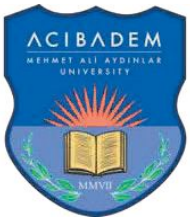

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BINDING TO INORGANIC SURFACES AS PHAGE DISPLAY LIGANDS

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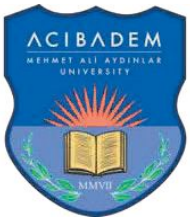

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DECLARATION

I hereby declare that; this thesis has been written by me based on the data obtained in line with the scientific rules and ethical principles of responsible conduct of research. All information, data, comments, analyses have been collected and processed through scientific, academic writing style, and literature used have been duly shown by giving reference to the sources by the publication ethics. I also announce and emphasize that I have not violated any rules secured by patent and copyrights whilst the conduct and writing of this research.

07.09.2020

Esma AYBAKAN

ACKNOWLEDGMENTS

With many thanks to my supervisor Dear Assist Prof. Dr. Erkan Mozioğlu and my co-advisor Dear Prof. Dr. Tanıl Kocagöz for their guidance, contributions, encouragement and supports during this research. Furthermore, I would like to thank my father, who is my greatest chance as a girl and thank my mother whom I took as a role model of her perseverance for their supports in my life. I am grateful to all my family, dear lab mates whom we worked together until night and my dear close friends for being with me in this process.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	Centigrade Degree
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometer
2X YT	Yeast Extract Tryptone
bp	Base Pairs
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
E. coli	Escherichia coli
F'+	Presence of a Fertility Factor
F^+	Donor Bacteria - Presence of a Fertility Factor
g	G-Force
g	Gram
gI	Gen I
IPTG	Iopropyl-Beta-D-Thiogalactopyranoside
kb	Kilobases
L	Liter
LB	Luria-Bertani Broth
LBA	Luria-Bertani Agar
M	Molar
mL	Milliliter
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
NaCI	Sodium Chloride
ng	Nanogram
nm	Nanometer
OD	Optic Density

ORTE	Observable Real-Time Electrophoresis
P8	Protein 8
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
RF	Replicative Form
RNA	Ribonucleic Acid
rpm	Rotation Per Minute
ssDNA	Single Stranded Deoxyribonucleic Acid
TAE	Tris Acetate EDTA
TBS	Tris Buffered Saline
Tet	Tetracycline
TSS	Transformation and Storage Buffer
UV	Ultraviolet Light
V	Volt
w/v	Weight/Volume
X-gal	5-Bromo-4-Chloro-3-Indolyl-Beta-D-Galacto-Pyranoside

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SUMMARY

In this thesis, it is aimed to design and obtain M13 filamentous bacteriophages specific to silica and biotin by using genetic engineering methods. For this purpose, silica and biotin-specific peptide sequences were fused into the pIII proteins of M13 bacteriophages, so they were displayed on the surfaces of the bacteriophages. Firstly, DNA sequences encoding the relevant peptide sequences were identified and primers containing DNA sequences complemented to the genomic DNA of M13 bacteriophage and coding specific peptide sequences were designed. Using these primers and inverse polymerase chain reaction, the peptide sequences were fused to the bacteriophage genome. After phosphorylation and ligation steps, they were transformed into *Escherichia coli* bacteria. Transformed bacteria were selected as blue colonies in solid media containing X-gal and IPTG. Phage production was performed by using the confirmed blue colonies. Phage infection abilities were verified by isolating phagemid DNA molecules after bacteria were infected by the recombinant phages. Instead of the standard protocols used for engineering of bacteriophages in the literature, modified strategies were developed in this thesis study. Therefore, we believe we provide a more rapid, easier, cheaper method for the engineering of bacteriophages with the rational design. These recombinant phages are intended to be used in the future to obtain new bifunctional or multifunctional new bacteriophages by functionalizing different peptides on the same phage. Then, as the phage display ligand, these phages will be used as tools for the development of new generation biosensors.

Keywords: Biotin-Binding, M13, Phage Display, Phage Engineering, Silica-Binding

ÖZET

İnorganik Yüzeylere Bağlanabilen Bakteriyofajların Faj Gösterim Ligandı Olarak Geliştirilmesi

Bu tez çalışmasında, genetik mühendisliği araçları kullanılarak, silika ve biyotine bağlanabilen, M13 filamentöz bakteriyofajlarının tasarımı ve üretilmesi amaçlanmıştır. Bu amaçla, silika ve biyotine özgü peptit ardışımlarının (*sekanslarının*) M13 bakteriyofajlarının pIII proteinlerine eklenmesi ve bu şekilde bakteriyofajların yüzeylerinde sergilenmesi hedeflenmiştir. İlgili peptit ardışımlarını kodlayacak DNA ardışımları belirlenmiş ve bir ucu genomik DNA ile eşlenik diğer ucu peptitleri kodlayacak DNA ardışımlarını içeren primerler tasarlanmıştır. Bu primerler ile bakteriyofajın genomu kalıp olarak kullanılarak ters polimeraz zincirleme tepkimesi (inverse PCR) yardımıyla ilgili peptit ardışımları, bakteriyofaj genomuna eklenmiştir. Bu doğrusal rekombinant DNA molekülleri, fosforlisayonun ve ligasyonun ardından *E. coli* bakterilerine aktarılmıştır. Gen aktarımlı bakteriler, X-gal ve IPTG içeren katı besiyerlerinde mavi koloniler olarak seçilmiş ve hedefe özgü primerler kullanılarak polimeraz zincirleme tepkimesi (PZT) yardımıyla doğrulanmıştır. Bu kolonilerden fajlar üretilmiş ve bu fajların enfeksiyon yetenekleri, bakterilerden fajmitlerin saflaştırılması yoluyla gösterilmiştir. Bu tez çalışması sırasında kullanılan yöntemler, faj gösterimi teknolojisinde kullanılan standart yöntemlerin değiştirilmesi ve iyileştirilmesinin yanı sıra bazı aşamalarda, tümüyle yeni tekniklerin geliştirilmesini kapsadığından, faj gösterimi ligandı olarak akılcı tasarım ile rekombinant bakteriyofajların üretimini hızlandıracak, basitleştirecek ve gerekli maliyeti azaltarak verimi arttıracak özgün teknikler geliştirilmiştir. Genetik mühendisliği ile elde edilmiş bu fajların, farklı peptitleri pVIII proteinlerinde sergileyecek şekilde iki işlevli veya çok işlevli olarak kullanılması tasarlanmaktadır. Bu sayede, faj gösterimi ligandı olarak bu fajlar, yeni nesil biyosensörlerin geliştirilmesinde araç olarak kullanılacaktır.

Anahtar Sözcükler: Biotin Bağlayıcı, Faj Gösterim, Faj Mühendisliği, M13, Silika Bağlayıcı

1. AIM OF THE STUDY AND BACKGROUND

In this thesis, it is aimed to design and obtain M13 filamentous bacteriophages specific to silica and biotin by using genetic engineering methods. For this purpose, silica and biotin-specific peptide sequences were fused into the pIII proteins of M13 bacteriophages, so they were displayed on the surfaces of the bacteriophages. In this phage display technique, phage coat proteins are used as the ligands and they are selected to any targets such as organic compounds or inorganic surfaces. Since phages naturally use their displayed peptides on their coat proteins to recognize and infect their host bacterial cells (1–3). In phage display technique, foreign peptides which mostly include 12 aminoacid sequences were displayed on the phage coat proteins (4). These modifications do not affect their specific sites which are important to recognize their host bacteria and they can infect the bacterial cells (4). All these features make them extremely useful biological tools (1,2,5). Bacteriophages can be subjected to the rational design for engineering of new, recombinant bacteriophages. In these cases, DNA molecules are modified by using genetically engineering methods and foreign DNA sequences coding target peptide sequences are cloned into bacteriophage DNAs.

Standard protocols used for engineering of bacteriophages with rational design have some limitations in practice. Therefore, in this study, different modified strategies about cloning, screening, concentration, and infection of phages were developed to improve the phage display technology. Phages developed in this study will be subjected to build multifunctional phages which may be used therapeutic and diagnostic purposes in the future.

2. INTRODUCTION

2.1. Bacteriophages

Bacteriophages (phages) were discovered and firstly named by Fèlix d'Herelle in 1917 (6–9). Bacteriophages are a type of virus which are obligate to bacteria (4,6,9). (4,6,9). They have only nucleic acids either DNA or RNA in their protein coats (6,9). They transfer the genetic material to bacteria and then, viral proteins are expressed in the bacteria (6,8,9). In the absence of any host, they can remain infection abilities for years, unless they are damaged by environmental conditions (6). Phages are susceptible to things like UV that damage the nucleic acid, but are very stable under different environmental conditions such as high temperature, DNases, proteases, extreme pH, and organic solvents (6). Bacteriophages can infect only their specific host bacterial cell (6,9). They cannot infect cells of more complex organisms due to large differences in intracellular machines, in addition to certain cell surface proteins where they must bind to host cell (6,9). Bacteriophages have a wide range of sizes and shapes, filamentous phages are the one type of them (6,9). They are classified by their host organisms, morphology, type of the genetic material and structure (6,9,10). Viral taxonomy does not require a phylogenetic relationship (6).

2.1.1. Filamentous phages

Filamentous phages have semi-flexible fibrous structures composed of capsid proteins around of a single-stranded DNA (ssDNA) (4,11,12). They do not lyse the bacteria for reproduction by meaning they use the bacterial cell like a machine (11). This process is called as a lysogenic cycle (11,13). DNA sequences of many filamentous phages such as bacteriophage f1, M13 and fd are 98% identical to each other (11,13,14).

2.1.2. M13 phage genome and structure

The genus of M13 phages is Inovirus which infects enterobacteria, especially *E. coli* (15). They have single-stranded DNA genomes in ~6400 bases length (15,16). Phage size is ~ 1 μm length (17,18). They infect only male bacteria that carry F pilus (F episome, F^+) and release from infected cells while bacterial cells continue to grow and divide (12,15,19,20). Single-stranded DNA of the M13 phage particle is packaged with 2700 copies of the main coat Protein 8 (P8) and 5 copies of minor protein P3, P6, P7, and P9 (Figure 2.1) (4,14–17,20,21).

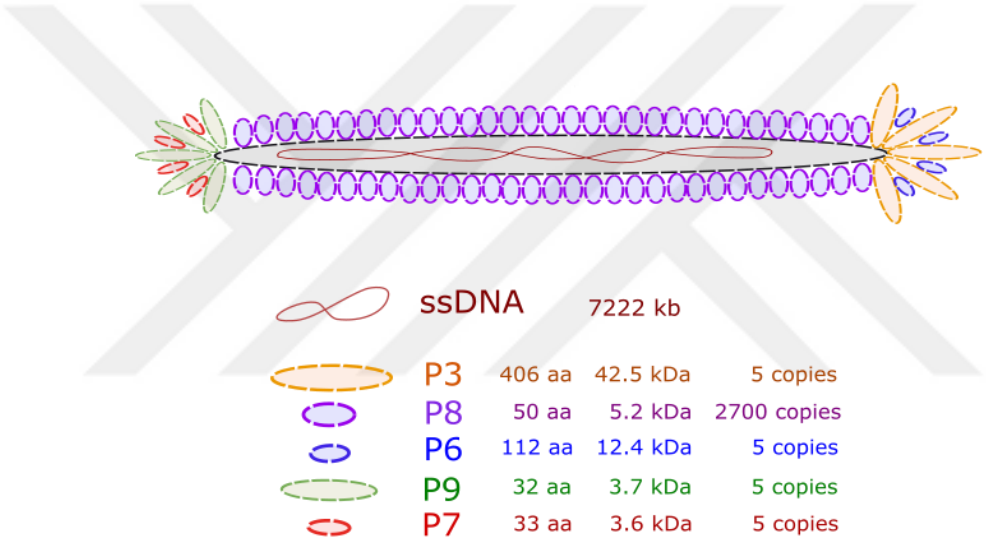

Figure 2.1: The structure of M13 Bacteriophage [Modified according to the Smeal et.al. 2017 (21), Slonczewski 2017 (19), Brasino 2016 (22).]

M13 bacteriophage capsid consists of a flexible protein cylinder (13,15,19). The cylinder is mounted around a supercoiled, circular ssDNA (12,15,19). Each subunit of M13 contains a short alpha-helical peptide which was encoded by gene 8 (Table 2.1) (2,5,15,19). These protein subunits have positively charged lysine residues at the internal end to bind the negatively charged DNA (15). Five copies of the P3 protein at one end of the tube (4,12,15). It is a much larger and more flexible protein that binds the cell surface receptor for phage adsorption (15). P7 and P9 proteins let the phage

particle leave the cell (12,15,17,19,20). P2, P5, and P10 play a role in viral DNA replication (15,19,20). M13 bacteriophage capsid is composed of P3, P6, P7, P8, P9 transmembrane proteins (15,19,20). These coat proteins are accumulated on the cell membrane and then DNA molecules are packaged as phage particles (15,20). These virus particles composed of DNA-protein structure are secreted from the cell (15,20). Packaging and secretion processes occur simultaneously and manage by P1, P4, P11 proteins that allow it to be produced in bacteria and does not participate in the phage structure (15,20).

Table 2.1: M13 Phage Proteins [Modified according to Sambrook 2001 (15), Ledsgaard and Kilstrup 2018 (20).]

Gene Name	Protein Name	Abbre-viation	Size (kDa)	Responsible For	Function
I	Protein 1	P1	39.6	Assembly	Spans the inner membrane of infected host bacteria, interacts with p4 and initiates the phage assembly.
II	Replication Associated Protein/ Protein 2	P2	46.2	Replication	Introduces a nick in the intergenic region of the (+) RF DNA specifically thereby initiates the rolling circle replication that generates new (+) strand progeny DNA molecules and also cleaves the ssDNA products.
III	Attachment Protein/ Protein 3	P3	44.7	Adsorption, Extrusion	It is a minor coat protein that is anchored to the membrane by a single membrane-spanning domain near the carboxyl terminus. Attaches to the proximal tip of phage. This tip enters a host first during infection and leaves last from the membrane when progeny particles are released. It is required for adsorption of phage to bacteria's sex pili and penetration
IV	Virion Export Protein/ Protein 4	P4	45.9	Assembly, Extraction	Multimeric protein that forms of gated channel interaction the bacterial cytoplasm to the exterior. Highly produced in infected bacteria and required for induction of bacterial phage shock protein operon that occurs during infection.

Table 2.1: M13 Phage Proteins [Modified according to Sambrook 2001 (15), Ledsgaard and Kilstrup 2018 (20).] (continue)

Gene Name	Protein Name	Abbre-viation	Size (kDa)	Responsible For	Function
V	DNA Binding Protein/ Protein 5	P5	9.7	Replication	Binds strongly and in a cooperative manner to new (+) DNA strand products. Thereby protein and DNA complex move to specialized packaging sites on membrane then stripes and recycles in the bacterial cell. It acts as a translational repressor for viral proteins by binding to the leader mRNAs.
VI	Head Virion Protein/ Protein 6	P6	12.4	Infection, Building	Located at the proximal end of phage and associated with P3. Before incorporation into a phage particle, located in the cytoplasmic membrane of infected bacteria.
VII	Tail Virion Protein/ Protein 7	P7	3.6	Assembly, Building	Interacts with the packaging signal located in the intergenic region of phage DNA. Located on the end of phage that emerges first from the infected bacterial cell.
VIII	Major Capsid Protein/ Protein 8	P8	7.6	Coat Protein	The major coat protein of phage and synthesizes like a preprotein called “precoat” which binds to the inner surface of the plasma membrane and translocate across the membrane in the presence of transmembrane potential.
IX	Tail Virion Protein/ Protein 9	P9	3.7	Assembly, Building	Located at the end of phage where assembly starts. Interacts with the packaging signal where the intergenic region of phage.
X	Protein 10	P10	12.7	Replication	Translation of it begins at the in-frame AUG codon of P2. Required for efficient accumulation of ssDNA and strong repressor of phage specific DNA synthesis and thought to limit the amount of replicative form DNA products in bacteria.
XI	Protein 11	P11	12.4	Assembly	Spans the cytoplasmic membrane but lacks a cytoplasmic domain. It may be taking a role in the assembly of phages.

The M13 genome is 6.4 kilobases and encodes 11 proteins with promoters, terminators, multiple overlapping genes, ribosome binding sites and a complex that regulates the infection of phage (15,16,21). The M13 genes and proteins are called G1-G11 and P1–P11 respectively (15,21). The phage genome is arranged in two different transcriptional units (15). Five promoters and three terminators produce an mRNA cascade that is contributed to phage protein concentration regulation (15,21). The non-coding region includes a promoter and terminator, the origins of positive and negative-strand DNA synthesis, in addition to the signal that is essential for the packaging of the ssDNA into phage particle (15,21). The M13KE genome is a derivative of M13mp19 which cloning sites introduced at the 5’ end of protein 3 gene and 7.222 kilobases in length (Figure 2.2) (23).

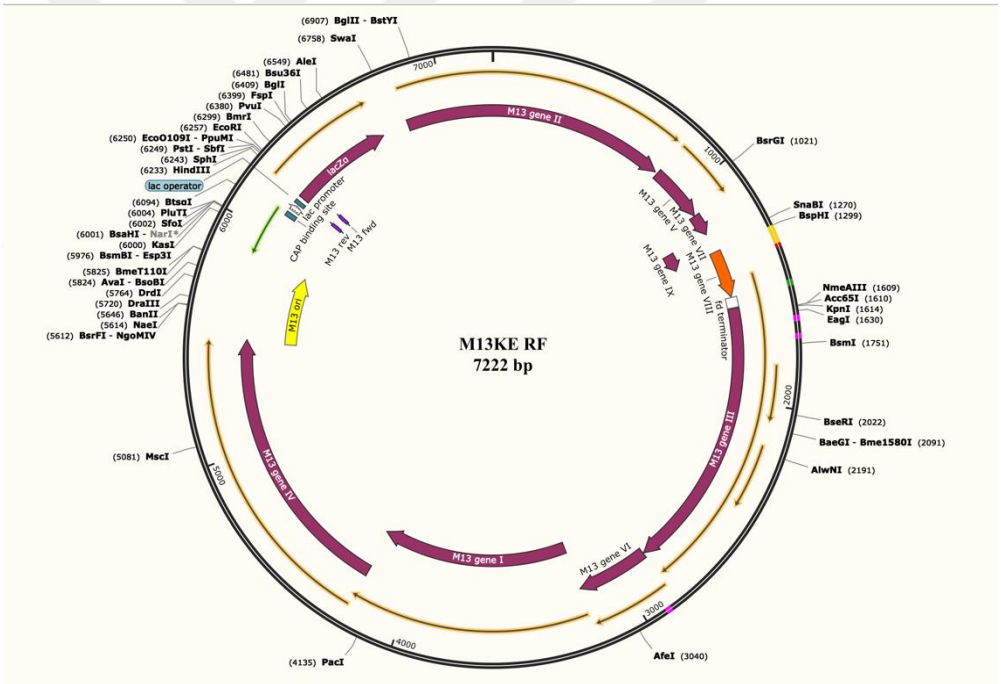

Figure 2.2: Replicative Form (RF) of M13KE Genome and Restriction Sites Map (Produced by Snapgene version 4.2.11)

2.1.3. M13 general life cycle

The M13 life cycle starts with the adsorption of P3 fusion proteins to the F episome of host bacteria (4,15). After the viral genome enters into bacteria, single-stranded DNA has rapidly replicated the circular double-stranded DNA form called replicative form (RF) by *E. coli* enzymes (4,15,24). Then RF molecules generate single-stranded viral progeny DNA molecules by a rolling circle mechanism (Figure 1.3) while the 11 phage genes are being expressed for replication, reproduction, packaging and releasing (4,15). In rolling circle model replication, genetic material encodes the replicon-specific initiator proteins (15,17,21). They initiate the replication of genetic material by binding to a specific site in the replication origin and create a nick specifically next to it (15,21). DNA polymerase enzymes use 3'OH prime of this site which was generated with nick (15,21). Polymerization is continued while each completed replicon is digested from the extending DNA strand (Figure 2.3) (15,21).

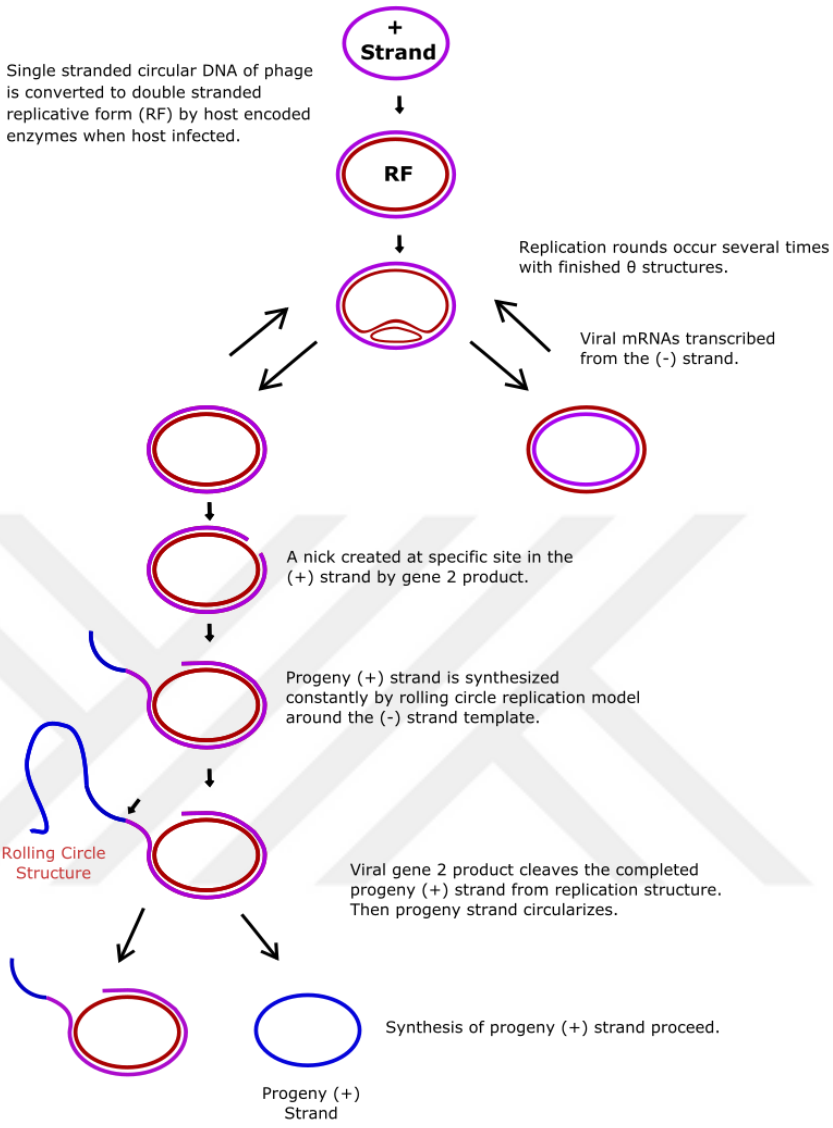

Figure 2.3: Replication of Bacteriophage M13 DNA in Infected Bacteria-The first, a nicking of closed circular RF DNA begins, occur during the first 15-20 minutes of infection and result in the accumulation of 100-200 circular RF DNA molecules per cell. The subsequent production of single-stranded DNA is a continuous process that leads to the morphogenesis of progeny particles [Modified according to the Sambrook 2001 (15) via Inkspace Software].

