion procedure using the *PstI* restriction enzyme to confirm its approximate size of 10 kb, with related band visible in lane 3 and next to plasmid (lane 2) and 1-kb ladder (lane 1); (D) the 6431-bp fragment from the modified plasmid was amplified using a PCR reaction for additional validation, as demonstrated in lanes 3 and 4 adjacent to the 1-kb ladder in lane 2 and the incorrect-size band in lane 1; (E) prior to the phage preparation process, the presence of the M13AMB1 plasmid in the *E. coli* TG1 bacterium was verified using the colony PCR method by amplification of a 181-bp fragment. The related band is visible in lanes 1 through 4 next to the 1-kb ladder in lane 5; however, it is not present in lanes 6 and 7. PCR, Polymerase chain reaction.

***Quantification and comparison of modified and wild-type phage titer***

After confirming the process of phage genome manipulation and separately transforming the M13KO7 and the M13AMB1 plasmids into *E. coli* TG1 bacteria, the process of phage preparation and extraction was done. Calculation of live phages was carried out after preparation of a serial dilution for wild-type and modified phages from $10^{10}$-$10^{15}$, and subsequently, transduction of *E. coli* TG1 bacteria with each of these phages. It should be noted that the number of colonies grown on the plate with dilutions $10^{10}$ and $10^{11}$ was so high that it was not possible to count the clones. Also, no colonies were grown on plate $10^{14}$ relating to bacteria transduced with the helper phage M13KO7, while colonies were visible on plates $10^{14}$ and $10^{15}$ of bacteria transduced with the modified phage M13AMB1. In general, the modified phage clones were smaller than the wild-type phage clones. Next, by averaging the number of calculated phages present in each plate, the titer of modified and helper phages was obtained, which equals $4.8 \times 10^{14}$ and $7 \times 10^{12}$ for the modified and wild-type phages, respectively. In other words, the M13AMB1 phage titer was ~2-log higher than the helper phage titer.

After extracting the genomes of the wild-type and modified phages, the phage titers were also calculated using the qPCR method (Fig. 4). The titer of modified and wild-type phages was calculated as $1.3 \times 10^{9}$ and $4.08 \times 10^{8}$, respectively, which indicates about a 3-fold increase in the titer. Figure 5 represents a comparison of the mean copy numbers of the amplified fragment of the modified M13 phage with those of the wild-type phage, as determined by qPCR analysis. The data indicate a significantly higher titration for the modified phage compared to the wild-type phage.