If phage protein concentrations are low, the single-stranded progeny copies are converted into additional RF DNA (21). On the other hand, if phage protein concentrations increase, especially P5, the rate of synthesis of the RF DNA from ssDNA decreases (21). P5 binds to progeny ssDNA genomes and prevent the conversion into RF DNA (21). This complex is recognized by the membrane phage assembly complex that P1, P4 and P11 (21). The assembly complex first binds to P7 and P9 then to P5-ssDNA complex (21). Then the complex is assembled around the ssDNA genome; P5 is removed, P3 and P6 are added (21). Finally, phage particles pass through cell membranes (Figure 2.4) (21).

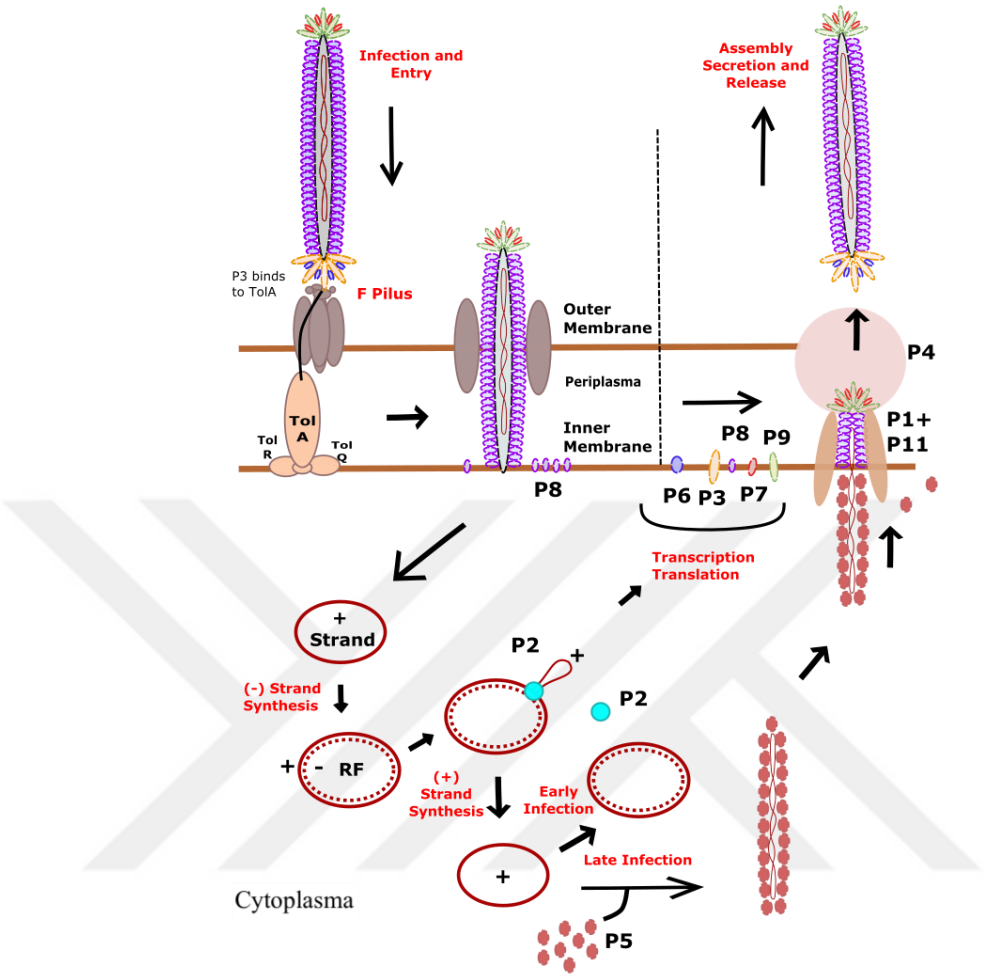

Figure 2.4: Life Cycle of M13 Bacteriophages- Phage attaches to host with P3 binding to the F pilus and interacts with the TolA receptor which localized at the inner membrane. Viral ssDNA (+ strand) enters into the bacterial cell while the P8 integrates into the inner membrane. RNA polymerase generates a primer from (+) strand and by the way complementary strand (-) synthesis begins from replication origin. Bacterial DNA polymerase 3 uses the primer for replication and completes the whole (-) strand. P2 initiates the (+) strand synthesis, therefore, creates a nick at the positive origin of replication on a replicative form (RF) of (+) strand. Then replication starts with a rolling circle model. New progeny (+) strands synthesize and used for a template to (-) strand synthesis during the initial viral infection stage. RF count increases and phage proteins produce from its. Viral P2, P5 ve P10 remain in cytoplasm and play role in viral genome replication, assembly and packaging of phage. P1, P4 and P11 create a transport complex in the inner and outer membrane. P7, P9, P8, P6 and P3 clump in membrane before packaging. Single (+) strands coat with P5 and this complex transports to the membrane export complex that consisting of P1, P11, P4 [*Modified according to the Rakonjac et al. 2017 (11) and Slonczewski et al. 2017 (19) via Inkspace Software*].

2.2. Phage Display Technology

Phage display was designed by George Smith in 1985 as a technique for presenting polypeptides on the surface of lysogenic filamentous phages (1,3,12). This technique has become the most effective ways for producing large scale of peptides, proteins and antibodies (Figure 2.5) (1–3,13,25). It also makes possible to work on protein-ligand interactions, receptor binding sites, therefore the phage display technique has been used in various biomedical applications such as identifying of epitopes and functional or accessible regions of biomolecules, developing of monoclonal antibodies, modeling of the autoantibodies in cancer disease, phage-ELISA, quartz crystal microbalance, surface plasmon resonance, and electrochemical biosensors, bioelectronics (1-5,25–33). Besides diagnostic approaches, selected/engineered phages can be used for therapeutic purposes and therefore they are called as theraneustic tools (1,2,37–39,3–5,12,16,34–36).

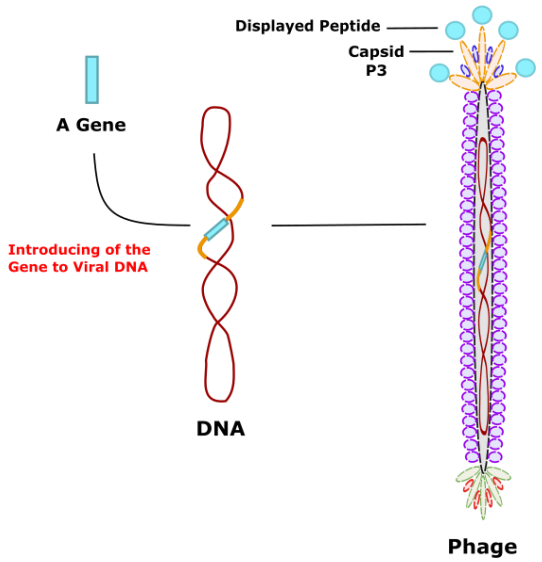

Figure 2.5: The Phage Display Technology- A gene introduces to the viral genome and DNA transformed to the bacterial cell by the way phage particles that displayed new peptide on capsid protein are produced [*Modified according to the Fernholm 2018 (3) via Inkspace Software*].

The phage display technique has been used to select peptide ligands specific to any targets such as organic compounds or inorganic surfaces (23). For that, first, a DNA library is designed. This DNA library includes approximately 10^{10} unique short DNA molecules (23). Each of them is flanked by primer sites at the both ends (23). These primer sites are used for cloning this DNA library into the bacteriophage DNA (23). Random nucleotide sequences are between the primer sites and synthesized by DNA synthesizers. In this synthesis, nucleotides are randomly chosen from a mixture including A, T, C, G nucleotides equally (23). Primer sites are complemented with N-terminus of pIII gene (23). Once this DNA library is cloned into the phage genome, peptides coded by these random DNA sequences is displayed on the P3 (23). Therefore, a peptide library on the phage which is called as a phage library is obtained (23). It means there are 10^{10} different displayed peptides in a phage library (23). By using this phage library, peptides specific to any targets can be selected (23). This selection process is named as the phage display (23). After binding and washing steps in cycles, some phages can be selected as ligands specific to the target (23). After this selection, phages bound specifically to the target, is amplified and their genomes are sequenced to learn peptide sequences specific to the target (23).

As another option, phages can be directly used as the phage display ligands to design new biosensors. Although P3, P6, P8, P7 or P9 proteins can be used for displaying (Figure 2.6) (1,2,12,34,40), P3 is the most used. P3 is a huge and most complex protein that includes three different domains (17). N-terminal domain of P3 protein is responsible for entering of the viral genome into host *E. coli* and the second domain binds to F pilus and provides the host recognition (17). For the integration of P3 with other coat proteins, the C-terminal domain are located in the cytoplasm and interacts with them, whereas N-terminals are located in the periplasm (17). The length of foreign peptides intended to be displayed on P3 is limited with 8-12 amino acids to protect infection abilities of the phages (1,12,41,42). For engineering of phages, peptide sequences are rationally calculated and then, they are verified experimentally. For that, DNA sequences coding these peptides are cloned into the phage genome and their binding affinities are tested (43).

There are some protocols for engineering of the phages as the phage display ligands, however they have some important limitations in practice (43).

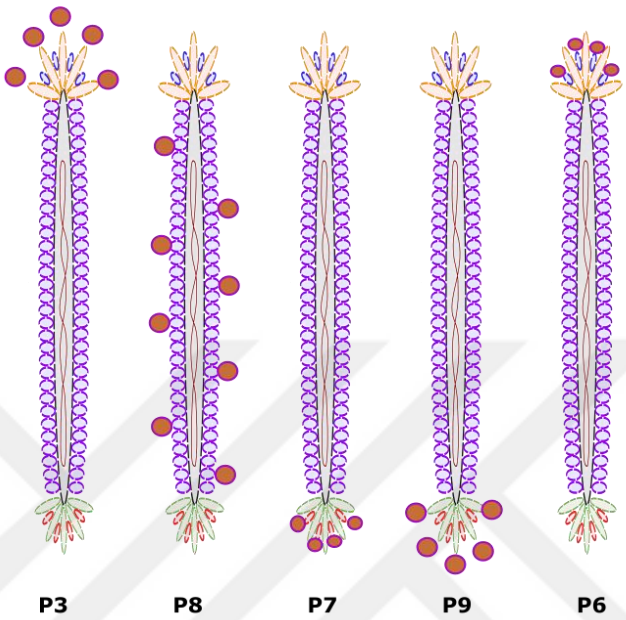

Figure 2.6: Schematic Presentation of M13 Phage Display Systems- The pink circle represents a molecule displayed on various phage proteins [*Figure modified according to Bazan et al. 2012 (1) and Petrenko 2018 (12) via Inkspace Software*].

By developing new strategies overcoming the difficulties encountered in these methods mentioned above, phages specific to biotin and silica were engineered in this study.

2.3. Engineering of Bacteriophage M13 Binding to Inorganic Surfaces

2.3.1. Biotin-binding phages

Biotin is an essential vitamin that group of B and must be taken by mammals because they cannot synthesize it (44). Biotinylated antibodies or antigens are presently used in numerous immunoassay systems in clinics for the detection of analytes or biomarkers with high selectivity and sensitivity (44). More immunoassays are based on (strept)avidin-biotin interaction system (44–46) These systems are used for thyroid markers, cancer markers, cardiac biomarkers, drugs. Biotin-binding peptides were studied by Saggio and Laufer (47). It was found that those peptides - **C**-S-**W**-R-**P**-**P**-**F**-**R**-A-V-**C**, **C**-S-**W**-A-**P**-**P**-**F**-**R**-A-S-**C**, **C**-N-**W**-T-**P**-**P**-**F**-**R**-T-R-**C** were successfully bound to biotin (47). It was also showed that CXWXPPF(K or R)XXC sequences still had the ability of binding to biotin (47). Therefore, in this study, CNWTPPFKTRC was selected for engineering of the M13KE phage as biotin-binding ligands.

2.3.2. Silica-binding phages

Solid-binding peptides show a high binding affinity and selectivity to the solid surfaces like polymers, metals and silica, etc. (48). These peptides are used for designing of different biosensors or bioassays (48). For this study, MSPHPHPRHHHT amino acid sequences published by Naik and et al. were used for engineering of the M13KE phage as silica-binding ligands (49).

3. MATERIAL AND METHODS

3.1. Materials and Equipments

The list of equipment and chemicals used in this thesis study is given in Table 3.1 and Table 3.2.

Table 3.1: Chemicals

Chemicals	From	Catalog ID
%30 Polyacrylamide Solution	Neofroxx / Germany	1106ML500
5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside / Xgal	Goldbio / Missouri	X4281C
Acetic Acid (glacial)	Sigma-aldrich / Missouri	1.00063
Agarose	Geneon /	604-001
Ammonium Per Sulphate / APS	Neofroxx / Germany	1610GR100
Bacteriological Agar	Biolife / Italia	4110302
Boric Acid	Merck / Germany	B6768-500G
BseRI Restriction Enzyme	NEB / Massachusetts	R0581S
CIAP	NEB / Massachusetts	M0290S
Dimethyl sulfoxide / DMSO	Neofroxx / Germany	1610GR100
DNA Ladder 10 kb	Genemarkbio / Taiwan	GLM11
DNA Ladder 3 kb	Genemarkbio / Taiwan	GM100
DNA Ladder 10 kb	Geneon / Germany	305-005
DNA Ladder 100 bp	Genedirex / USA	DM001-R500
DNA Polymerase Master Mix	Genemarkbio / Taiwan	RP02-II
Escherichia coli K12 ER2738	NEB / Massachusetts	E4104

Table 3.1: Chemicals (continue)

Chemicals	From	Catalog ID
E.Z.N.A.® M13 DNA Mini Kit	Omega / Germany	D6900-01
E.Z.N.A.® Plasmid Midi Kit	Omega / Germany	D6904-03
Eag I Restriction Endonuclease	NEB / Massachusetts	R05055
EtBr	Sigma- Aldrich / Missouri	E1510-10ML
Ethylenediaminetetraacetic acid / EDTA	Bioshop / Canada	EDT002
Zymoclean Gel DNA Recovery Kit	Zymo / California	D4007
Glycerol	Neofroxx / Germany	1073LT001
KpnI Restriction Enzyme	Takarabio / Japan	1068A
Phusion™ High Fidelity DNA Polymerase 2U/µL	Thermofisher / Massachusetts	F-530XL
HPLC Grade EtOH	Sigma-aldrich / Missouri	09-0851
Isopropyl β- d-1-thiogalactopyranoside / IPTG	Genemarkbio / Taiwan	GMSJ0012-0005
Kpn I Restriction Endonuclease	Takarabio / Japan	1068A
Luria Bertani Liquid Medium (Miller)	Caisson / USA	LBP01-500GM
MyTaq™ HS DNA Polymerase	Bioline / London	BIO-21111
N,N,N′,N′-Tetramethyl ethylenediamine / TEMED	Bio-Rad / California	1610800EDU
NaCI	Merck / Germany	106406
NaOH	Merck / Germany	UN1832
PCR Clean-Up Kit	Genemarkbio / Taiwan	DP04
Polyethylene glycol /PEG-8000	Sigma-aldrich / Missouri	89510
Ph.D.™ Peptide Display Cloning System	NEB / Massachusetts	E8101S
T4 DNA Ligase	Genemarkbio / Taiwan	GDL001LS(1000U)
T4 DNA Ligase	Takarabio / Japan	2011A
T4 Polynucleotide Kinase	NEB / Massachusetts	M0201S
Trizma Base	Sigma-aldrich / Missouri	T1503-1KG
Tryptone	Neogen / UK	Q42758/352
Yeast Extract (YT)	Neogen / UK	Q43100/011

Table 3.2: Instruments

Instruments	
-80C Refrigerator	Kirsch
+4, -20C Refrigerator	Kirsch
Agarose Gel Electrophoresis Equipment	TIBO
Autoclave	Witeg
Top bench Centrifuge	Thermo Scientific
Centrifuge	Beckman
Chemidoc Imaging System	Bio-Rad
Energy Supplier	Bio-Rad
Heat Block	Biosan
Incubator	Thermo Scientific
Large Size Incubator with Shake	N-Biotek
Magnetic Mixer	Thermo Scientific
Microwave oven	Samsung
Minispine	Biosan
Nanodrop	Thermo Scientific
ORTE (Observable Real-Time Electrophoresis)	TIBO
pH Meter	Thermo Scientific
Polyacrylamide Gel Electrophoresis Equipment	Bio-Rad
Shake Incubator	Thermo Scientific
Spectrophotometer	HITACHI
Thermal Cycler	Bio-Rad
Vortex	VWR
Water Bath	Commat

Solutions, it’s ingredients and concentrations are listed in Table 3.3.

Table 3.3: Chemical Solutions and Preparations

Solutions	Preparation	Sterilization
%20 PEG-8000 + 2.5 M NaCI	20 gr PEG-8000, 2.5 M NaCI in 100 mL ddH_2O	0.22 μm Filtered
2X YT Medium	16 gr Tryptone, 10 gr Yeast Extract, 10 gr NaCI in 1L ddH_2O	Autoclaved at 121°C and 1 atm for 20 minutes
50X TAE	121 gr Tris base, 28.55 mL glacial acid, 50 mL 0.5 M EDTA pH:8 to 500 mL with ddH_2O	--
5X TBE	54 gr Tris base, 27.5 g Boric acid, 20 mL 0.5 M EDTA pH:8 to 1L with ddH_2O	--
1% Agarose Gel (w/v)	1 gr agarose in 100 mL 1X TAE Buffer	--
10% APS (w/v)	10 mg APS in 100 mL ddH_2O	--
EtBr Staining Solution	3.8 μg/mL – 19 μL100 mg/mL EtBr in 500 mL ddH_2O	--
1 M IPTG	2.38 gr IPTG in 10 mL ddH_2O	0.22 μm Filtered
LB Medium	25 gr LB powder in 1L ddH_2O	Autoclaved at 121°C and 1 atm for 20 minutes
LBA/ Tet/ X-gal/ IPTG	25 gr LB powder, 15 gr bacteriologic agar in 1L with 20 μg/mL Tetracycline, 0.02 or 0.2 mg/mL Xgal, 0.1 or 1 mM IPTG	Autoclaved at 121°C and 1 atm for 20 minutes without + supplements
TBS Buffer	6.05 gr Tris base, 8.76 gr NaCI in 800 mL ddH_2O adjust pH: 7.5 with HCI and up to 1L	Autoclaved at 121°C and 1 atm for 20 minutes without + supplements
Tetracycline Stock (10 mg/mL)	500 mg Tetracycline in 50 mL %70 EtOH HPLC Grade	--
TSS Buffer	5 gr PEG-8000, 1.5 mL 1M $MgCI_2$, 2.5 mL DMSO in LB to final volume 50 mL	0.22 μm Filtered
X-gal Stock(20 mg/mL)	20 mg in 1 mL DMSO	--

3.2. M13KE Plasmid Amplification

M13KE replicative form as a plasmid was purchased (NEB, UK). Many cloning experiments were tried, and these steps were required to a lot of the M13KE plasmid amount. For this reason, the plasmid was transformed to bacteria then amplified and isolated.

3.2.1. The preparation of competent cells by chemically

Competent cells were prepared according to Sambrook et. al. (2001) protocol (15). *E. coli* ER2738 bacterial cells were inoculated in LB medium including 20 µg/mL tetracycline at 180 rpm, 37°C for overnight. Growth culture was diluted with 1:100 volume of the fresh LB media including 20 µg/mL tetracycline and incubated at 180 rpm, 37°C. In the meantime, TSS Buffer and microcentrifuge tubes were put on the ice. Once cell concentration reached to OD 0.3 at 600 nm, culture was put on the ice for 10 minutes. The culture was centrifuged at 3.000 rpm, 4°C for 10 minutes. The supernatant was removed, and the cell pellet was resuspended with 10% volume of TSS Buffer and gently pipetted. The cell suspension was aliquoted to microcentrifuge tubes as 50 µL and tubes were frozen at cold ethanol (-80°C). Tubes were stored at -80 °C until used.

3.2.2. Transformation

Sambrook et. al. (2001) transformation protocol was modified in this study (15). Briefly, competent *E. coli* ER2738 cell suspension was thawed on ice. 50 ng of M13KE plasmid DNA (pM13KE) was added into cells and mixed by tapping. The tubes were incubated at 42°C water bath for 90 seconds. Then tubes were put on ice for 5 minutes. 800 µL of LB Medium pre-warmed at 37°C was added into tubes and inoculated at 180 rpm, 37°C for 1 hour. Then 100 µL of bacterial culture was plated

on LBA/ Tet (20 μg/mL)/ X-gal (0.02 mg/mL)/ IPTG (0.1 mM) agar plates pre-warmed at 37°C. Finally, plates were incubated at 37°C for overnight.

3.2.3. Plasmid isolation and DNA purification

One of the blue colonies was cultured in 2X YT Medium including 20 μg/mL tetracycline at vigorous aeration (250 rpm), 37°C for overnight. Then culture was centrifuged at 10.000xg, 4°C for 5 minutes. The supernatant was discarded. The plasmid was isolated by using Zymo Maxi Prep Plasmid Isolation Kit. The concentration of plasmids were determined spectrophotometrically (50). Plasmids were analyzed by agarose gel electrophoresis.

3.3. Engineering of bacteriophage M13 binding to inorganic surfaces

For engineering of M13KE, the construction protocol of phage display peptide libraries was modified in this study (23). CNWTPPFKTRC which was published by Saggio and Laufer was used as Biotin-binding peptide sequence, whereas MSPHPHPRHHHT which was published by Naik and et.al was used as Silica-binding peptide sequence in this study (23,48,49). Peptide sequences were designed to fuse at N-terminus of P3 protein where shortly after signal sequence as described previously at Noren and Noren (2001) (23). For that, peptide sequences were converted to nucleotide sequences and codon optimization for *E. coli* species was via IDT DNA Codon Optimization Tool (Figure 3.1) (51).

A: For Silica Binding

```
    M   S   P   H   P   H   P   R   H   H   H   T
5′ ATG TCT CCT CAT CCC CAT CCC CGC CAC CAC CAC ACT  3′
```

B: For Biotin Binding

```
    C   N   W   T   P   P   F   K   T   R   C
5′ TGC AAC TGG ACT CCG CCG TTT AAA ACG CGC TGC 3′
```

Figure 3.1: Amino Acid and Codon Sequences- (A) For Silica-binding peptide (B) For Biotin-binding peptide.

Two different types of cloning methods were performed for fusion of the DNA sequences coding peptides specific to silica and biotin: Restriction enzyme-based cloning and Inverse Polymerase Chain Reaction (PCR)-based cloning method.

3.3.1. Restriction enzyme-based cloning

3.3.1.1 Preparation of vector

M13KE replicative form was digested with two restriction enzymes (Figure 3.2). First, DNA was subjected to the digestion of the *Kpn* I restriction enzyme and treatment of the calf intestinal alkaline phosphatase (CIAP) at 37°C for 30 minutes (Table 3.4). Then the enzymes were inactivated at 65°C for 20 minutes.

Table 3.4: Kpn I Digestion

Reaction Ingredients	Stock Concentration	Final Concentration	Volume (µL)
Smart Buffer	10 X	1 X	5
Kpn I Restriction Enzyme	10 U/µL	10 U	1
CIAP			1
DNA	887 ng/ µL	887 ng	1
Nuclease free ddH_2O			42

Digested DNA bands were cut and purified from the 0.8% agarose gel by using E.Z.N.A Gel Elution Kit. Purified DNA concentration was determined via nanodrop. The prufied DNA was digested with the second enzyme *Eag* I and dephosphorylated with CIAP at 37°C for 30 minutes. Then the enzymes were inactivated at 65°C for 20 minutes.

Table 3.5: Eag I Digestion

Reaction Ingredients	Stock Concentration	Final Concentration	Volume (µL)
Cut Smart Buffer	10 X	1 X	5
Eag I Restriction Enzyme	10 U/µL	10 U	1
CIAP			1
Kpn I Digested DNA	8 ng/ µL	256 ng	32
Nuclease free ddH_2O			11

Digested DNA bands were cut and purified from the 0.8% agarose gel by using E.Z.N.A Gel Elution Kit. The purified DNA concentration were determined via nanodrop.

3.3.1.2 Duplication and phosphorylation of oligomers coding silica and biotin-binding peptides

Oligomers with sticky ends were designed given in Table 3.6. 100 μM of forward and reverse oligomers were mixed in $dddH_2O$. The samples were incubated at 95°C for 4 minutes and then at 76°C for 4 minutes to obtain duplicated oligomers. The oligomer duplexes were phosphorylated with T4 Polynucleotide Kinase (Takara) at 37°C for 30 minutes (Table 3.6). Then the enzyme was inactivated at 65°C for 20 minutes.

Table 3.6: Oligomers

Oligomer	Sequence	Size (bp)
Positive strand (coding silica-binding peptide)	CTT TCT ATT CTC ACT CTA TGT CTC CTC ATC CCC ATC CCC GCC ACC ACC ACA CTT C	55
Negative strand (coding silica-binding peptide)	**GGC C**GA AGT GTG GTG GTG GCG GGG ATG GGG ATG AGG AGA CAT AGA GTG AGA ATA GAA AG**G TAC**	63
Positive strand (coding biotin-binding peptide)	CTT TCT ATT CTC ACT CTT GCA ACT GGA CCC CGC CGT TTA AAA CCC GCT GCT C	52
Negative strand (coding biotin-binding peptide)	**CAT G**GA AAG ATA AGA GTG AGA ACG TTG ACC TGG GGC GGC AAA TTT TGG GCG ACG AG**C CGG**	60

Bold cases are sticky end of oligomers

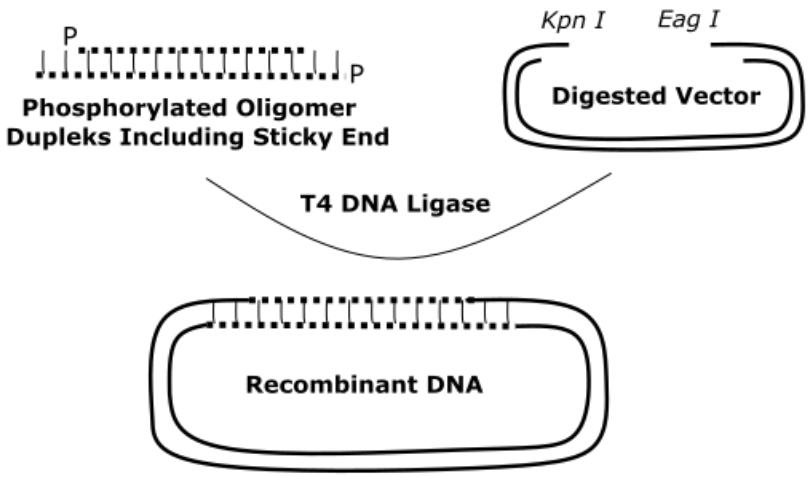

Figure 3.2: Restriction Enzyme-Based Cloning (*Illustrated by using Inkspace Software*)

Table 3.7: Phosphorylation Reaction

Reaction Ingredients	Stock Concentration	Final Concentration	Volume (µL)
Buffer	10X	1X	5
Polynucleotide Kinase			1
ATP	10 mM	1 mM	5
DNA	250 pmol	5 pmol/µL	5
Nuclease free ddH_2O			34

After diluted in ratio 1:1000 volume of phosphorylated DNA duplexes with nuclease-free ddH_2O, the vector/insert molar ratio was calculated by using the Ligation Calculator of NEB Biocalculator Tool (52).

Table 3.8: Molar Ratio of Insert and Vector

Vector/Insert	Vector:Insert Molar Ratios		
	1:3	**1:5**	**1:10**
Required Mass Insert	0.6 ng	0.9 ng	1.8 ng
The volume of biotin-binding peptide DNA sequence duplex insert	2.2 µL	3.7 µL	7.4 µL
The volume of silica-binding peptide DNA sequence duplex insert	2.3 µL	3.8 µL	7.6 µL

3.3.1.3 Ligation of oligomers coding silica and biotin peptides

M13KE vector and the oligomer duplexes of peptides were ligated according to Table 3.8 volumes with T4 DNA Ligase at 16°C for 1 hour.

Table 3.9: Ligation Reaction

Reaction Ingredients	Stock Concentration	Final Concentration	Volume
Ligase Buffer	10 X	1X	5 µL
T4 DNA Ligase Enzyme			1 µL
Vector DNA	2 ng/ µL	22 ng	11 µL
Insert DNA			From Table 3.8
Nuclease free ddH_2O			To 50 µL total volume

3.3.2. Inverse PCR-based cloning

DNA sequences of target peptides were fused to M13KE by using the Inverse Polymerase Chain Reaction which was modified as described in Dong Qi et. al. (2008) (43). Briefly, the silica or biotin-binding peptide DNA sequences were at 5' of the forward primers whereas, 3' of the forward primers and reverse primer were complement to M13KE DNA (Table 3.10 and Figure 3.3).

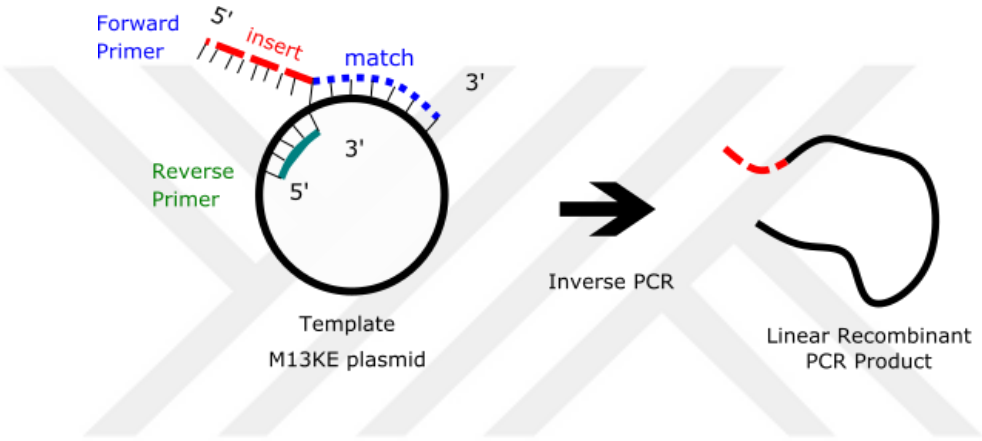

Figure 3.3: Primer Design of Inverse PCR (*Illustrated by using Inkspace Software*)

Table 3.10: Inverse PCR Primers

Oligomer/Primer Name	Nucleotide Sequence	Size	%GC	Tm (ºC)
Silica-Binding Peptide DNA Sequence	TCT ATG TCT CCT CAT CCC CAT CCC CGC CAC CAC CAC AC**T TCG GCC GAA ACT GTT GAA AGT TG**	62 bp	55	73
Biotin-Binding Peptide DNA Sequence	TCT TGC AAC TGG ACC CCG CCG TTT AAA ACC CGC TG**C TCG GCC GAA ACT GTT GAA AGT TG**	59 bp	54	73
Reverse Primer	GTG AGA ATA GAA AGG TAC CAC TAA AG	26 bp	39	54

Sequences annealing to templates are in the bold case, foreign sequences for insertion are in normal case.

Inverse PCR was performed by using the high-fidelity DNA polymerase (Thermo, UK) and designed primers above in order to obtain the recombinant double-strand linear DNA molecules. After phosphorylation step with polynucleotide kinase enzyme, the blunt ends were ligated by using T4 DNA Ligase Enzyme (Figure 3.4).

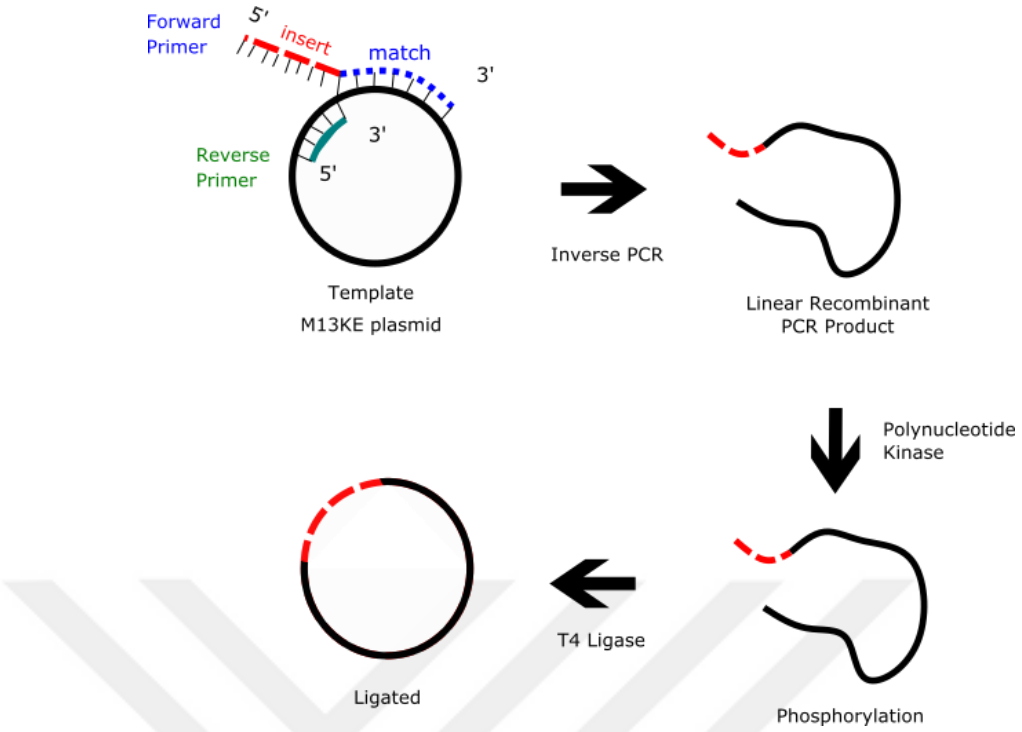

Figure 3.4: Inverse PCR-Based Cloning (*Illustrated by using Inkspace Software*)

Table 3.11: Inverse PCR

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
Phusion HF Buffer	5X	1X	10
dNTPs mix	10 mM	0.2 mM	1
Thermo High Fidelity Taq Polymerase Enzyme	2 U/µL	1 U	0.5
Forward Primer (Insert Oligomer)	10 µM	0.2 µM	1
Reverse Primer	10 µM	0.2 µM	1
Template DNA	8.87 x 10^{-4} ng/µL	26.61 ng	5
Nuclease free water			31.5
Total Volume			50

Table 3.12: Inverse PCR Conditions

Initial Denaturation	98°C	2 minutes
*Denaturation	98°C	10 seconds
*Annealing	60°C	20 seconds
*Extension	72°C	2.5 minutes
Final Extension	72°C	5 minutes
Final Temperature	4°C	∞

* 34 Cycle

Amplicons were purified from the 1% agarose gel by using E.Z.N.A Gel Elution Kit. DNA concentration was determined via nanodrop. Then purified PCR products were phosphorylated by using T4 Polynucleotide Kinase (NEB) Enzyme (Table 3.13, Table 3.14).

Table 3.13: The Composition of Phosphorylation Reaction

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
Genermarkbio 5X T4 Ligase Buffer	5 X	1 X	5
NEB Polynucleotide Kinase Enzyme	10 U/µL	10 U	1
DNA	133 ng for Silica-binding peptide		19
	285 ng for Biotin-binding peptide		19
Total Volume			25

Table 3.14: Phosphorylation Reaction Conditions

Incubation	37°C	30 minutes
Inactivation	65°C	20 minutes
Final Temperature	4°C	∞

Finally, the DNA was ligated by using 3U of T4 DNA Ligase Enzyme (Genemarkbio) at room temperature for 1 hour.

3.4. Transformation

Sambrook et. al. (2001) transformation protocol was modified in this study (15). Briefly, the competent *E. coli* ER2738 cell suspension was thawed on ice. Recombinant DNAs as 96 and 246 ng for Silica and Biotin respectively were mixed with competent cells. The negative control was an equal volume of sterile ddH_2O and the positive control was 888 ng of pM13KE as a wild type. The tubes were incubated at 42°C in water bath for 90 seconds. Then tubes were put on ice for 5 minutes. 200 µL of LB Medium pre-warmed at 37°C was added into tubes. In the meantime, soft agar LB Medium (0.7% agar) was melted at microwave oven and cooled down. Then 3 mL of the medium was mixed with the transformed bacterial cell culture and the mixture was poured onto LBA/ Tet (20 µg/mL)/ X-gal (0.02 mg/mL)/ IPTG (0.1 mM) agar plates pre-warmed at 37°C. After waited for solidification of the agar at room temperature for 5-10 minutes, the cultures were incubated at 37°C for overnight.

3.5. Colony Screening

The M13KE vector carrying lacZα gene used in this study was used for obtaining blue and white colonies. *E. coli* ER2738 which is lacZΔM15 cannot utilize the 5-Bromo-4-Chloro-3-Indolyl Beta-D-Galactopyranoside (X-gal) which is a colorless derivative form of lactose. After transferred the M13KE vector into the bacterial cells, they produce blue colonies in the presence of the Isopropyl β- d-1-thiogalactopyranoside (IPTG) and X-gal (53–55). Therefore, transformed cells can be screened as blue colonies. Blue colonies were analyzed with colony PCR.

3.5.1. Polymerase Chain Reaction

PCR was performed for colony screening. For this purpose, forward and reverse primers were designed as Table 2.15. Gradient PCR between 63-72°C was used for annealing temperature optimization. PCR products were analyzed by gel electrophoresis. Some of the blue colonies were picked up with a sterile loop. The sample was firstly plated on the fresh LBA/ Tet (20 µg/mL)/ X-gal (0.02 mg/mL)/ IPTG (0.1 mM) agar plates and then subjected to the PCR.

Table 3.15: Colony Screening Primers

Primer Name	Nucleotide Sequence	Size	%GC	Tm (ºC)
P3 Forward	CCTCGAAAGCAAGCTGATAAACC	23 bp	48	56
F3 Reverse	GTATGGGATTTTGCTAAACAAC	22 bp	36	50

Table 3.16: PCR Ingredients for Colony Screening

Components	Stock Concentration	Final Concentration	Volume (µL)
Mytaq Reaction Buffer	5X	1X	5
Bioline MyTaq Hot Start Polymerase Enzyme	5 U/µL	2.5 U	0.5
P3 Forward Primer	10 µM	0.4 µM	1
F3 Reverse Primer	10 µM	0.4 µM	1
Template DNA			Touched to colony
Nuclease free water			17.5
Total Volume			25

Table 3.17: PCR Conditions for Colony Screening

Initial Denaturation	95°C	3 minutes
*Denaturation	95°C	15 seconds
*Annealing	63-72°C	10 seconds
*Extension	72°C	5 seconds
Final Extension	72°C	5 minutes
Final Temperature	4°C	∞

* 34 Cycle

3.5.2. Gel electrophoresis

Colony PCR products were analyzed by PAGE (Table 3.18) and agarose gel (0.8-2%) electrophoresis.

Table 3.18: PAGE

%12 (w/v) Acrylamide Gel	**2 Gel / 1cm**
%30 (w/v) Acrylamide-Bisacrylamide Solution (29:1)	4.8 mL
5X TBE Buffer	2.4 mL
ddH_2O	4.8 mL
TEMED*	10 µL
%10 APS*	200 µL

* Added last and APS used freshly

3.6. Phage Amplification

E. coli ER2738 bacterial cells were inoculated in 5 mL of LB medium including 20 µg/mL tetracycline at 180 rpm, 37°C for overnight. Then culture was centrifuged at 4.500xg for 15 minutes. The supernatant was discarded, 5 mL of fresh LB medium including 20 µg/mL Tet was added onto cell pellet and gently mixed. The culture was incubated at 180 rpm, 37°C for 30 minutes. Then the growth culture was infected with 30 µL of phage solution in 200 mL of 2X YT medium including 20 µg/mL Tet and 5% (v/v) glycerol. Then the culture was incubated with vigorous shake at 250 rpm, 37°C for 4 hours.

3.6.1. Obtaining phages

3.6.1.1 PEG precipitation method

After cooled down on the ice, the phage culture was centrifuged at 10.000xg, 4°C for 10 minutes. The supernatant including phages was transferred into the clean tubes and mixed with 1:5 volume of cold 20% (w/v) PEG 8000 including 2.5 M NaCl solution. The tubes were incubated on ice for 1 hour and centrifuged at 10.000xg, 4°C for 20 minutes, then supernatant was discarded. Pellet was briefly centrifuged for removing the residual cells at 4.000xg, 4°C for 4 minutes and supernatant debris was discarded. Each phage pellet was resuspended in 1 mL of 1X TBS Buffer and aliquoted to sterile microcentrifuge tubes as 1 mL. Tubes were briefly centrifuged by top bench centrifuge at 14.000xg for 1 minute. The supernatant was transferred into clean microcentrifuge tubes, then 200 µL of 20% (w/v) PEG 8000 including 2.5 M NaCI solution was added. Tubes were inverted many times and incubated on ice for 1 hour. The tubes were centrifuged at 4°C, 14.000 rpm for 10 minutes and the supernatant was discarded. Pellet was resuspended in 200 µL of TBS Buffer. For long term storage, the equal volume of 80% sterile glycerol was added and stored at -20°C until used.

3.6.1.2 MyMagiCon method

MyMagiCon (Gigabiomol, Turkey) beads were used in order to concentrate phages rather than following PEG and centrifugation steps. Briefly, concentration of phages in supernatant was increased by using MyMagiCon beads after the removal of bacteria from the solution. Then, supernatant was collected and stored by adding sterile glycerol at -20 °C.

3.6.1.3 Reverse-flow filter method

The phage culture was put on the ice and centrifuged at 4.500xg, 4°C for 15 minutes. The supernatant was transferred into clean tubes and 60 mL of supernatant was filtered by using a 0.22 µm sterile filter. Then the filter was inverted and 3 mL of 1X TBS Buffer passed through the filter in the reverse direction as shown at Figure 3.5. The phages were collected in clean tubes and equal volume of 80 % (v/v) glycerol was added for long-term storage at -20°C.

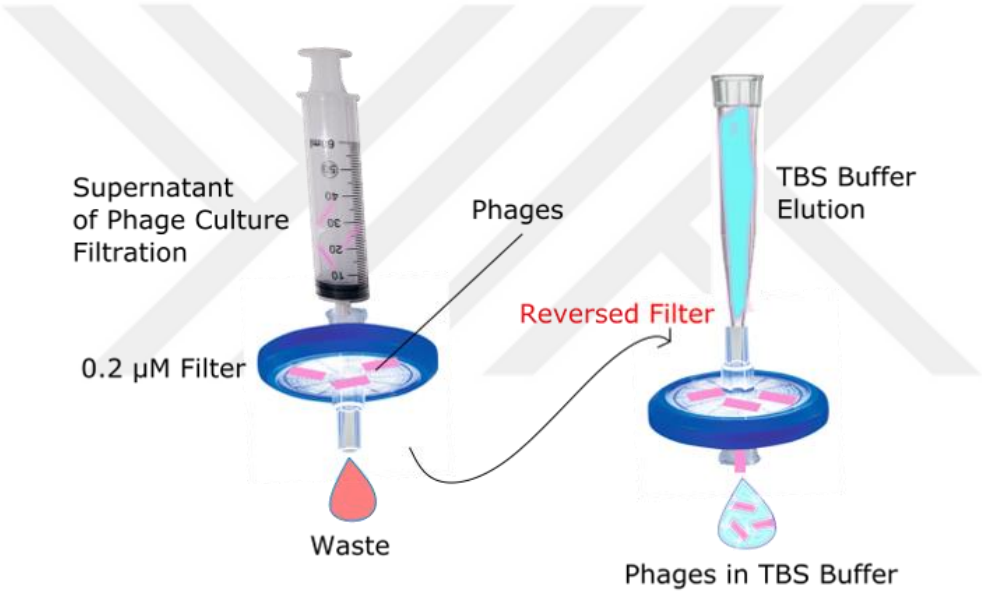

Figure 3.5: Reverse-Flow Filter Method

3.7. Analysis of Phages

3.7.1. Verification of phage infection capability

In order to verify phage infection to different methods were performed: Top agar method as a standard protocol and the Spreading method as the modified strategy (15,56). For top agar method, soft agar LB Medium (0.7% agar) was melted at microwave oven and cooled down. Then 3 mL of the medium was mixed with the 200 µL mid-log host bacterial culture and 10 µL phage stock solution. The mixture was poured onto LBA/ Tet (20 µg/mL)/ X-gal (0.02 mg/mL)/ IPTG (0.1 mM) agar plates pre-warmed at 37°C. After waited for solidification of the agar at room temperature for 5-10 minutes, the cultures were incubated at 37°C for overnight.