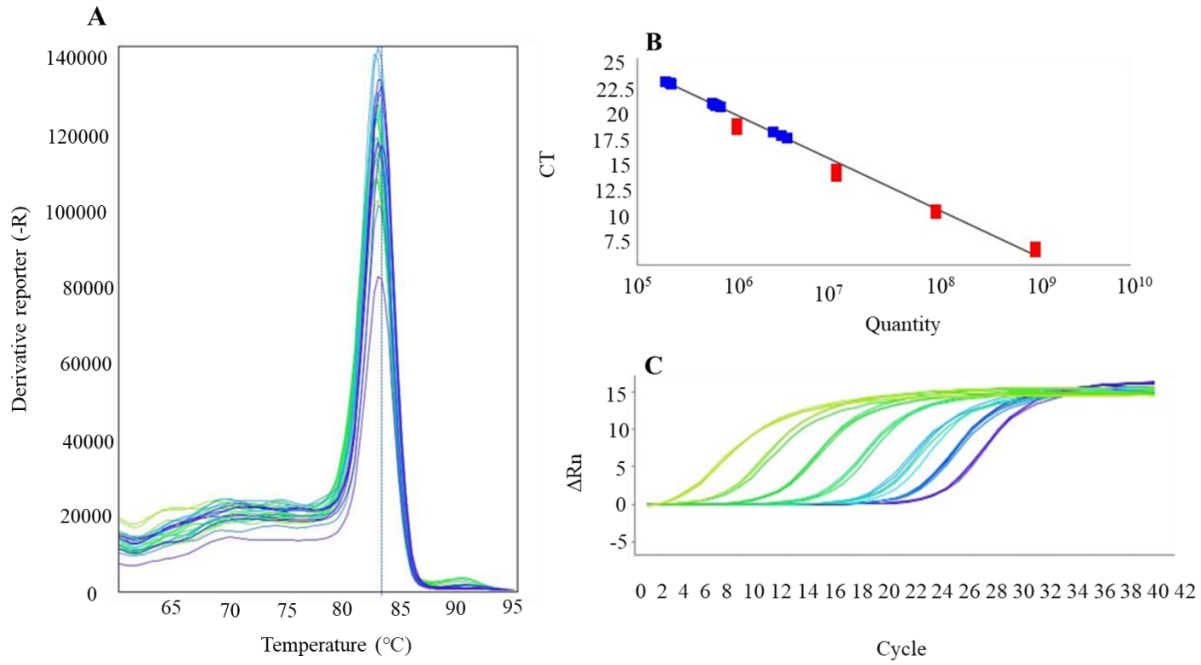


**Fig 4.** qPCR plots. (A) Melt curve. Melt curves of the amplified fragment from the genomes of M13AMB1 and M13KO7 phages (blue and purple curves) and the standard sample (181-bp fragment) amplified from the M13KO7 plasmid (yellow and green curves) are shown. (B) Standard curve. The graph shows a linear decrease in the average Ct value (the cycle number) with increasing phage quantity (the frequency of phage) in a qPCR reaction. The blue squares represent the 181-bp fragment amplified from the M13AMB1 and M13KO7 phage samples, while the red square represents the 181-bp fragment amplified from the M13KO7 plasmid, which was used as a standard sample to calibrate the qPCR procedure. The Ct value measures PCR cycle requirements for DNA amplification, with lower values indicating higher phage titers. (C) Amplification plot. This graph, plotted as delta-Rn per cycle, illustrates the quantification of the 181-bp fragment amplified from bacteriophages M13AMB1 and M13KO7 (blue and purple curves, respectively) in comparison to the amplification of the same fragment from serial dilutions of a standard sample (yellow to green curves). The plot indicates that the concentration of the target DNA in the M13AMB1 samples is significantly higher than the concentration in the M13KO7 samples.

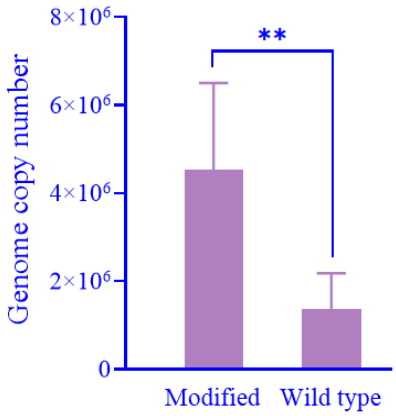


**Fig. 5.** Comparison of the wild-type and modified M13 phage titration. The column chart, based on qPCR data analysis, showcases the mean copy numbers of the amplified fragment of the modified M13 phage in comparison to the wild-type phage. The chart reveals a significant increase ($P < 0.01$) in the modified phage's titration over the wild-type phage. Data are presented as means ± SD.

## DISCUSSION

The M13 phage is a filamentous bacteria-infecting virus that has gained significant attention in recent years for its potential applications in various fields, including biotechnology and medicine. By modifying the wild-type M13 phage, scientists have been attempting to find ways to produce altered strains of M13, which have enhanced capabilities and applications (2). One area of research involves modifying the M13 genome to introduce new functionalities or improve existing ones. For instance, M13 has been successfully engineered to display foreign peptides or proteins on its surface. This modification allows the phage to become a powerful tool for vaccine development, drug delivery, and bioimaging. Researchers have also investigated the potential of using M13 as a scaffold for the assembly of functional materials, such as metallic nanoparticles, quantum dots, or enzymes. These modifications can provide the phage with new properties and enable applications in fields like nanotechnology (1,6).

In this study, we engineered the M13KO7 phage to enhance its yield by modifying its origin of replication. By deleting the origin of replication of p15A in the helper phage M13KO7 and replacing it with the pMB1 origin of replication, which has a higher copy number, the study aimed to increase the phage yield. For evaluating the success of the engineering process, both serial dilution and qPCR methods were utilized. Our results mention that the titer of the modified phage obtained through the serial dilution method was $4.8 \times 10^{14}$, while the titer of the helper phage was $7 \times 10^{12}$. This indicates a significant increase (~2-log) in phage production for the engineered strain. The qPCR method yielded a titer of $1.3 \times 10^{9}$ and $4.08 \times 10^{8}$ for the modified and helper phages, respectively, further confirming the higher abundance (~3-fold) of the engineered phage.

In comparison with other related studies, the research presents some advantages. For example, the genetic modification led to a substantial increase in the phage titer, as evidenced by the significant difference in titer between the modified phage and the helper phage. This demonstrates the potential of genetic engineering to enhance phage yield, which is consistent with the findings of other studies. For instance, Kadiri *et al.* demonstrated the formation of nanonets using genetically modified M13 bacteriophages, highlighting the potential of genetic engineering to create novel phage-based structures with unique properties (16). Additionally, the study by Gosh *et al.* aimed to improve the versatility of the M13 bacteriophage as a platform for multiple peptide display, showcasing the diverse applications of genetic engineering in modifying phage properties (17).