In order to improve phage tittering protocol used commonly as top agar method. A modified spreading protocol inspired from Luria Delbrück experiment was developed (56). For this purpose, phage solutions were diluted with 1:10 volume of ddH_2O then spread onto LBA/ Tet (20 µg/mL)/ X-gal (0.02 mg/mL)/ IPTG (0.1 mM) agar plates via sterile cotton ecuvion stick. Then mid-log host bacterial culture was diluted with 1:100 volume of LB medium and spread onto the same plate. One of the negative controls was only host culture (no phage) and another one was the only phage (no host bacteria). The plates were incubated at 37°C for overnight.

In order to increase the sensitivity of the modified spreading protocol, X-gal and IPTG concentrations were used in 10 times higher concentration than the standard protocol such as LBA/ Tet (20 µg/mL)/ X-gal (0.2 mg/mL)/ IPTG (1 mM) agar plates. Phage solutions were diluted with 1:10 (10^{-2}, 10^{-3}, 10^{-4}) volume of ddH_2O and verified by using the spreading method as mentioned above.

3.7.2. Plasmid isolation from infected cells

The phage infection culture was put on the ice and centrifuged with 4.500xg at 4°C for 15 minutes. Plasmids were isolated by using E.Z.N.A.® Plasmid Midi Kit. The plasmid concentration was determined via nanodrop.

3.7.3. Single strand DNA isolation from M13 phages

The single-strand DNA was isolated by using standard E.Z.N.A.® M13 DNA Mini Kit protocol. Samples were analyzed by the Nanodrop Spectrophotometer and the gel electrophoresis.

3.7.4. Polymerase chain reaction

3.7.4.1 P8-P3 polymerase chain reaction

PCR product including fused DNA was obtained with PCR of P8-P3 primers (Table 3.19). The DNA fragments were analyzed by gel electrophoresis.

Table 3.19: PCR Primers

Primer Name	Nucleotide Sequence	Size (bp)	%GC	Tm (ºC)
P8 Forward	GGTTGGTGCCTTCGTAGTGG	20	60	58
P3 Reverse	GCCCAATAGGAACCCATGTACC	22	55	57

Table 3.20: PCR Ingredients

PCR Ingredients	Stock Concentration	Final Concentration	Volume (µL)
5X Genemarkbio Pcr Master Mix	5X	1X	5
P8 Forward Primer	10 µM	0.4 µM	1
P3 Reverse Primer	10 µM	0.4 µM	1
Template ssDNA	0.9 ng/µL	4.4 ng	5
Nuclease free water			13
Total Volume			25

Table 3.21: PCR Conditions

Initial Denaturation	95°C	3 minutes
*Denaturation	95°C	30 seconds
*Annealing	65°C	30 seconds
*Extension	72°C	40 seconds
Final Extension	72°C	5 minutes
Final Temperature	4°C	∞

* 30 Cycle

3.7.4.2 *PCR specific to DNA sequences coding silica/biotin-binding peptides*

PCR was performed for cloning analyze. For this purpose, specifically forward and reverse primers were designed for Biotin-binding and Silica-binding peptide sequences (Table 3.22). Gradient PCR between 55-65°C was used for annealing temperature optimization. Wild type DNA was used as a negative control. PCR products were analyzed by gel electrophoresis. PCR products were sent to Eurofins Genomics Company for Sanger DNA sequencing.

Table 3.22: PCR Primers

Primer Name	Nucleotide Sequence	Size (bp)	%GC	Tm (ºC)
Forward (for silica)	TGTCTCCTCATCCCCATCCC	20	60	60
Reverse (for silica)	ACAACGCCTGTAGCATTCCA	20	50	60
Forward (for biotin)	CTGGACCCCGCCGTTTA	17	64	59
Reverse (for biotin)	AGCAAGCCCAATAGGAACCC	20	55	60

Table 3.23: PCR Ingredients

PCR Ingredients	Stock Concentration	Final Concentration	Volume (µL)
5X Genemarkbio PCR Master Mix	5X	1X	5
Forward Primer	10 µM	0.4 µM	1
Reverse Primer	10 µM	0.4 µM	1
Template plasmid DNA/ssDNA		1.7 ng	5
Nuclease free water			13
Total Volume			25

Table 3.24: PCR Conditions

Initial Denaturation	95°C	3 minutes
*Denaturation	95°C	30 seconds
*Annealing	55°C	30 seconds
*Extension	72°C	40 seconds
Final Extension	72°C	5 minutes
Final Temperature	4°C	∞

* 30 Cycle

3.7.5. Determination of phage concentrations

The titer protocol which given in literature (23,57) was modified in this study to analyze the number of virus particles capable of forming plaques per unit volume Briefly in this study phage spreading technique was tried for an alternative to the top agar technique. For this purpose, 10^{-2} dilution of phage stocks in sterile ddH_2O were mixed by vortex and spread onto LBA/ Tet (20 µg/mL)/ X-gal (0.2 mg/mL)/ IPTG (1 mM) agar plates with sterile cotton ecuvion stick. Then OD_{600} 0.5 *E. coli* ER2738 culture was 1.10^{-4} diluted with LB and plated on the same petri dish. Plates were incubated at 37°C for overnight. Blue and white bacterial colonies were counted.

To optimize phage concentrations for the spreading method, mid-log bacterial culture was infected with 10^{-5} and 10^{-4} dilutions of recombinant phages spread on LB agar plates including Tet (20 µg/mL)/ X-gal (0.2 mg/mL)/ IPTG (1 mM). Additionally, phage concentration was determined by absorption at 269 nm and 320 nm (22). Values were calculated via Abdesignlab Online Phage Concentration Calculator (58).

3.8. Re-Infection Analysis

For the re-infection experiments, three different culture media were prepared: First LB medium contained 20 µg/mL Tet, whereas the second LB medium contained 20 µg/mL Tet, 0.2 mg/mL X-gal, 1 mM IPTG. Both of them were inoculated with bacteria from blue colonies. The third LB medium contained 20 µg/mL Tet and it was inoculated *E. coli* ER2738 which had not been infected by a phage before. All cultures were incubated at 180 rpm, 37°C for overnight. Then, 1.10^{-5} dilutions in fresh LB media of each culture were infected by 10^{-1} dilutions of phages and cultured by using top agar method. Blue and white colonies were counted.

4. RESULTS

4.1. M13KE Plasmid Amplification

After amplified M13KE plasmid in bacterial cells, they were purified. The concentration of M13KE plasmid was found as 887 ng/µL. 800 ng of plasmid DNA (pM13KE) was run on 0.8% agarose gel and bands were visualized as given in Figure 4.1.

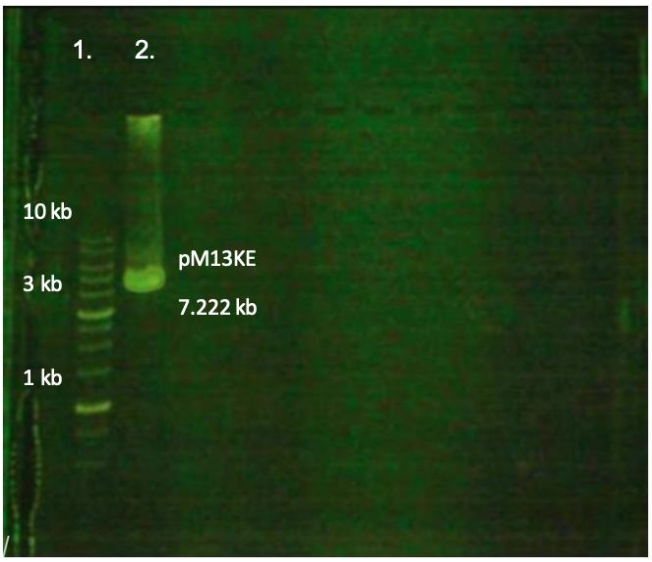

Figure 4.1: M13KE Plasmid DNA. Lane 1, 1 kb DNA Ladder; Lane 2, M13KE plasmid DNA. 0.8% agarose gel with SYBR.

4.2. Engineering of bacteriophage M13 binding to inorganic surfaces

Translation reading frames of cloned DNA sequences were controlled with online EXPASY Translation Tool as given in Table 4.1 and in Figure 4.2.

Table 4.1: Translation of Cloned Sequences in Frame

Sequence Name	Aminoacid Sequence
Silica-binding peptide	L * V V P F Y S H S **M S P H P H P R H H H T**
Biotin-binding peptide	L * V V P F Y S H S **C N W T P P F K T R C**

* Stop codon. Wild type sequences are in normal font, foreign sequences for insertion are in bold (EXPASY Translation Tool).

A

B

Figure 4.2: Frame Controls- Frame of the Silica-binding (A) and Biotin-binding (B) peptide sequences fused DNA. DNA sequence in upper case letters, black letters were wild type sequence, green and blue letters were sequences of fused peptides (*Snapgene Viewer*, *version 4.2.11*).

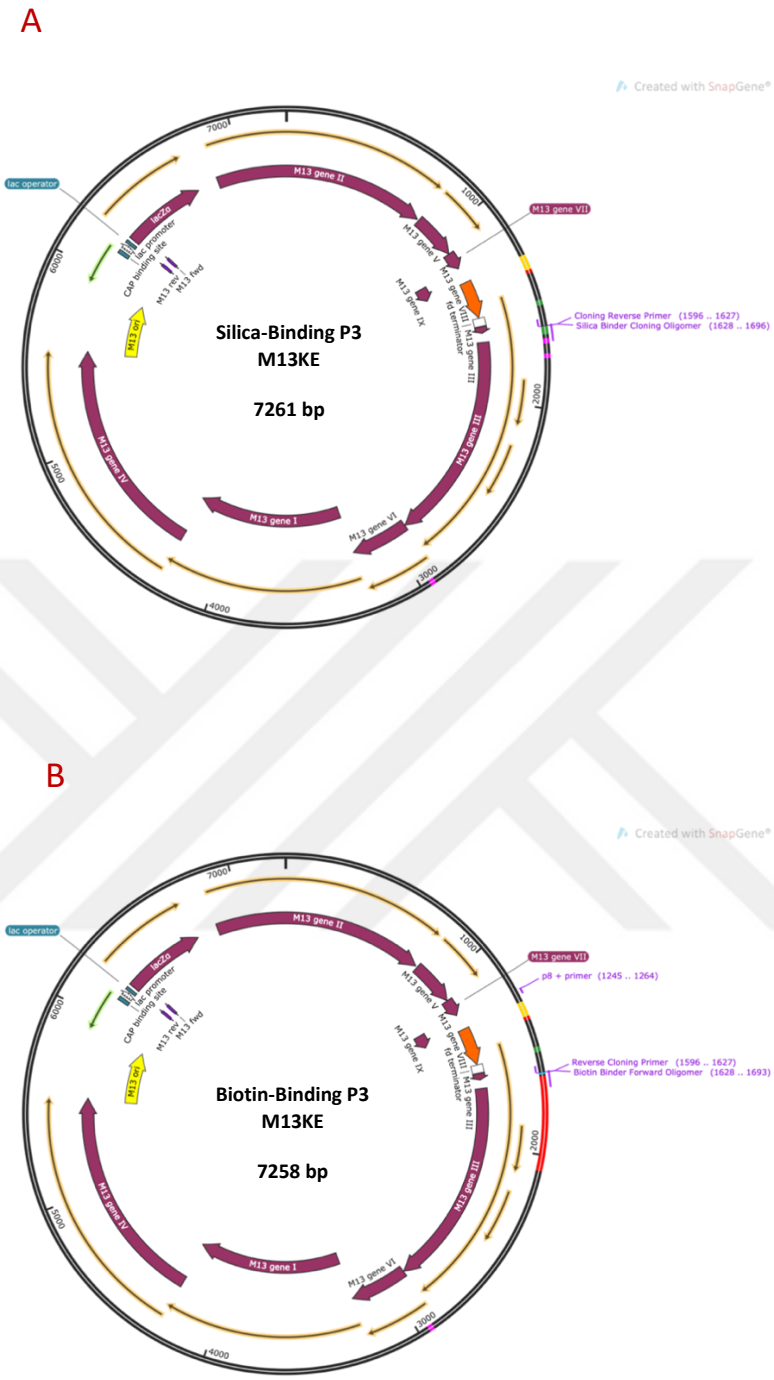

Figure 4.3: Genome Cassettes of Recombinant DNAs. Silica-binding (A) and Biotin-binding (B) P3 recombinant M13KE phage genomes.

4.2.1. Restriction enzyme-based cloning method

Products of *Kpn I* restriction enzyme digestion were analyzed by 0.8% agarose gel as given in Figure 4.4.

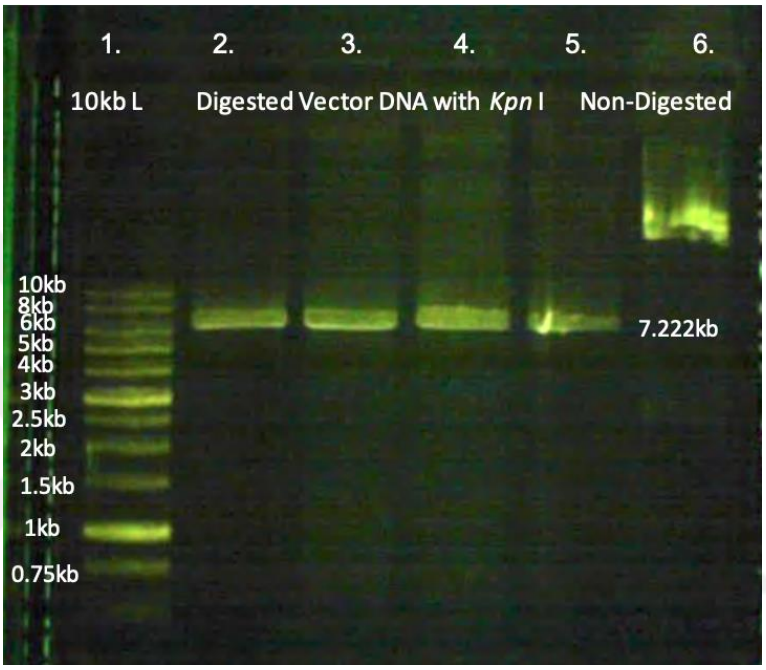

Figure 4.4: Digestion of vector DNA with Kpn I. Lane 1, 1kb DNA Ladder; Lane 2-5 digested pM13KE; Lane 6, non-digested pM13KE in 0.8% agarose gel with SYBR.

Then, products were purified from the gel and cut by *Eag I* restriction enzyme. Results were analyzed by agarose gel electrophoresis as given in Figure 4.5.

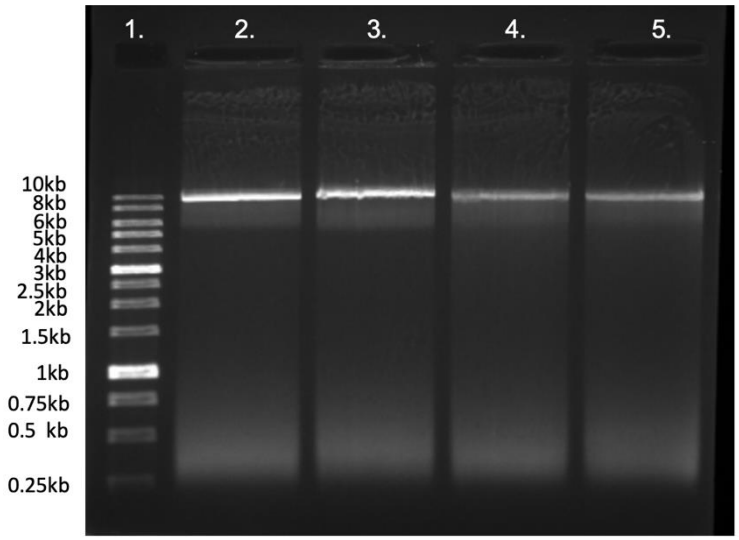

Figure 4.5: Double Digestion of the Vector DNA. Lane 1, 1kb DNA Ladder; Lane 2-5, double digested pM13KE in 0.8% agarose gel with SYBR.

Transformed bacterial cells were chosen as blue colonies on agar plates by using top agar method as given in Figure 4.6. The vector/insert molar ratio were successful in ratio of 1:5 and 1:3 for biotin-binding and silica-binding sequences were fused respectively, but there were very few colonies. There were not any blue colonies in the negative control.

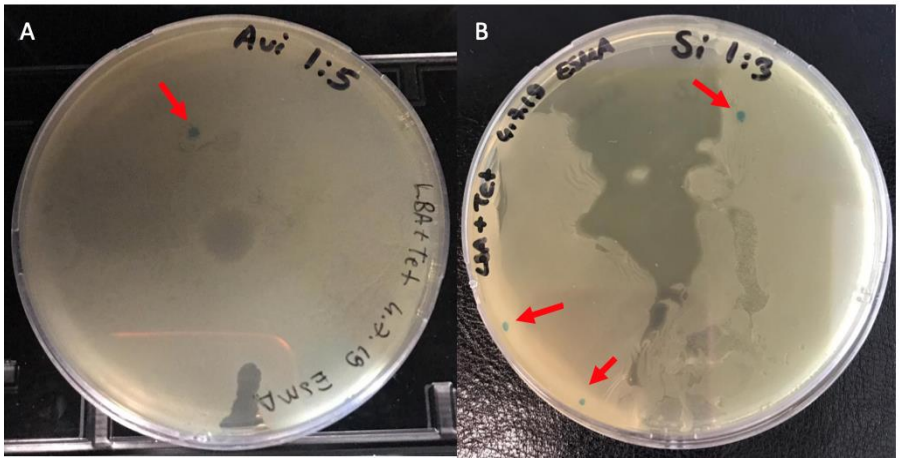

Figure 4.6: Colony Screening by Top Agar Method. Red arrows show blue colonies for (A) biotin-binding and (B) silica-binding peptide sequence encoding.

Amplicons obtained colony PCR with P3/ F3 primers were analyzed by 2% agarose gel as given in Figure 4.7.

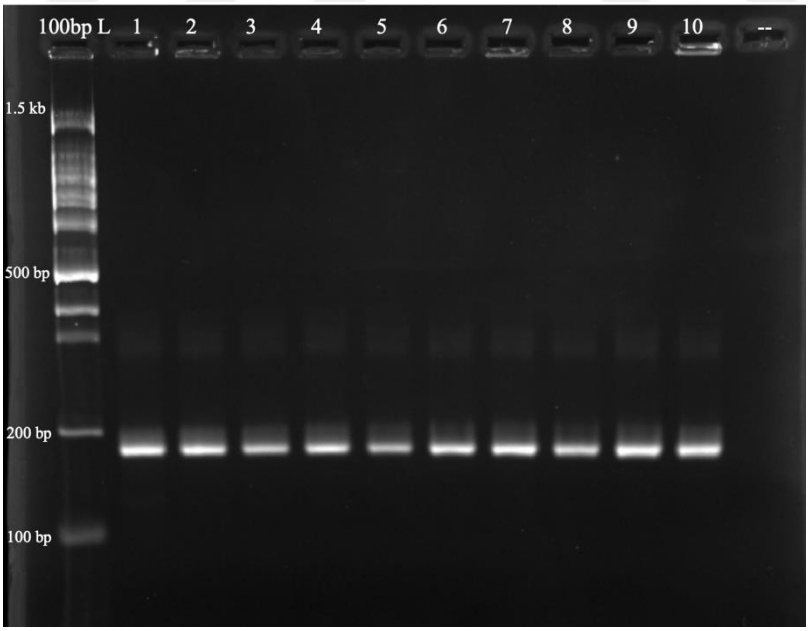

Figure 4.7: Colony PCR. Lane 1, 100 bp DNA Ladder. PCR products of wild type M13KE DNA (Lane 2), coding silica-binding peptide sequence (Lane 3-5), coding biotin-binding peptide sequence (Lane 6-8), coding silica-binding peptide sequence from another petri (Lane 9-10), no-DNA as the negative control (Lane 11). 2% agaraose gel.

4.2.2. Inverse PCR-based cloning method

Amplicons obtained from the inverse PCR, were analyzed by 1% agarose gel as given in Figure 4.8. After purification from the gel, the concentrations of DNA molecules were 7 ng/µL and 15 ng/ µL for silica-binding and biotin-binding peptide sequences encoded respectively.

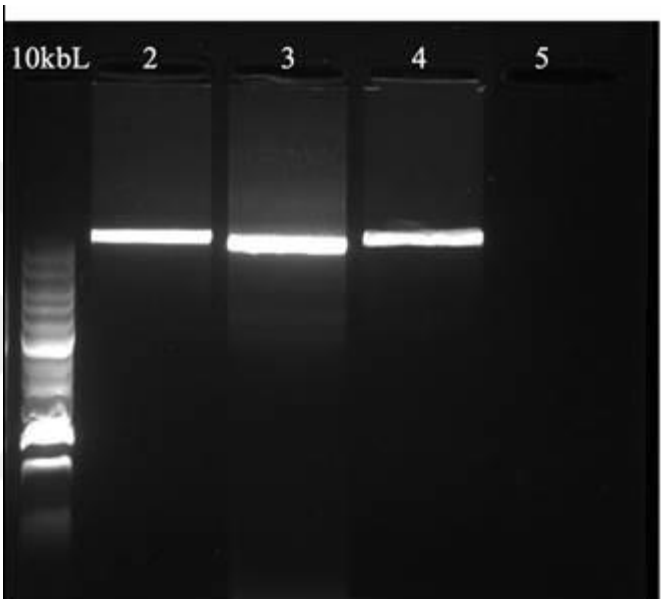

Figure 4.8: Inverse PCR. Lane 1, 10 kb DNA Ladder; Amplicons of DNA coding silica-binding peptide sequence (Lane 2), biotin-binding peptide sequence (Lane 3-4), negative control (Lane 5). All samples were stained with SYBR Gold and visualized by using Chemidoc Image System (Bio-Rad). 1% agarose gel.

Transformed bacterial cells were chosen as blue colonies on agar plates as given in in Figure 4.9. No blue colonies were obtained on the negative controls.

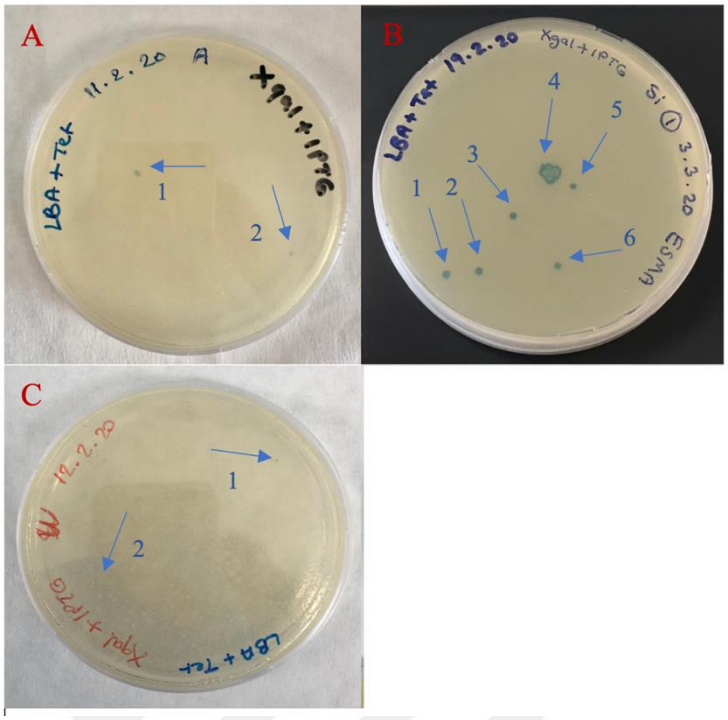

Figure 4.9: Colony Screening by Top Agar Method. Red arrows show blue colonies for (A) biotin-binding, (B) silica-binding peptide sequences encoded, (C) wild type as the positive control.

After the colony PCR, amplicons were analyzed by using PAGE as given in Figure 4.10.

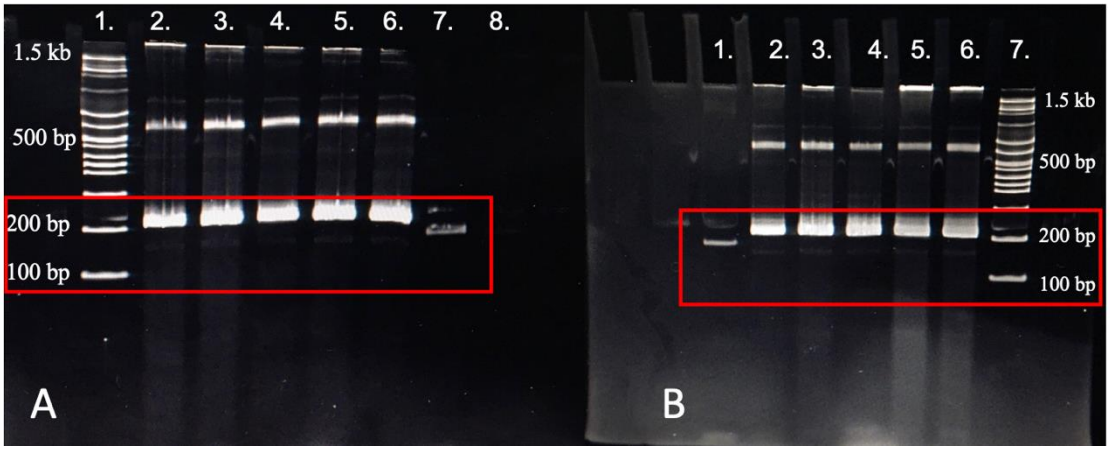

Figure 4.10: Colony PCR. Amplicons for DNA coding biotin-binding peptide sequence (A); Lane 1, 100 bp DNA Ladder; Lane 2-6, biotin-binding peptide sequence; Lane 7, wild type. Amplicons for DNA coding silica-binding peptide sequence (B); Lane 1, wild type; Lane 2-6, silica-binding peptide sequence; Lane 7, 100 bp DNA Ladder. Red box shows the range of the expected amplicon lengths. All samples were stained with EtBr and visualized by using Chemidoc Image System (Bio-Rad). 12% DNA PAGE.

To decrease non-specific bands, annealing temperature was optimized by the gradient PCR. At 63°C, there was not any non-specific bands in the agarose gel, whereas there was in the PAGE which is better resolution than agarose as given in Figure 4.11.

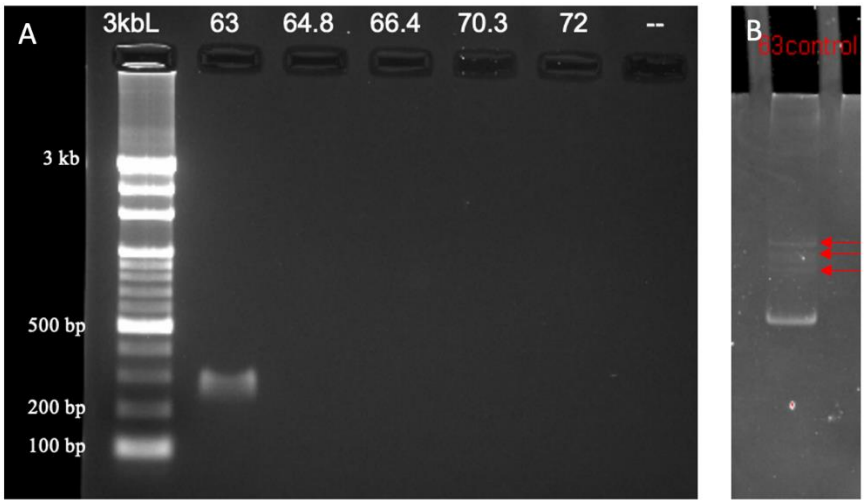

Figure 4.11: P3-F3 Primers Gradient PCR. (A) Lane 1, 3 kb DNA Ladder. PCR products of different annealing temperature (Lane 2-6), no-DNA as the negative control (Lane 7). 2% agarose gel. (B) Lane 1, PCR product of 63°C annealing temperature.12% DNA PAGE. All samples were stained with EtBr and visualized by using Chemidoc Image System (Bio-Rad).

Colony PCR performed at 63°C as annealing temperature were also analyzed by PAGE and there were non-specific bands as given in Figure 4.12.

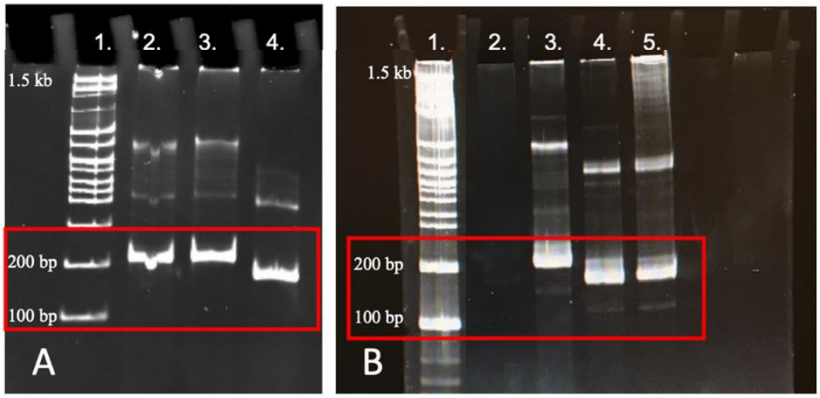

Figure 4.12: Colony PCR. PCR products of 63°C annealing temperature. (A) Lane 1, 100 bp DNA Ladder; Lane 2, Amplicon coding biotin-binding peptide sequence; Lane 3, silica-binding peptide sequence; Lane 4, wild type. (B) Lane 1, 100 bp DNA Ladder; Lane 2, no-DNA as the negative control; Lane 3, biotin-binding peptide sequence; Lane 4-5, wild type. Red box shows the range of the expected amplicon lengths. 12% DNA PAGE.

To overcome this problem about non-specific amplification, primer optimization assay was done. As given in Figure 4.13, a homodimer formation was seen when used only forward primers without DNA templates in the reaction mixture.

Figure 4.13: PCR Optimization. Lane 1, 100 bp DNA ladder; 1-4 tagged lanes, PCR samples of DNA. PCR samples of just P3 Forward Primer and DNA (1), just F3 Reverse Primer and DNA (2), both primers and DNA (3), just DNA without any primer (4). 5-8 tagged lanes, PCR samples of without DNA. Red box shows the primer dimers. 12% DNA PAGE.

4.3. Phage Amplification

After cultured phages in the liquid media including X-gal and IPTG, culture media were changed into the blue-green colors from yellow as given in Figure 4.14. After precipitation and washing steps, phages were obtained and stored until used.

Figure 4.14: Phage Amplification. (A) Overnight culture, (B) The culture after centrifugation, (C) Isolated phages after PEG precipitation, (D) Phage stocks in glycerol.

4.4. Analysis of Phages

4.4.1. Verification of Phages

To verify infection capabilities, the phages were spread on LBA agar media including Tet (20 µg/mL)/ X-gal (0.02 mg/mL)/ IPTG (0.1 mM), and then bacteria were spread on them. After incubation, blue colonies were observed as given in Figure 4.15. The blue color of colonies was quite pale despite colony density. There were not any blue colonies when plated only host bacterial cells without phages. Only phages without any bacterial cells as the second negative control was not given any colonies.

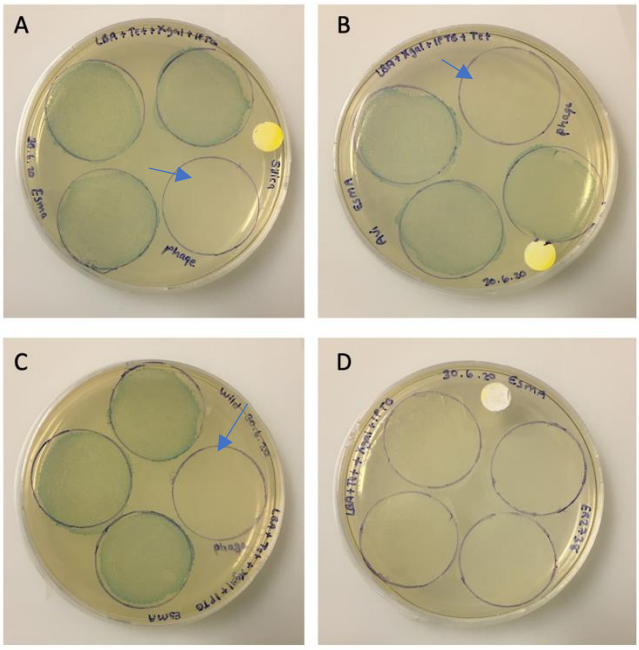

Figure 4.15: Phage Infection. (A) Silica-binding, (B) biotin-binding, (C) wild type phages; (D) E. coli ER2738.

To increase detection sensitivity of the blue colonies, the X-gal and IPTG were used in a higher concentration as X-gal (0.2 mg/mL)/ IPTG (1 mM). A serial dilution of the bacterial cells growth overnight was done as 10^{-2}, 10^{-3}, 10^{-4}. After subjected to the spreading method, blue colonies were evaluated. The color of colonies was quite dark blue as given in Figure 4.16 and the number of colonies were proportional according to the dilutions.

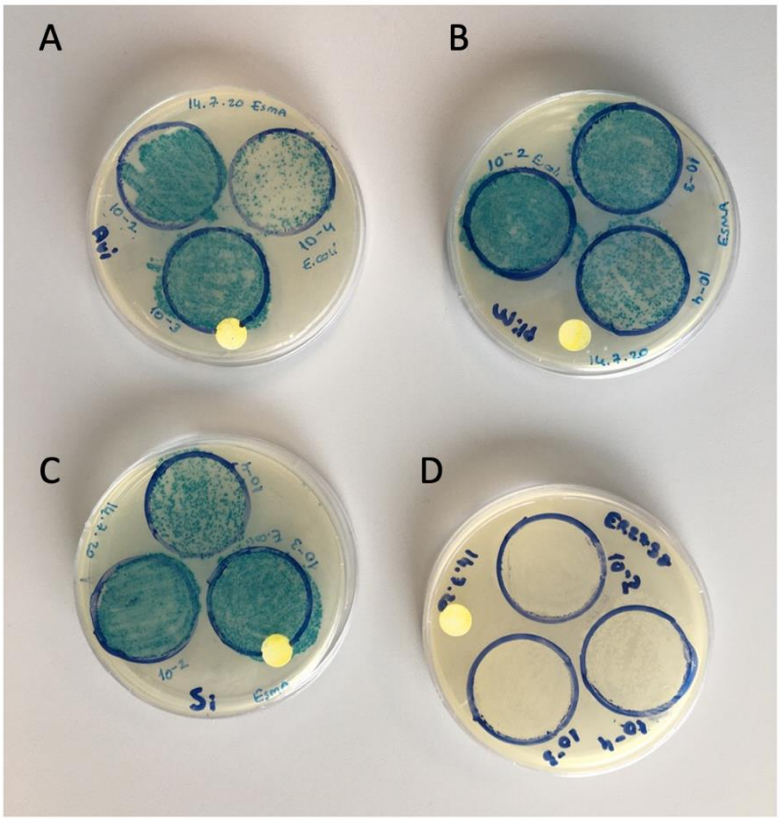

Figure 4.16: Infection of Host Cell Dilutions. (A) Biotin-binding, (B) wild type, (C) silica-binding phages; (D) E. coli ER2738.

4.4.2. Single strand DNA isolation from M13 phages

The single-strand DNAs from phages were isolated by using E.Z.N.A.® M13 the phage supernatant was equally divided into the two tubes. One tube was used directly according to the kit protocol. The second one was first subjected to concentration process by using MyMagiCon (Gigabiomol, Türkiye). Then, the kit protocol was followed for this concentrated liquid. The concentrations of ssDNA obtained from two methods were given in Table 4.2.

Table 4.2: Concentrations of ssDNA Obtained with PEG and MyMagiCon Methods

Concentrated ssDNA with:	Concentration (ng/µL)
MyMagiCon (for biotin)	71
MyMagiCon (for silica)	30
MyMagiCon (for wild type)	20
PEG (for biotin)	22
PEG (for silica)	29
PEG (for wild type)	18

The 150 ng of ssDNAs were analyzed by 0.8% agarose gel as given in Figure 4.17. The intact ssDNA was obtained from the concentrated samples, whereas there was a smear when used samples directly.

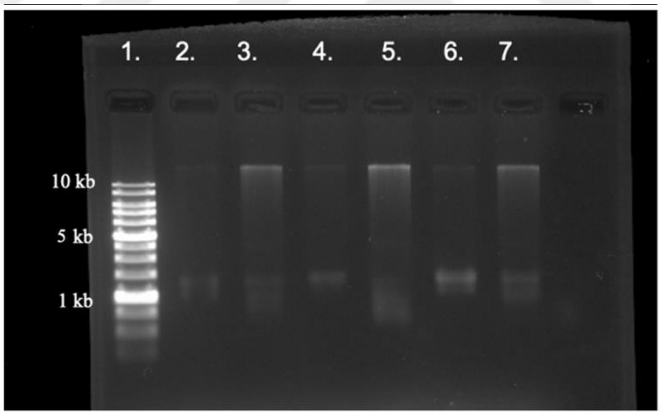

Figure 4.17: ssDNA Samples. Lane 1, 10 kb DNA Ladder. Concentrated ssDNA samples with MyMagiCon (for biotin) (Lane 2), PEG (for biotin) (Lane 3), MyMagiCon (for silica) (Lane 4), PEG (for silica) (Lane 5), MyMagiCon (for wild type) (Lane 6), PEG (for wild type) (Lane 7). All samples were stained with EtBr and visualized by using Chemidoc Image System (Bio-Rad). 0.8% agarose gel.

To increase phage concentration yield, a new method called as the reverse-flow was developed as illustrated in Figure 3.5. After concentrated phages by using the reverse-flow method, the amount of ssDNAs were spectrophotometrically determined

as given in Table 4.3. When compared to results obtained with standard in Table 4.2 used, the recovered phage yield was approximately 20 times more in this new method.

Table 4.3: Concentrations of ssDNA Obtained with the Reverse-Flow Filter Method

ssDNA from:	**Concentration (ng/µL)**
Biotin-binding phages (first elution)	880
Biotin-binding phages (second elution)	829
Silica-binding phages (first elution)	800
Silica-binding phages (second elution)	919
Wild type phages	717

The 300 ng of the ssDNA samples were analyzed by 0.7% agarose gel as given in Figure 4.18.

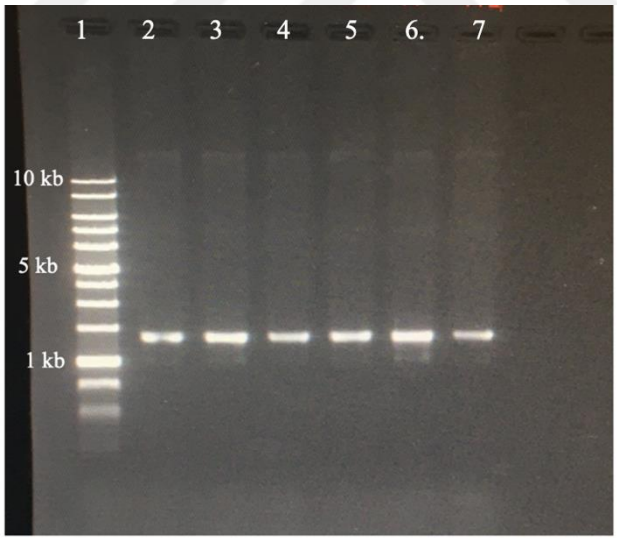

Figure 4.18: ssDNA Samples. Lane 1, 10 kb DNA Ladder. ssDNA samples with filter method for coding biotin-binding peptide sequence (Lane 2-3), silica-binding peptide sequence (Lane 4-5), wild type M13KE DNA (Lane 6-7). 0.8% agarose gel with SYBR.

Furthermore, plasmids from the bacteria infected phages were isolated and the plasmid concentrations were spectrophotometrically determined as given in Table 4.4.

Table 4.4: Concentrations of Plasmids

Plasmid DNA obtained bacteria infected by:	**Concentration (ng/µL)**
Biotin-binding phages (first elution)	349
Biotin-binding phages (second elution)	335
Silica-binding phages (first elution)	403
Silica-binding phages (second elution)	327
Wild type phages	362

The 750 ng of plasmids were analyzed by 1.2% agarose gel as given in Figure 4.19.

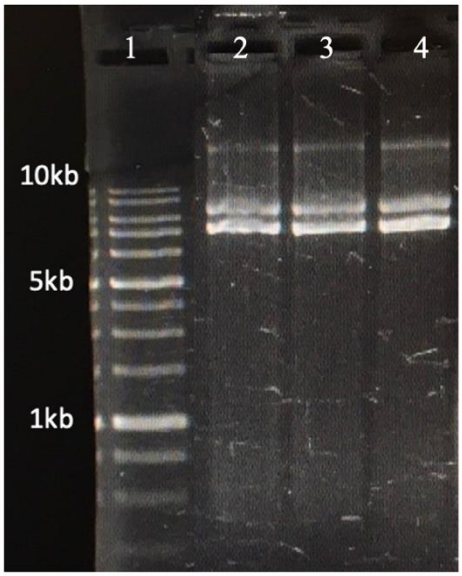

Figure 4.19: Plasmid DNAs. Lane 1, 10 kb DNA Ladder. Plasmid DNAs coding biotin-binding peptide sequence fused (Lane 2), silica-binding peptide sequence fused (Lane 3), wild type (Lane 4). All samples were stained with EtBr and visualized by using Chemidoc Image System (Bio-Rad). 1.2% agarose gel.

4.4.3. Polymerase Chain Reaction

4.4.3.1 P8-P3 Polymerase Chain Reaction

After gradient PCR with P8/P3 primers, amplicons were analyzed by 2% agarose gel as given in Figure 4.20 and Figure 4.21. DNA bands was in 571 bp length as expected and optimum temperature for annealing was found as 65°C.

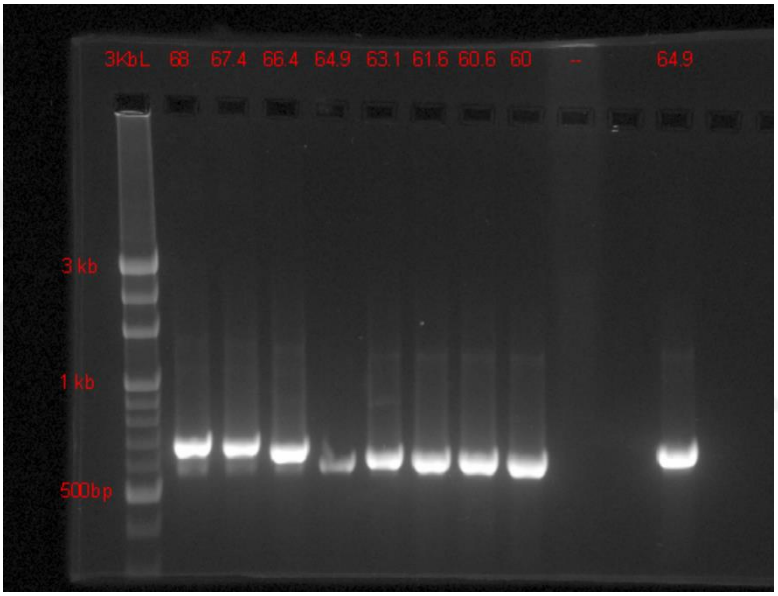

Figure 4.20: P8-P3 Primers Gradient PCR. Lane 1, 3 kb DNA Ladder. PCR products of different annealing temperatures (Lane 2-9 and 12), no-DNA as the negative control (Lane 10). 2% agarose gel with SYBR.