However, the research also presents certain limitations that should be considered. One potential limitation is the impact of genetic modification on phage properties, as highlighted in the study by Wong *et al.* (18). They investigated the possibility of enhancing M13-based phagemids for transgene delivery through the elimination of the bacterial backbone, emphasizing the need to carefully consider the impact of genetic modifications on phage behavior and performance. Furthermore, a genetically engineered recombinant M13 phage containing a series of CRISPR-Cas13a systems has been developed to target antibiotic resistance genes and highlight the complexity and potential challenges associated with phage genetic engineering (19).

Compared to our study attempting to enhance phage yield through genetic engineering, Kok *et al.* took a different approach to achieve the same goal (20). They present a protocol to increase the titers of difficult bacteriophages. The rapid adaptive mutation of phage-up (RAMP-UP) protocol, a liquid infection approach, significantly improved phage titers, allowing the evolution of high-titer bacteriophages within a short timeframe of just four days. The resulting titers were adequate for extracting and sequencing DNA from the bacteriophages, demonstrating the practical implications of the protocol.

The results obtained from the titer determination methods indicated the successful engineering of the M13KO7 phage and validated the effectiveness of replacing the origin of replication. The increased copy number achieved through this modification can have significant implications for large-scale phage production and applications in biotechnology and medicine. Overall, the results demonstrated that small modifications at the genetic level might lead to notable gains in experimental performance, paving the way for innovative research avenues in molecular biology and biotechnology. Notably, ori optimization not only improves phage titration efficiency but also likely has implications for other domains, including gene cloning and nanotechnology.

The strengths of our study include demonstrating the effectiveness of genetic modification in enhancing phage yield, as well as providing a detailed description of the genetic engineering process, which can serve as a valuable reference for similar research endeavors. Also, using both serial dilution and qPCR methods for titer determination enhances the robustness of the findings, providing a comprehensive assessment of the impact of genetic modification on phage yield. While the study presents promising results, it is important to consider the potential effects on phage properties and the need for further validation to fully assess the practical implications of the genetic modification. The detailed methodology and the use of multiple titer determination methods are notable strengths of the study, providing valuable insights into the field of phage genetic engineering.

## CONCLUSION

In conclusion, this study highlights the importance of optimizing the origin of replication in the M13 phage to enhance phage titration. Through our exploration of replacing the pMB1 origin with p15A, a significant increase in phage titers was achieved, as validated by both the serial dilution and qPCR methods. The modified M13AMB1 phage exhibited notably higher titers compared to the M13KO7 phage, showcasing the effectiveness of the replication origin replacement approach. These findings underscore the importance of understanding and manipulating phage replication mechanisms for various molecular biology applications, such as phage display and recombinant protein expression. Moreover, this research paves the way for future investigations into further optimizing M13 phage and exploring novel applications of modified phage variants. In essence, this study represents a substantial advancement in leveraging bacteriophages for diverse scientific and technological endeavors.

***Acknowledgments***

The authors express sincere gratitude to those who contributed to this study, highlighting their collaborative spirit and dedication. They also acknowledge the financial support provided by the Research Department of Isfahan University of Medical Sciences through Grant No. 3401733 and the Behyaar Zist Company.

***Conflicts of interest statement***

The authors declared no conflict of interest in this study.

***Authors' contributions***

MH. Darvishali developed the research and helped with the manuscript's drafting, editing, and development; H. Khanahmad supervised the study; and M. Fadaie assisted with editing the article. The finalized article was read and approved by all authors.