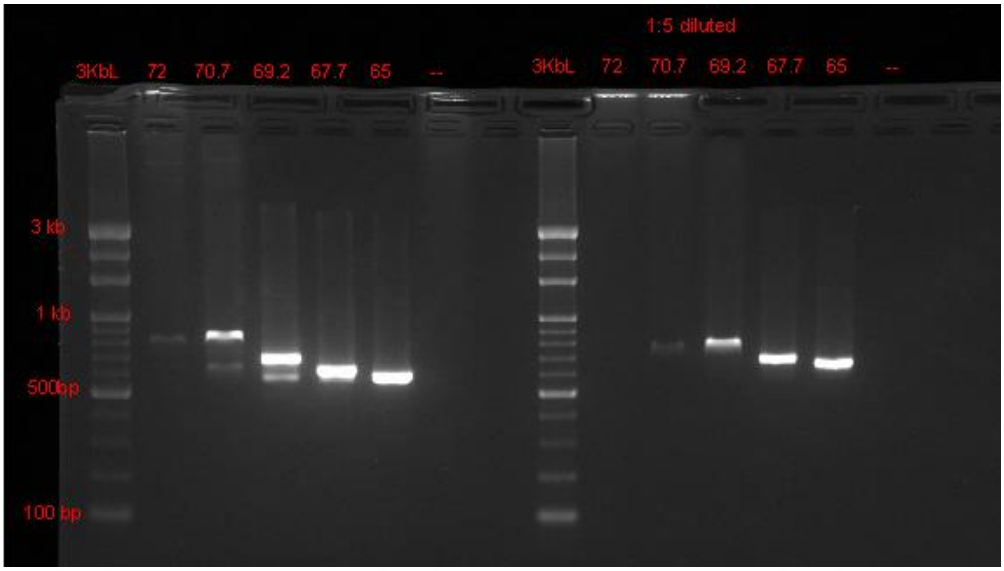

Figure 4.21: P8-P3 Primers Gradient PCR. Lane 1 and Lane 9, 3 kb DNA Ladder. PCR products of different annealing temperatures (Lane 2-6), no-DNA as the negative control (Lane 7), 1:5 dilution volume of PCR samples (Lane 10-14). 2% agarose gel with SYBR.

After PCR, amplicons of recombinant ssDNAs and plasmids were analyzed by 2% agarose gel as given in Figure 4.22. DNA bands were in 571, 607, 610 bp length for wild type, biotin-binding peptide and silica-binding peptide sequences fused DNAs respectively as expected.

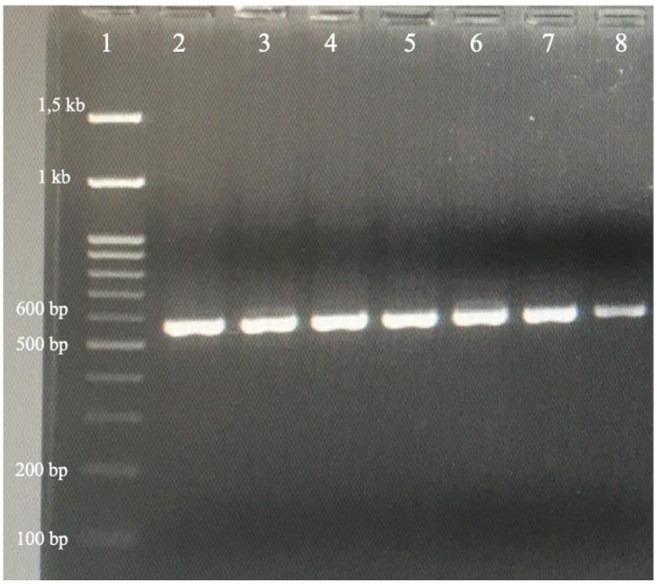

Figure 4.22: P8-P3 PCR. Amplicons from plasmid: Lane 1, 100 bp DNA Ladder; Lane 2, biotin-binding peptide sequence coding; Lane 3, silica-binding peptide sequence coding; Lane 4, wild type. Amplicons from ssDNAs: Lane 5, biotin-binding peptide sequence coding; Lane 6, silica-binding peptide sequence coding; Lane 7-8, wild type. 2% agarose gel.

4.4.3.2 *PCR specific to DNA sequences coding silica/biotin-binding peptides*

PCR with primers specific to biotin-binding peptide and silica-binding peptide sequences were optimized and amplicons were analyzed by 2% agarose gel as showed in Figure 4.23. Optimal annealing temperature was determined as 55°C for both of silica and biotin. The DNA sequences were confirmed by Sanger Sequencing Method. Sequences data including biotin-binding peptide and silica-binding peptide were given in appendix 1-2 and 3-4, respectively.

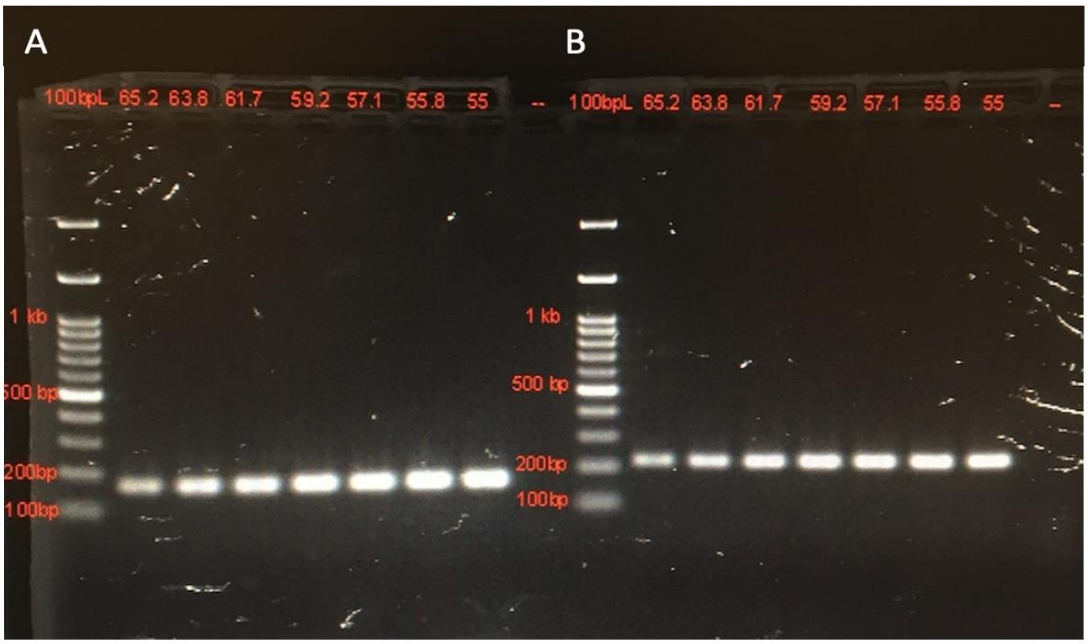

Figure 4.23: Gradient PCR. Amplicons coding silica-binding peptide sequence (A); Lane 1, 100 bp DNA Ladder; Lane 2-8, PCR product of different aneealing temperature, Lane 9, no-DNA as the negative control; Amplicons coding biotin-binding peptide sequence (B); Lane 1, 100 bp DNA Ladder; Lane 2-8, PCR product of different aneealing temperature, Lane 9, no-DNA as the negative control. 2% agarose gel.

After PCR specific to silica-binding peptide sequence with plasmid DNAs and single-strand DNAs by using 55°C as the annealing temperature, amplicons were analyzed by 2% agarose gel as shown in Figure 4.24. DNA bands were in 169 bp length as expected and there were not any bands when used wild type DNA as the negative control.

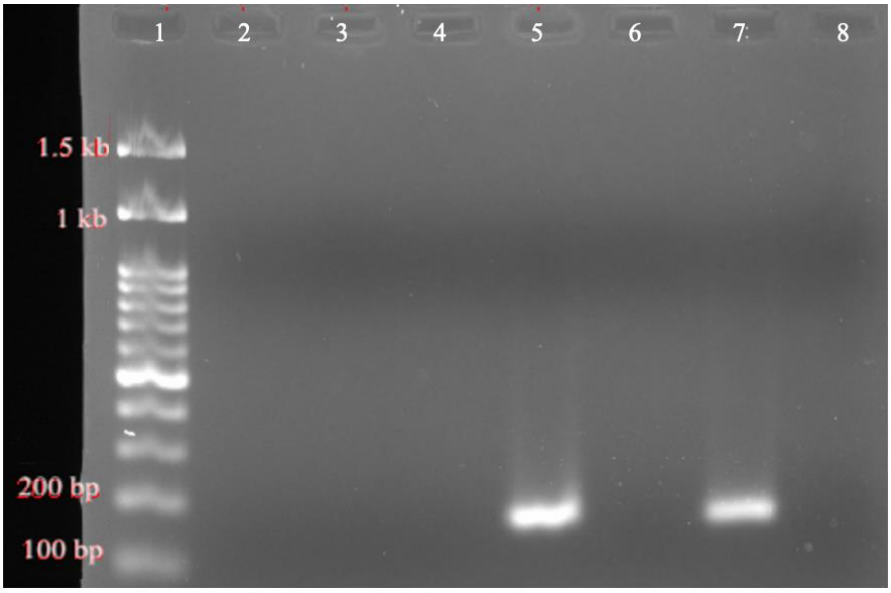

Figure 4.24: PCR. Lane 1, 100 bp DNA Ladder; PCR samples of biotin-binding peptide sequence fused plasmid (Lane 2), wild type plasmid (Lane 3), biotin-binding peptide sequence fused ssDNA (Lane 4), silica-binding peptide sequence fused plasmid (Lane 5), wild type ssDNA (Lane 6), silica-binding peptide sequence fused ssDNA (Lane 7), no-DNA as the negative control (Lane 8). 2% agarose gel.

After PCR specific to biotin with plasmid DNAs and single-strand DNAs by using 55°C as the annealing temperature, amplicons were analyzed by 2% agarose gel as shown in Figure 4.25. DNA bands were in 220 bp length as expected and there were not any bands when used wild type DNA as the negative control.

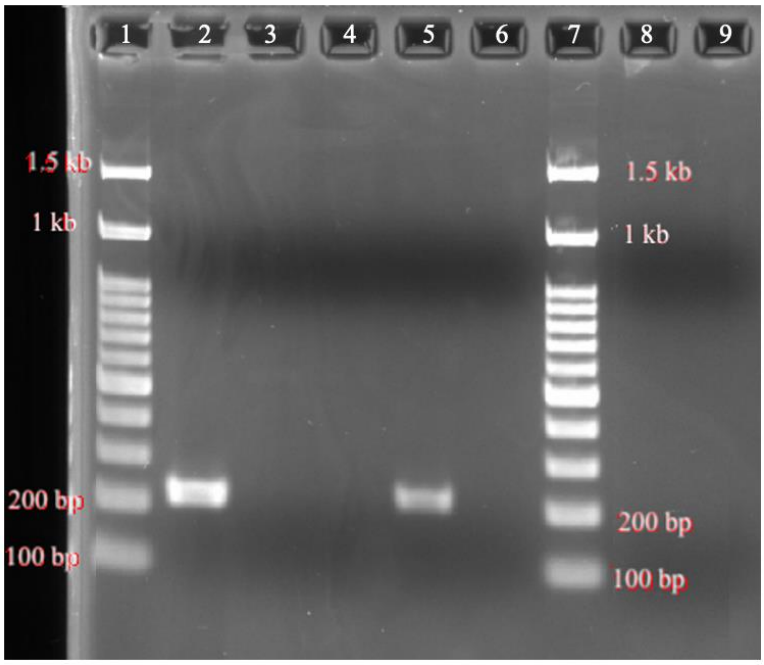

Figure 4.25: PCR. Lane 1, 100 bp DNA Ladder; PCR samples of biotin-binding peptide sequence fused plasmid (Lane 2), wild type plasmid (Lane 3), silica-binding peptide sequence fused plasmid (Lane 4), biotin-binding peptide sequence fused ssDNA (Lane 5), wild type ssDNA (Lane 6), 100 bp DNA Ladder (Lane 7), silica-binding peptide sequence fused ssDNA (Lane 8), no-DNA as the negative control (Lane 9). 2% agarose gel.

4.4.4. Determination of phage numbers

The number of phages were determined by counting blue and white colonies to compare PEG precipitation method and the reverse-filter method, as the new phage concentrating method as given in Figure 4.26 and Table 4.5. The number of blue colonies was twice as much than ones obtained from PEG precipitation.

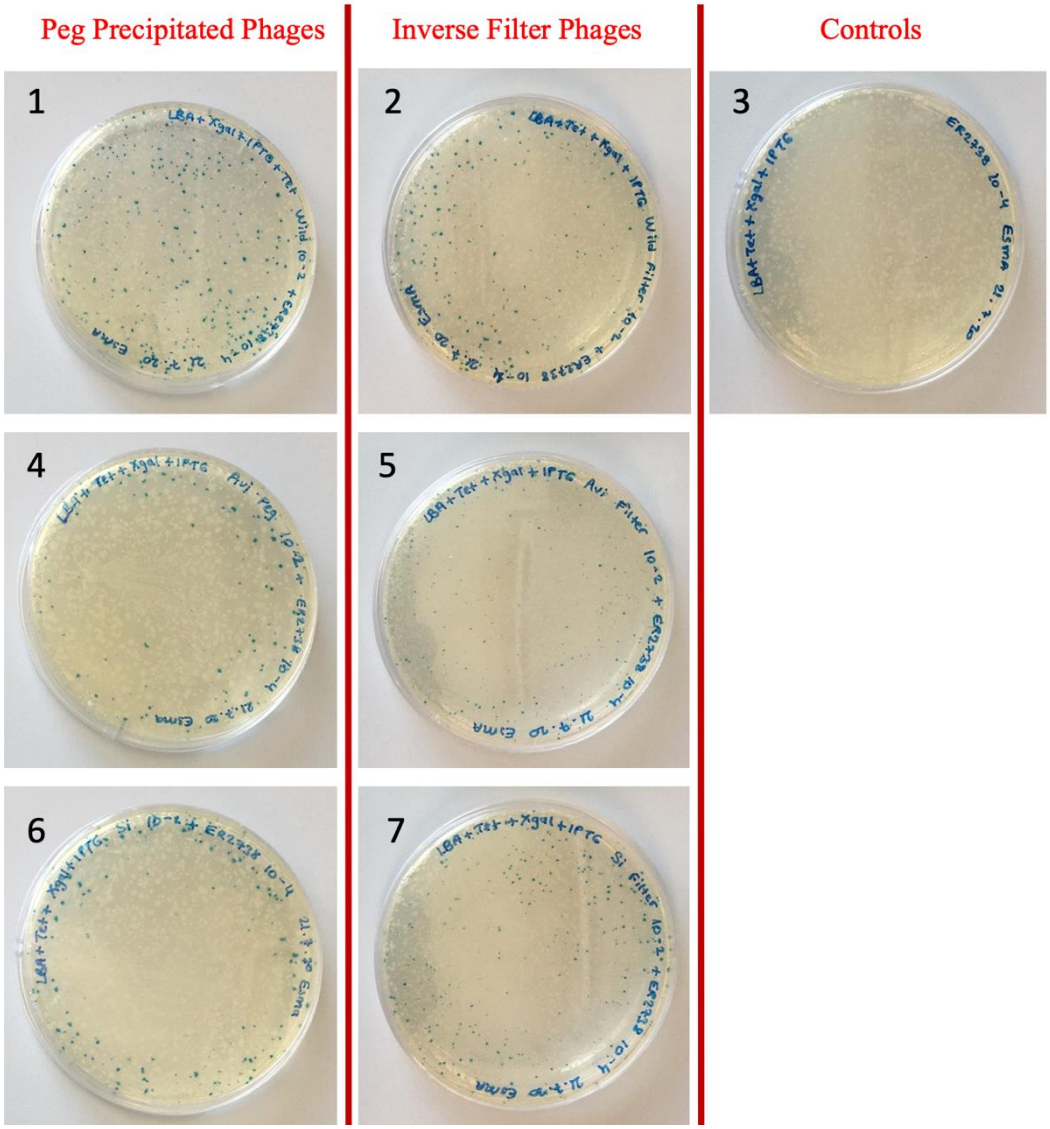

Figure 4.26: Comparison of PEG and the Reverse-Flow Filter Methods. 10^{-4} dilution of E. coli ER2738 host bacterial culture and 10^{-2} dilution of wild type phages (1), Biotin-binding phages (4) and Silica-binding phages (6) with PEG method; 10^{-2} dilution of wild type phages (2), Biotin-binding phages (5) and Silica-binding phages (7) with filter method. For the negative control of infection, only the same bacterial dilution culture was cultured (3).

Table 4.5: Phage Infection Colony Counts of PEG and Filter Methods

Phage Treatments	White Colonies Count	Blue Colonies Count
ER2738 (no phage)	1.752	No Blue Colony
Wild type phages with PEG method	1.000	120
Wild type phages with filter method	1.010	280
Biotin-binding phages with PEG method	1.250	129
Biotin-binding phages with filter method	1.270	241
Silica-binding phages with PEG method	1.200	173
Silica-binding phages with filter method	1.100	314

The concentrations of the virion were also determined by spectrophotometrically as given in Table 4.6. The concentrations of the virion were 67.600 and 18.000 virion/mL for wild-type phages when used the reverse-filter method and PEG precipitation method respectively.

Table 4.6: Concentrations of Virions

Phage Treatments	Size (bp)	Virion Concentration (virions/mlx10^{10})	Isolated Culture Volume (mL)	Suspend Volume (1XTBS) (mL)	Equation Factor	The Concentration After Normalization
Biotin-Binding Phages with Filter Method	7.258	16.800	100	8	X 4	67.200
Silica-Binding Phages with Filter Method	7.261	16.800	100	8	X 4	67.200
Wild Type Phages with Filter Method	7.222	16.900	100	8	X 4	67.600
Wild Type Phages with PEG Method	7.222	18.000	200	4	X 1	18.000

To optimize phage concentrations for the spreading method, mid-log bacterial culture was infected with 10^{-5} and 10^{-4} dilutions of recombinant phages spread on LB agar plates including Tet (20 µg/mL)/ X-gal (0.2 mg/mL)/ IPTG (1 mM). Wild-type phages were also used as the positive control. The number of phages were determined by counting blue colonies given in Figure 4.27 and in Table 4.7.

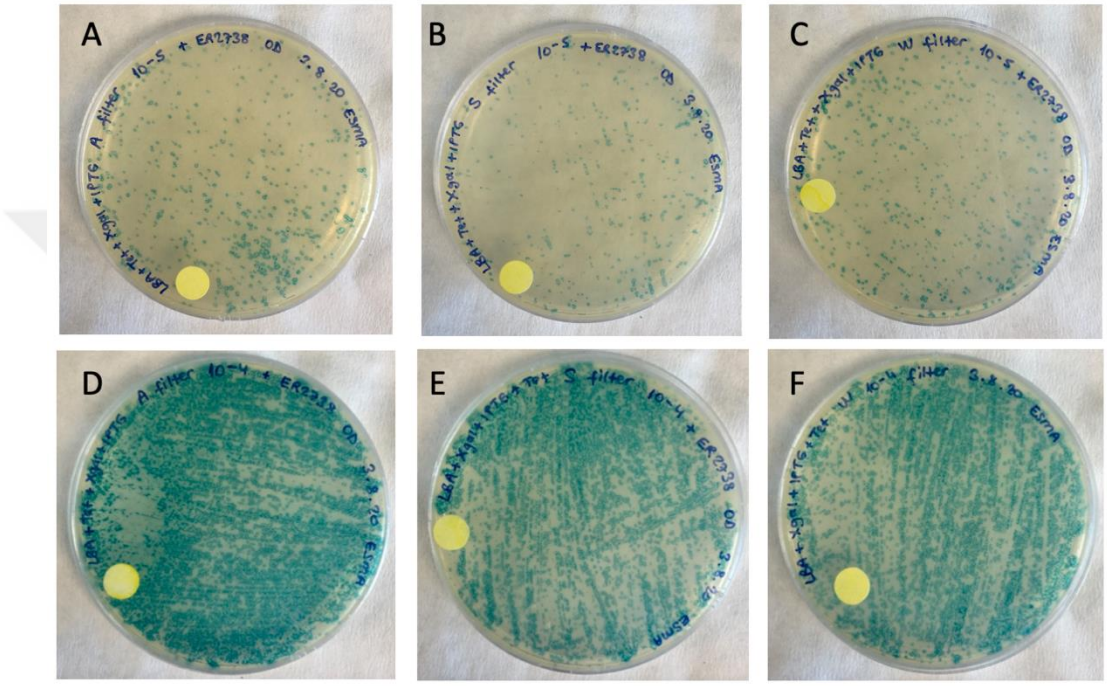

Figure 4.27: Infection Cultures. 10^{-4} dilutions of Biotin-binding (A), Silica-binding (B), Wild type (C) phages infections; 10^{-5} dilutions of phage infections (D, E, F).

Table 4.7: Phage Infection Blue Colony Counts of Filter Method

Phage Treatments (10^{-5} dilutions of)	**Blue Colonies Count**
Biotin-binding phages	628
Silica-binding phages	436
Wild type phages	688

4.5. Re-Infection Assay

To understand whether infected bacteria might lose lac alpha complementation after in serial passages, different starter cultures were done as explained in Figure 4.28.

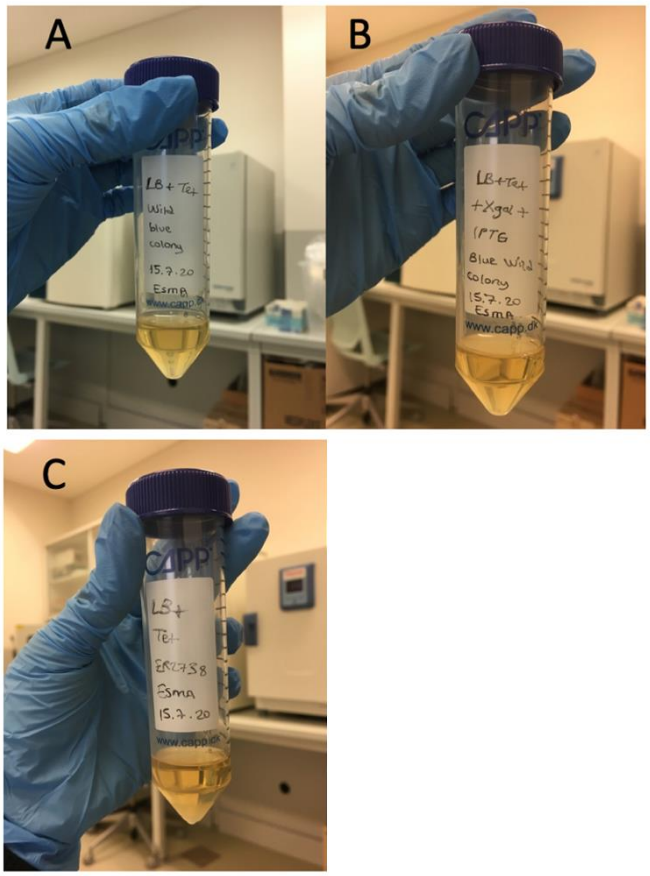

Figure 4.28: Starter Cultures. (A) Wild type phage infected colony was inoculated in LB medium including 20 µg/mL Tet, (B) in LB medium including 20 µg/mL Tet, 0.2 mg/mL X-gal and 1 mM IPTG. (C) E. coli ER2738 colony (white colony) was inoculated in LB medium including 20 µg/mL Tet.

Then, starter cultures were incubated for overnight as given in Figure 4.29.

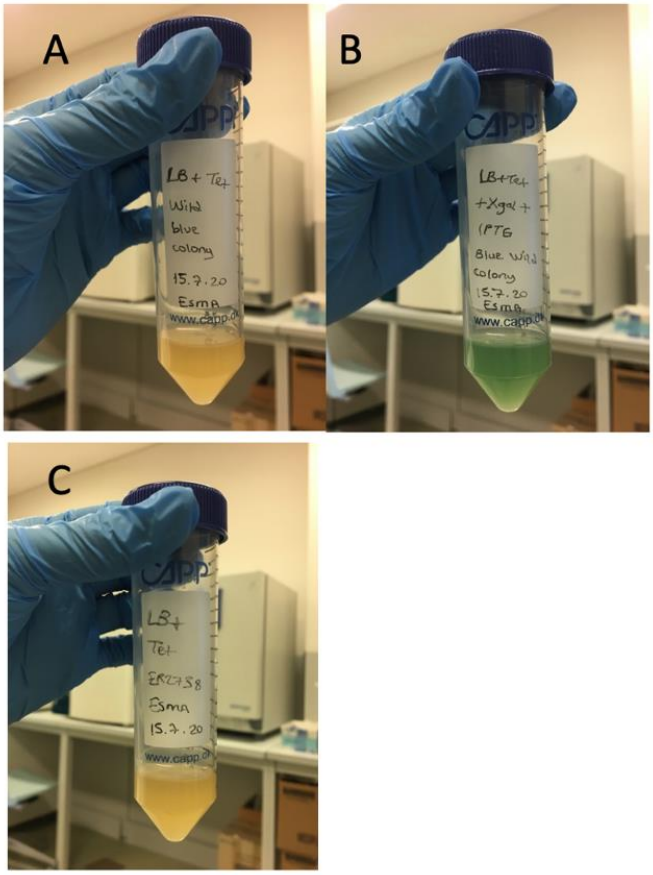

Figure 4.29: Overnight Cultures. (A) Wild type phage infected colony was inoculated in LB medium including 20 µg/mL Tet, (B) in LB medium including 20 µg/mL Tet, 0.2 mg/mL X-gal and 1 mM IPTG. (C) E. coli ER2738 colony (white colony) was inoculated in LB medium including 20 µg/mL Tet.

For re-infection assays, 10^{-2} dilution of phages were spread on LB agar including Tet (20 µg/mL)/ X-gal (0.2 mg/mL)/ IPTG (1 mM), then the 10^{-4} dilution of the overnight cultures mentioned in Figure 4.29 was spread on phages. After incubation, plates were observed as given in Figure 4.30. There were not any blue colonies when bacterial cells were not treated with phages. No colonies were not observed when phages without bacterial cells were spread as the second negative control. For the first time infection group, many blue colonies were observed when used bacterial cells which were not previously infected by phages. For the re-infection group, only a few blue colonies were observed when used bacterial cells which were previously infected. The number of white/blue colonies were given in Table 4.8.

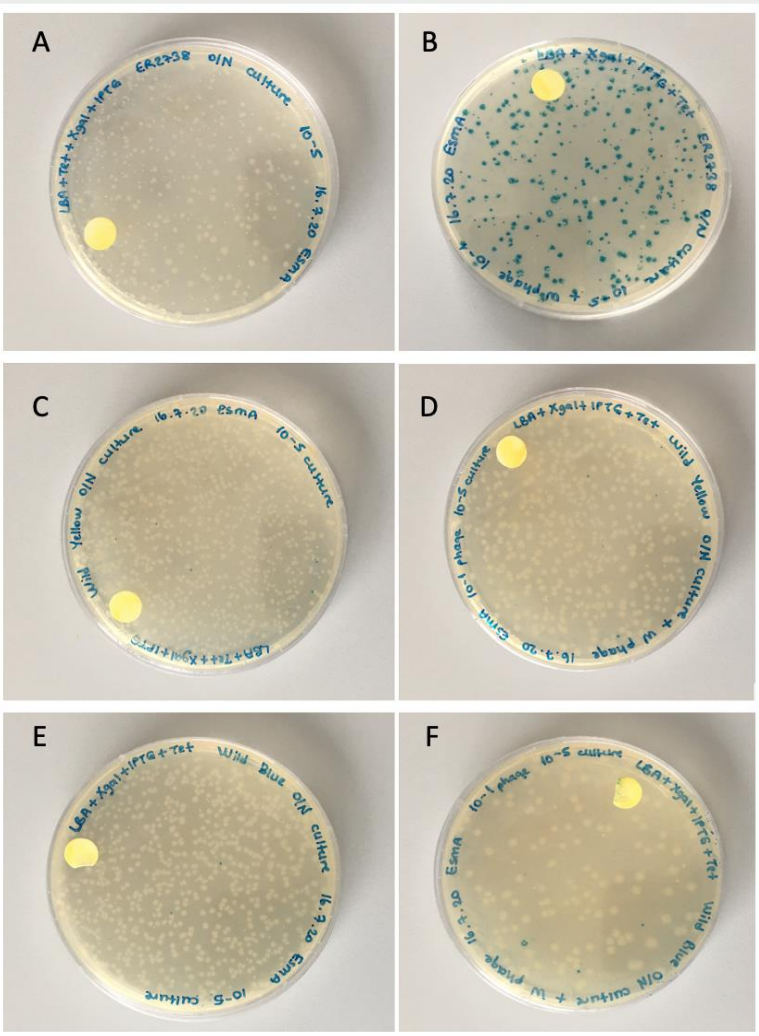

Figure 4.30: Re-Infection Cultures. Infection culture of fresh host bacteria (B); Culture of E. coli ER2738 (A), pre-infected and non-colored overnight culture (C), blue colored overnight culture (E); Re-infection of pre-infected and non-colored overnight culture (D), blue colored culture (F).

Table 4.8: Blue and White Colony Counts

Petri	**White Colonies Count**	**Blue Colonies Count**
E. coli ER2738 Cultured (A)	340	No Blue Colony
Infected Positive Control (B)	7	320
Uncolored Culture (C)	800	8
Uncolored Culture Re-Infection (D)	500	8
Colored Culture (E)	700	3
Colored Culture Re- Infection (F)	116	4

5. DISCUSSION AND CONCLUSION

The M13 phage is a filamentous bacteriophage which is composed of 11 structural proteins and ssDNA as the genomic material. An M13KE phage is a derivative form of the M13mp19 and its genome is 7.222 kilobases in length (23). Its P3 proteins can recognize and infect F^+ *E. coli* bacteria (4,12,14–17,19–21). Tetracycline resistance in *E. coli* ER2738 bacteria is related to F' pilus, therefore bacteria are cultured in M9 minimal medium including tetracycline. Tetracycline helps both F' pilus to continue on bacterial cells and to prevent contamination of other bacterial species. Foreign peptide sequences in 8-12 amino acid length can be fused on P3 proteins and these modifications do not affect to infectivity. Because M13 bacteriophages are very stable under different environmental conditions such as high temperature, DNAses, proteases, extreme pH, and organic solvents, they can be used as phage display ligands. Phage display technique is used for selection of peptides specific to any targets or producing the proteins, antibodies, and large scale of peptides (1–3,13,25). These phages can be used for theranostic purposes by meaning for both therapy and diagnostics (20, 23, 30). The phage display method can also be used as a tool for the development and reproduction of solid binder peptides in a large scale(1–4,13,25,42,48). Monoclonal antibodies are a significant group of recombinant protein therapeutics on the global market, and their market share was 75 billion US$ in 2013 from only therapeutic antibodies specific to cancer and autoimmune diseases (30). In vitro selection of antibodies with phage display technology has a growing interest, because the advantages of using phages are low-cost and large-scale production in a short time (30). In 2002, adalimumab was approved first as the human antibody on the market and as of the start of 2016, six human antibodies for treatment have been developed by using phage display technology (30,59). Since M13 phages can only infect bacteria without affecting eukaryotic cells, there is a great advantage in comparison to other therapeutics such as antibiotics (5,59,60) The use of phages also provides host specificity, simple amplification, cost-effectiveness, and safety (5,59,60). The biodegradability of M13 phages in tissues and various body fluids such as human blood, saliva, urine, artificial gastric juice was in vitro studied by Celec et al. (2012) (59). Their studies showed that while the M13 phages were decreased in rate

of 44%, 66% and 88% after 45 min in blood, urine, saliva and the most rapid phage degradability was occurred in jejunum homogenate with nearly 100% degradation after 45 min, because of proteolytic enzymes abundance (59). James W. Gillespie et al. (2019) showed that phage libraries are a unique nanobioparticle that can be used to identify functional organ and tissue-binding elements specific to cancer tissues, especially breast cancer (2). These results suggest that the phages have the potential to be used for diagnosis and directed drug delivery.

The use of peptides specific to inorganic surfaces such as silica and biotin has made great progress in the field of nanobiotechnology (48). These peptides have been used for making functional surfaces like metals, semiconductors, magnetic materials, polymers, minerals, and silica surfaces via self-assembly (48). Biotin and silica are inexpensive materials, and low-cost biotechnological tools can be developed. The silica are widely used in many biosensor surfaces (61), therefore it is important to develop ligands specific to the silica. Besides biosensor applications, ligands specific to silica was used as adsorbent to design a novel protein purification technique (62–64). The biotin has been used in sandwich assays for the signal transduction (45,65) and also in the detection of biotin levels directly as the signal molecules showing the proliferation of cancer cells (66). Furthermore, there are intelligent therapies targeting of biotin for drug delivery to cancer cells (67,68). Biotins in the blood affect the results of biotin-avidin based immunoassay significantly and cause false negative/positive results. (44,69). To eliminate biotin from the test samples is important (44). As another strategy, phages specific to biotin developed in this study might be used to remove biotins from blood samples. Phages chemically biotinylated have been used for the detection of the microorganisms via fluorescein-conjugated avidin by using confocal laser scanning microscopy and electron microscopy (70). Instead of the chemical modification, in our study, genetically engineered phages were developed to use them as biotin ligands. Together phages specific to silica, they will be used to design new biosensor showing multifunctional activities in one.

Phage display ligands can be selected by using phage libraries by following binding and washing cycles. As the second option, rational design is preferred to fuse peptides desired (1,4,30,41,42). For engineering of phages, two different cloning methods were performed such as restriction enzyme-based cloning and inverse PCR-based cloning. For restriction enzyme-based cloning method, *Eag* I or *Acc*65 and *Kpn* I restriction enzymes are recommended at Noren (2001) M13KE cloning system for P3 phage displaying (23). Although *Eag* I and *Acc*65 are isomer enzymes, they are not suitable for double digestion because both together do not work in the same reaction buffer with maximum efficiency in the same reaction buffer, which means that there is not a buffer compatibility. *Eag* I restriction enzyme is 100% active in NEB Buffer 3.1 and %10 active in Cut Smart Buffer, whereas *Kpn I* is 100% active in NEB Buffer 1.1. and %50 active in Cut Smart Buffer, which might exhibit star activity. To overcome this limitations, *Eag* I and *Kpn* I enzymes should be used separately in different buffer solutions. However, despite purification requirements in each step, DNA samples are lost, and the efficiency is decreased. After restriction enzyme digestion, the amount of DNA for ligation is obtained quite low. Another option, *Acc*65 restriction enzymes instead of *Eag* I can be used for the double enzyme digestion, which works in the same buffer with high efficiency (71). So, yield might be higher. Furthermore, the digested fragments of the vector DNA are too small to be observed in the agarose gel. Therefore, digested vector DNA with the double enzyme or cut with single enzyme and DNA in the uncut linear form cannot be differentiated from each other in the gel electrophoresis. After ligation and transformation, colonies carrying the wild type DNA are also obtained besides recombinant colonies. Since reproductive success of colonies carrying the wild type DNA is higher than colonies carrying recombinant phage DNA, it is not easy to select target bacterial colonies (72). There is not marker gene showing cloning in the P3 region, all colonies carrying target DNAs or wild-type DNAs show X-gal complementation and are observed as blue colonies. Therefore, the colony PCR has to be performed for verification. When used restriction-enzyme based cloning method to engineer a phage library according to routine protocol in the literature (Noren and Noren 2001), the ratio of blue and white colonies after transformation is used to calculate the success of cloning, because blue colonies show bacteria carrying recombinant M13KE DNA as well as wild-type ones (23). However,

the amount of X-gal and IPTG recommended to use in this protocol can give false negative results. In this study, different concentrations of the X-gal and IPTG were tried to understand if the detection sensitivity of blue colonies is increased, or not. When used 0.2 mg/mL of X-gal and 1mM of IPTG, positive colonies were better distinguished as dark blue colonies as expected (73).

As another method, the inverse PCR makes required time and steps considerably reduced for cloning of the M13KE phages. In the literature, for this purpose, inverse PCR was used to add restriction enzyme digestion regions for cloning (43). In this study, the inverse PCR was used for cloning of phages, but there were not any need to add any restriction enzyme sites. Instead of that, primers including DNA sequences coding peptides specific to biotin and silica were used to fuse target sequences into the M13KE genomic DNA by using whole-genome PCR. This method makes cloning easier and more rapid, and efficient, when compared to other methods in the literature (23,43). Any purification steps were not needed, because restriction enzymes were not required to use. Also, a small amount of vector DNA was enough for cloning, ligation, and transformation with high efficiency.

In the phage display technique, recombinant phages are screened by using top agar method. This method allows phages to infect only one cell by providing a solid matrix such as agar. It was developed like that because bacterial cells start to produce phages after transformation step and these phages can have a chance to reach to all bacterial cells equally if given a time enough. Therefore, transformed bacterial cells are directly mixed with melted agar media and then poured onto the agar media. However, it is not practical, and it needs well-trained people, because the agar must remain in molten state as well as warm enough not to kill the bacteria/phages during the process. To overcome this limitation of the top agar method, the spreading method was modified for it in this study. The spreading method for the phages was developed by inspiring from Luria Delbrück (56). After M13KE phages were spread onto the agar plates and in a short incubation like a couple of minutes, bacteria were spread on the phages by

using the sterile ecuvion sticks. So, phages were limited on the agar plate and provided to meet with only a certain number of bacteria. This new method was easier and rapid to apply when compared to the top agar method. The number of the phage and bacteria necessary for the method were optimized in this study.

In the phage display technique, concentrating phages is one of the most critical steps after selection or production (74). For that in routine applications, phages are cultured and bacterial cells are removed by centrifugation, then supernatant including phages are concentrated (15,23). A number of methods for concentrating of phages have been published (74). Most used one among them is PEG precipitation method (15,23).

PEG precipitation as a standard method is very time consuming which takes two days, because there are many washing and centrifugation steps to precipitate phages and to remove PEG residues from the final suspension. For large-scale productions, bigger rotors are required for centrifugation, and so it is not practical and cheap for the small laboratories unless samples are divided into the small volumes for centrifugation. Considering the many washing requirements, each additional step causes the loss of phage and therefore the yield of the method is low. To overcome these limitations in the use of PEG precipitation, the reverse-filter method as a new strategy was developed to concentrate phages in this study. In this method, the supernatant containing the phages was filtered by using a 0.22 μm filter and a buffer solution was passed through from the filter in the reverse direction to elute phages. After elution and purification of the ssDNAs of phages, it was found that 20 times more phages were obtained by using the reverse-filter method in a short time such as 30 minutes, when compared to the PEG precipitation method. Besides high efficiency, it makes the concentrating of phages easier, rapid, and more practical for each laboratory which is not well-equipped such as student research laboratories.

In this study, it was observed that when blue colonies were sub-cultured on agar plates including X-gal and IPTG, they started to produce white colonies. After many

sub-culturing, almost all the colonies were white. In the literature, it was suggested that the infected bacterial cells should not be stored as stock cultures to produce phages (15). Instead of bacterial cells, isolated phages should be stocked (15). Another suggestion was that phage culture should not be incubated more than four hours to amplify phages (72). To understand if returning of blue colonies to white colonies in this study is associated with this information given in the literature above, a re-infection assay was designed in this study. Results showed that when bacteria obtained from blue colonies were freshly sub-cultured in LB media including tetracycline, X-gal, and IPTG for overnight, most of them could not be formed blue colonies; they were lost their abilities of the complementation. When the overnight cultures were re-infected by phages, results were the same; no blue colonies, except only a few one. Considering prokaryotic immunity such as Crispr-Cas9 system (75), re-infection assay helped us to understand why infected bacteria should not be stored as "phage factory" and long incubation during phage production results in low yield.

As a conclusion, M13 bacteriophages specific to silica and biotin were engineered in this study and they are planning to design new multifunctional biosensors in future. Standard methods used for engineering of phages have some limitations in practice. In this study, modified strategies were used to improve engineering of phages: 1) The inverse PCR which does not require the use of restriction enzymes were designed for cloning. 2) Instead of the top agar, the spreading method was devised. 3) For concentrating of phages, a new, the reverse-filter method was developed. All these modifications made the method easier, more rapid, and more efficient in comparison the standard methods.

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7. APPENDICES

Appendix 1. Reverse DNA Sequence of PCR Specific to Biotin-Binding Peptide Sequence

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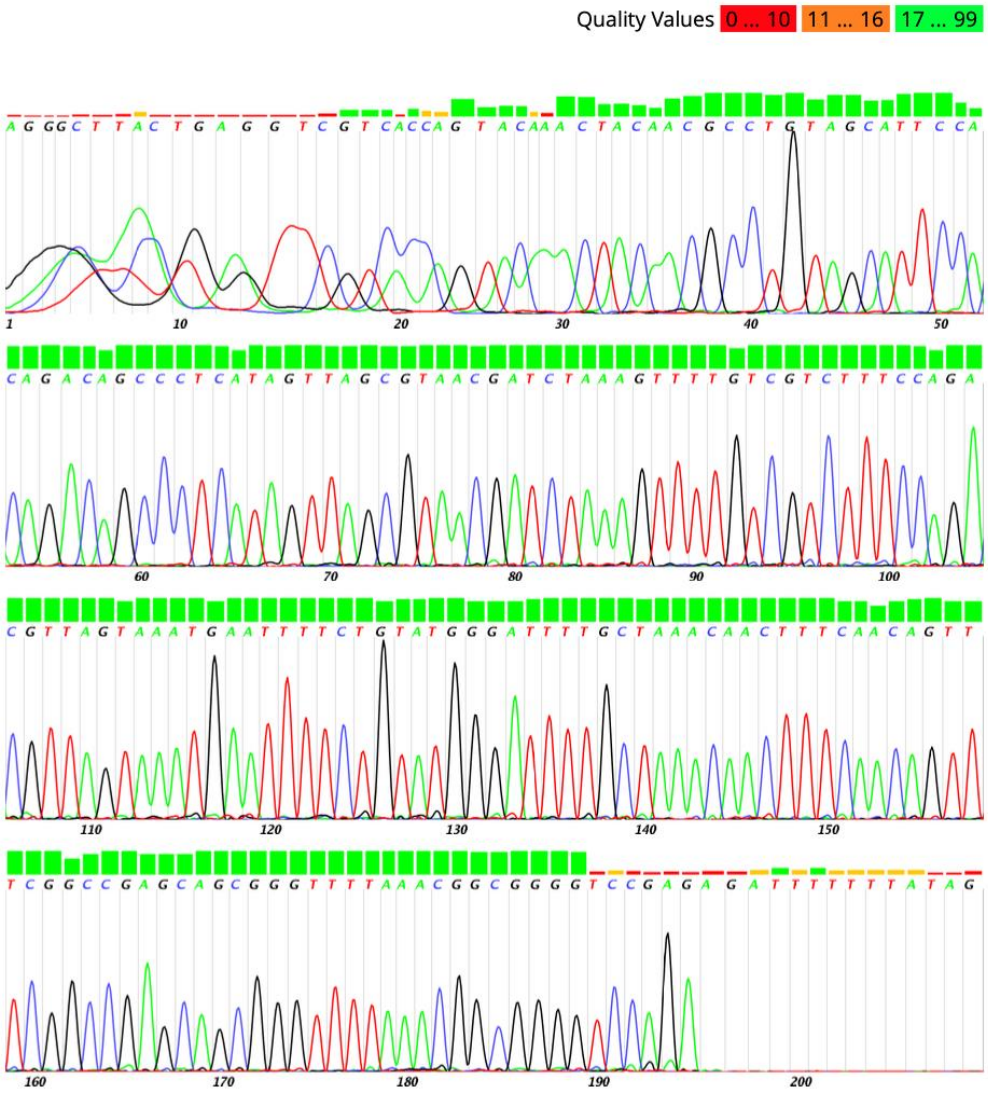