## REFERENCES


1. Chang C, Guo W, Yu X, Guo C, Zhou N, Guo X, *et al*. Engineered M13 phage as a novel therapeutic bionanomaterial for clinical applications: from tissue regeneration to cancer therapy. Mater Today Bio. 2023;20:1006121,1-16. DOI: 10.1016/j.mtbio.2023.100612.
2. Wang R, Li HD, Cao Y, Wang ZY, Yang T, Wang JH. M13 phage: a versatile building block for a highly specific analysis platform. Anal Bioanal Chem. 2023;415(18):3927-3944. DOI: 10.1007/s00216-023-04606-w.
3. González-Mora A, Hernández-Pérez J, Iqbal HMN, Rito-Palomares M, Benavides J. Bacteriophage-based vaccines: a potent approach for antigen delivery. Vaccines. 2020;8(3):504,1-24. DOI: 10.3390/vaccines8030504.
4. Jaroszewicz W, Morcinek-Orłowska J, Pierzynowska K, Gaffke L, Węgrzyn G. Phage display and other peptide display technologies. FEMS Microbiol Rev. 2022;46(2):1-25. DOI: 10.1093/femsre/fuab052.
5. Bazan J, Całkosiñski I, Gamian A. Phage display a powerful technique for immunotherapy: introduction and potential of therapeutic applications. Hum Vaccines Immunother. 2012;8(12):1817-1828. DOI: 10.4161/hv.21703.
6. Fadaie M, Dianat-Moghadam H, Ghafouri E, Naderi S, Darvishali MH, Ghovvati M, *et al*. Unraveling the potential of M13 phages in biomedicine: advancing drug nanodelivery and gene therapy. Environ Res. 2023;238(P1):117132. DOI: 10.1016/j.envres.2023.117132.
7. Méndez-Scolari JE, Florentín-Pavía MM, Mujica MP, Rojas N, Sotelo PH. A qPCR targeted against the viral replication origin designed to quantify total amount of filamentous phages and phagemids. Indian J Microbiol. 2019;59(3):365-369. DOI: 10.1007/s12088-019-00798-x.
8. Camps M. Modulation of ColE1-like plasmid replication for recombinant gene expression. Recent Pat DNA Gene Seq. 2010;4(1):58-73. DOI: 10.2174/187221510790410822.
9. Kendall Morgan MP. Plasmids 101. In: Plasmid 101. Adgene.2023. Available from: addgene.org.
10. Selzer G, Som T, Itoh T, Tomizawa J. The origin of replication of plasmid p15A and comparative studies on the nucleotide sequences around the origin of related plasmids. Cell. 1983;32(1):119-129. DOI: 10.1016/0092-8674(83)90502-0.
11. Chang A, Chau V, Landas J, Pang Y. Preparation of calcium competent Escherichia coli and heat-shock transformation. J Exp Microbiol Immunol. 2017;1:22-25.
12. Hosseini N, Khanahmad H, Nasr Esfahani B, Bandehpour M, Shariati L, Zahedi N, *et al*. Targeting of cholera toxin A (ctxA) gene by zinc finger nuclease: pitfalls of using gene editing tools in prokaryotes. Res Pharm Sci. 2020;15(2):182-190. DOI: 10.4103/1735-5362.283818.
13. Reddy P, McKenney K. Improved method for the production of M13 phage and single-stranded DNA for DNA sequencing. Biotechniques. 1996;20(5):854-860. DOI: 10.2144/96205st05.
14. Ács N, Gambino M, Brøndsted L. Bacteriophage enumeration and detection methods. Front Microbiol. 2020;11:594868,1-7. DOI: 10.3389/fmicb.2020.594868.
15. Boshtam M, Asgary S, Rahimmanesh I, Kouhpayeh S, Naderi J, Hejazi Z, *et al*. Display of human and rabbit monocyte chemoattractant protein-1 on human embryonic kidney 293T cell surface. Res Pharm Sci. 2018;13(5):430-439. DOI: 10.4103/1735-5362.236836.
16. Kadiri VM, Alarcón-Correa M, Ruppert J, Günther JP, Bill J, Rothenstein D, *et al*. Genetically modified M13 bacteriophage nanonets for enzyme catalysis and recovery. Catalysts. 2019;9(9):1-10. DOI: 10.3390/catal9090723.
17. Ghosh D, Kohli AG, Moser F, Endy D, Belcher AM. Refactored M13 bacteriophage as a platform for tumor cell imaging and drug delivery. ACS Synth Biol. 2012;1(12):576-582. DOI: 10.1021/sb300052u.
18. Wong S, Jimenez S, Slavcev RA. Construction and characterization of a novel miniaturized filamentous phagemid for targeted mammalian gene transfer. Microb Cell Fact. 2023;22(1):124,1-14. DOI: 10.1186/s12934-023-02135-w.
19. Kiga K, Tan XE, Ibarra-Chávez R, Watanabe S, Aiba Y, Sato'o Y, *et al*. Development of CRISPR-Cas13a-based antimicrobials capable of sequence-specific killing of target bacteria. Nat Commun. 2020;11(1):1-11. DOI: 10.1038/s41467-020-16731-6.
20. Kok DN, Turnbull J, Takeuchi N, Tsourkas PK, Hendrickson HL. *In vitro* evolution to increase the titers of difficult bacteriophages: RAMP-UP protocol. Phage. 2023;4(2):68-81. DOI: 10.1089/phage.2023.0005.