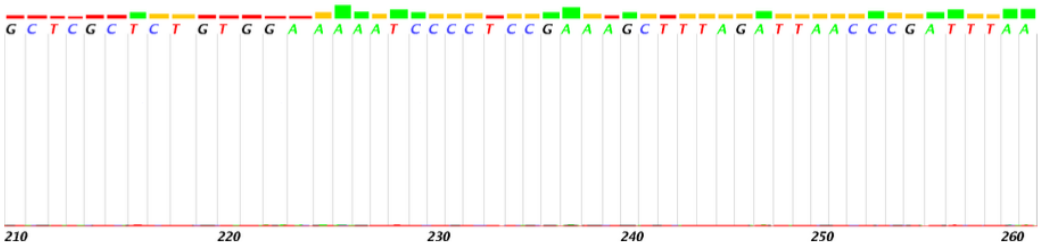
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250
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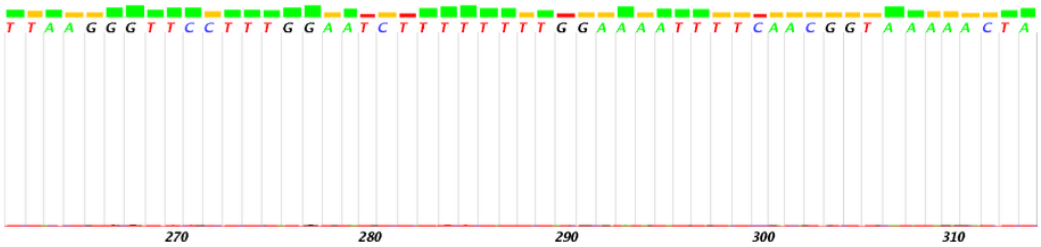
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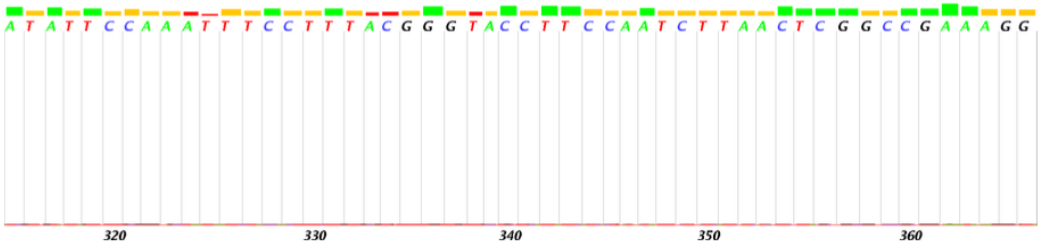
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350
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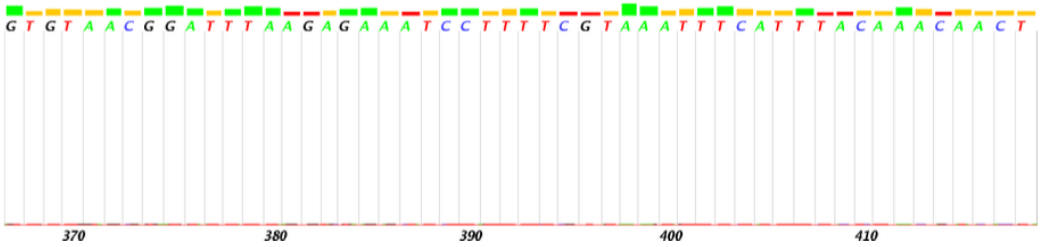
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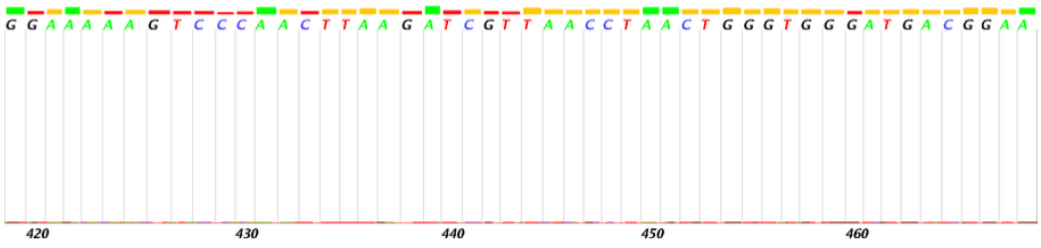
390
400
410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450
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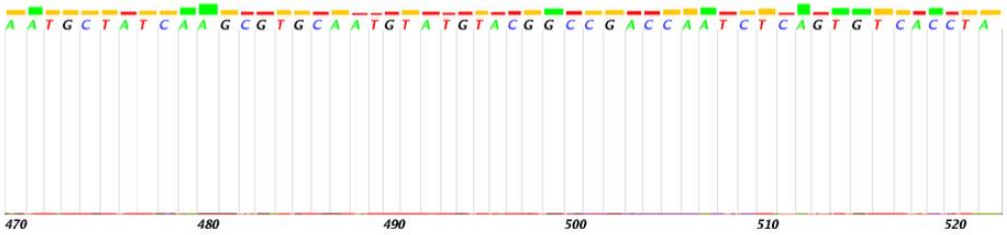
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480
490
500
510
520

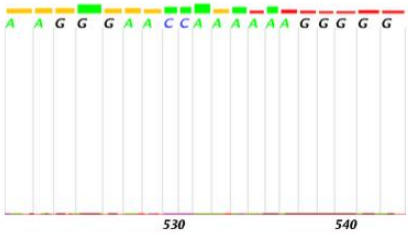
530
540

Table 1: ABI Information

Source File:	8-AR
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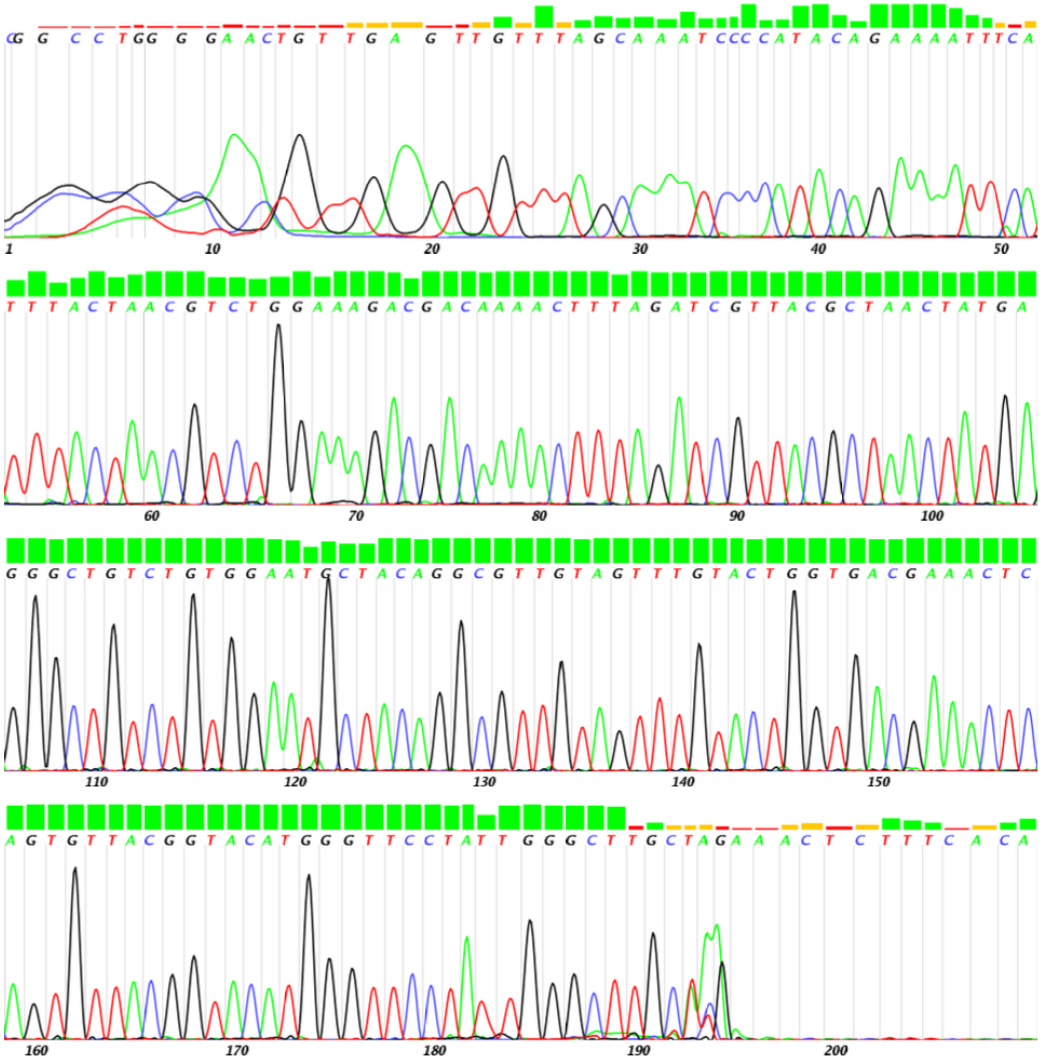
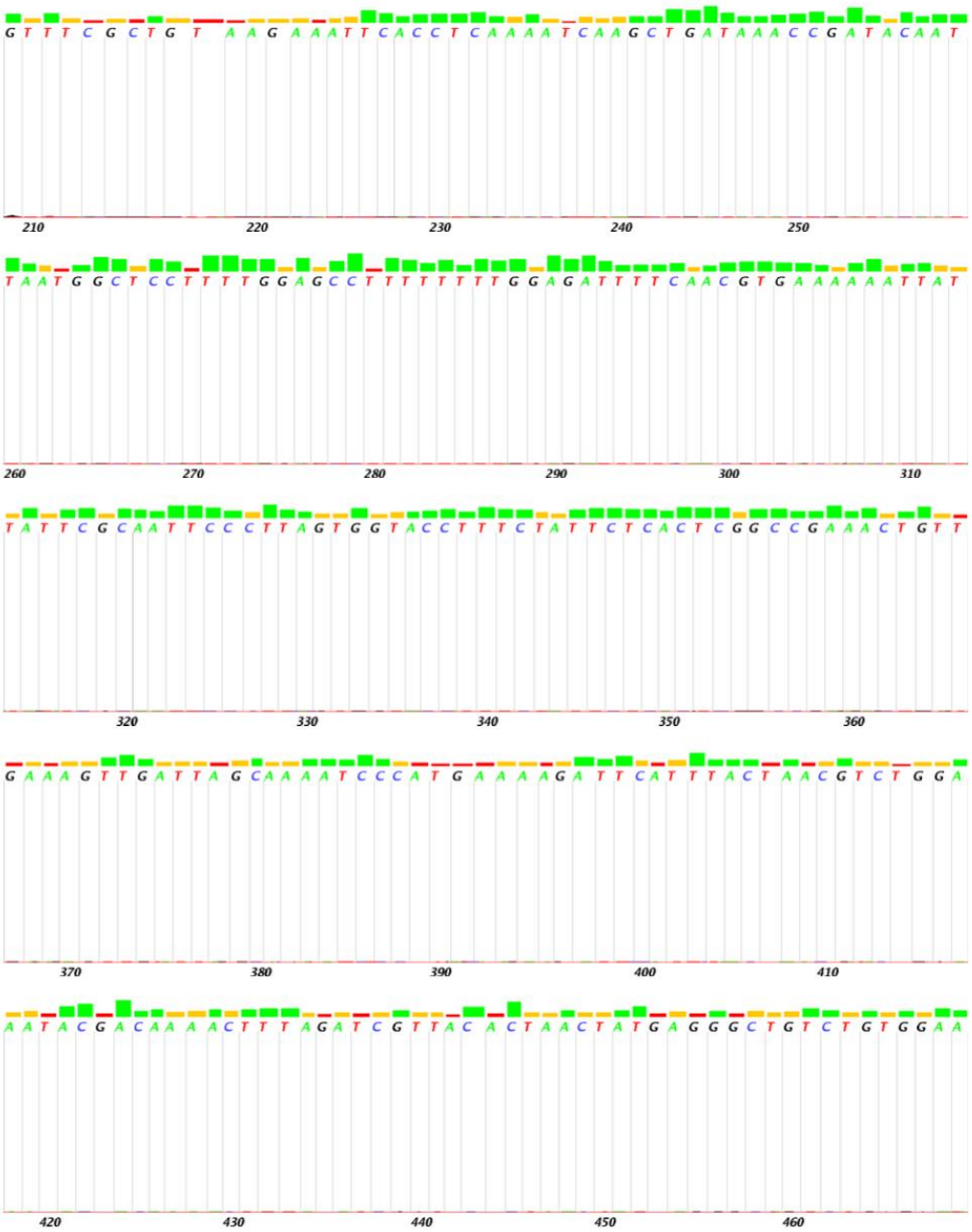
GATC Job Reference:	45137472
Template:	8
GATC Template Reference:	2AI418
Primer:	AR
GATC Primer Reference:	-
Run Start:	04.Sep.2020 02:24:18 AM
Run Stop:	04.Sep.2020 04:38:43 AM
Basecaller Version:	KB 1.4.1.8
Dye Primer Set:	KB_3730_POP7_BDTv3.mob
Read Length	166
Average Signal:	G(540) A(568) T(595) C(615)

Appendix 2. Forward DNA Sequence of PCR Specific to Biotin-Binding Peptide Sequence

Source File: 8-AF
Creation Date: 04.Sep.2020 05:24:12 AM

Quality Values 0 ... 10 11 ... 16 17 ... 99

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210 220 230 240 250
TAATGGCTCCTTTTGGAGCCTTTTTTTTGGAGATTTTCAACGTGAAAAAATTAT
260 270 280 290 300 310
TATTCGCAATTCCCCTTAGTGGTACCTTTCTATTCTCACTCGGCCGAAACTGTT
320 330 340 350 360
GAAAGTTGATTAGCAAAATCCCATGAAAAGATTCATTTACTAACGTCTGGA
370 380 390 400 410
AATACGACAAAACTTTAGATCGTTACACTAACTATGAGGGCTGTCTGTGGAA
420 430 440 450 460

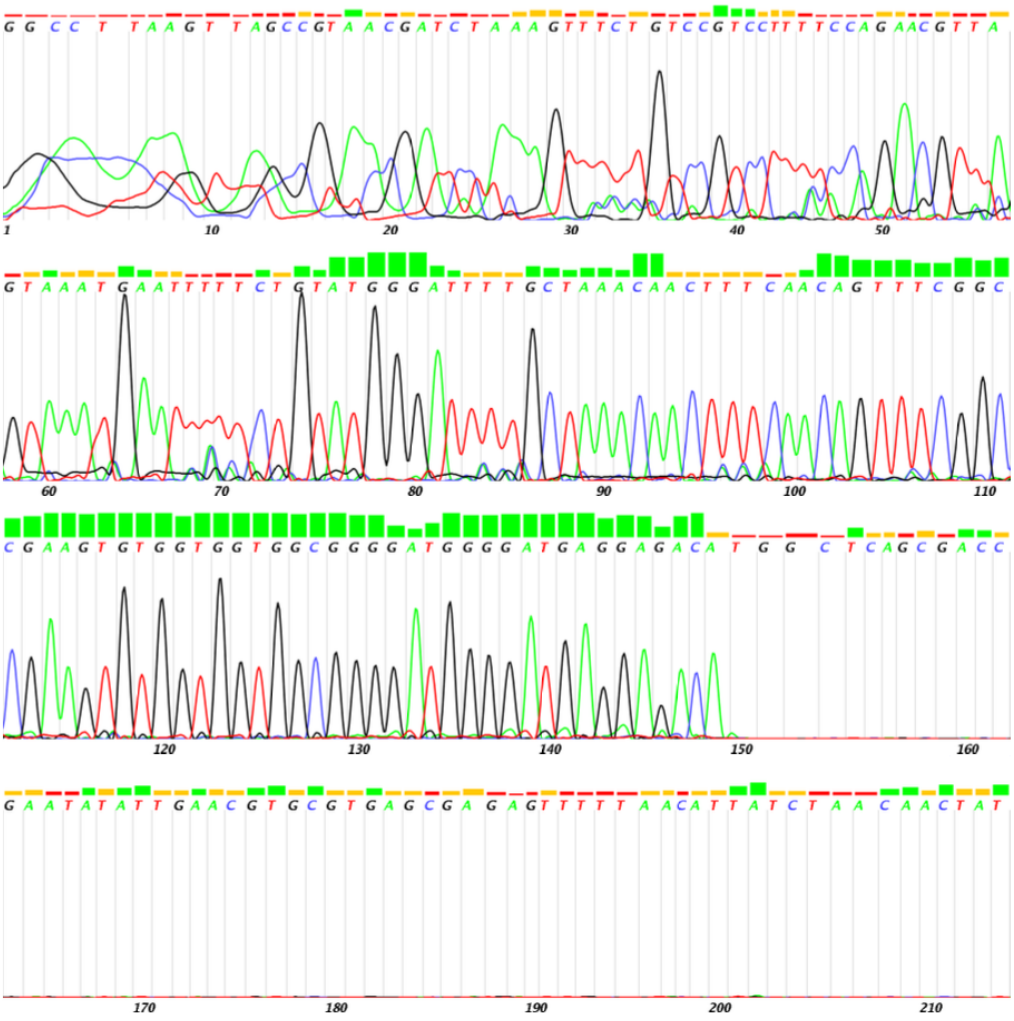
Table 1: ABI Information

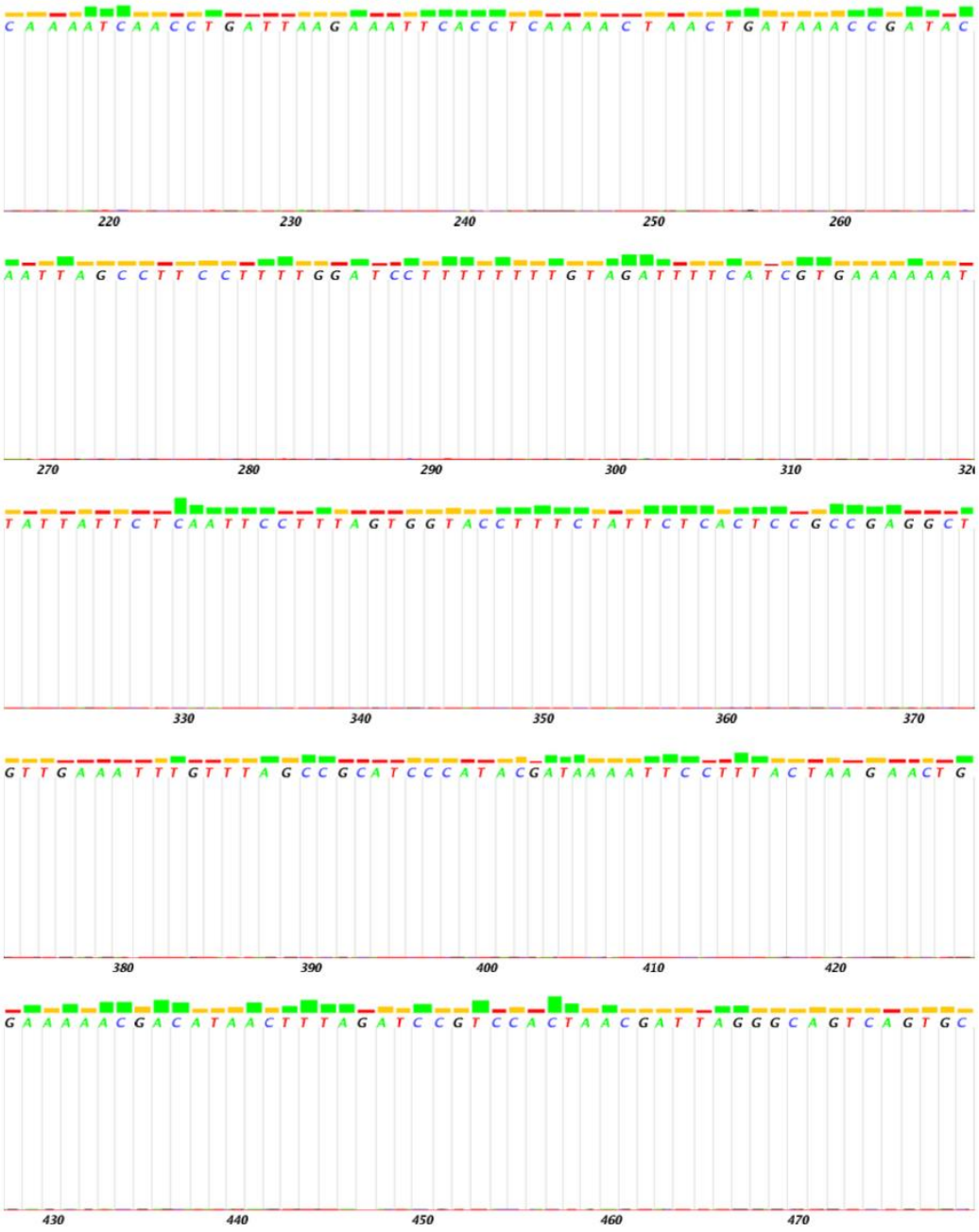
Source File:	8-AF
Watchbox:	3279346
Job Type:	SUPREMERUN tube | Barcode
GATC Job Reference:	45137474
Template:	8
GATC Template Reference:	-
Primer:	AF
GATC Primer Reference:	-
Run Start:	04.Sep.2020 02:24:18 AM
Run Stop:	04.Sep.2020 04:38:43 AM
Basecaller Version:	KB 1.4.1.8
Dye Primer Set:	KB_3730_POP7_BDTv3.mob
Read Length	191
Average Signal:	G(540) A(568) T(595) C(615)

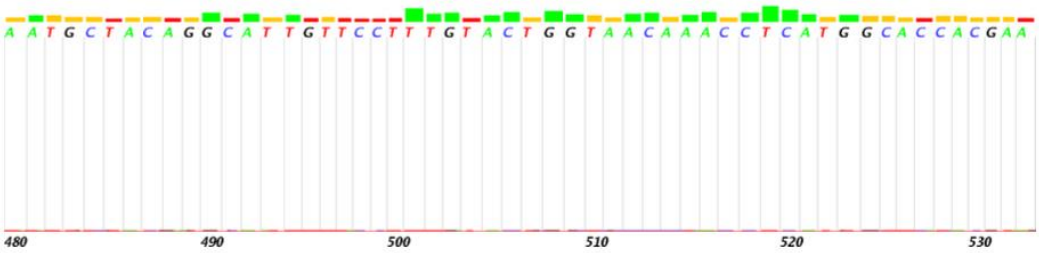
Appendix 3. Reverse DNA Sequence of PCR Specific to Silica-Binding Peptide Sequence

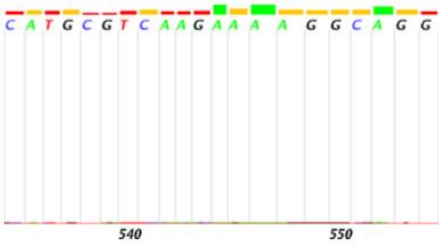
Source File: 9-SR
Creation Date: 04.Sep.2020 09:49:16 AM

Quality Values 0 ... 10 11 ... 16 17 ... 99




C A A A A T C A A C C T G A T T A A G A A A T T C A C C T C A A A A C T A A C T G A T A A A C C G A T A C
220
230
240
250
260
A A T T A G C C T T C C T T T T G G A T C C T T T T T T T T G T A G A T T T T C A T C G T G A A A A A A T
270
280
290
300
310
T A T T A T T C T C A A T T C C T T T A G T G G T A C C T T T C T A T T C T C A C T C C G C C G A G G C T
330
340
350
360
370
G T T G A A A T T T G T T T A G C C G C A T C C C C A T A C G A T A A A A T T C C T T T A C T A A G A A C T G
380
390
400
410
420
G A A A A A C G A C A T A A C T T T A G A T C C G T C C A C T A A C G A T T A G G G C A G T C A G T G C
430
440
450
460
470





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GATC Template Reference:	2AI420
Primer:	SR
GATC Primer Reference:	-
Run Start:	04.Sep.2020 02:24:18 AM
Run Stop:	04.Sep.2020 04:38:43 AM
Basecaller Version:	KB 1.4.1.8
Dye Primer Set:	KB_3730_POP7_BDTv3.mob
Read Length	76
Average Signal:	G(540) A(568) T(595) C(615)

Appendix 4. Forward DNA Sequence of PCR Specific to Silica-Binding Peptide Sequence

Source File: 9-SF
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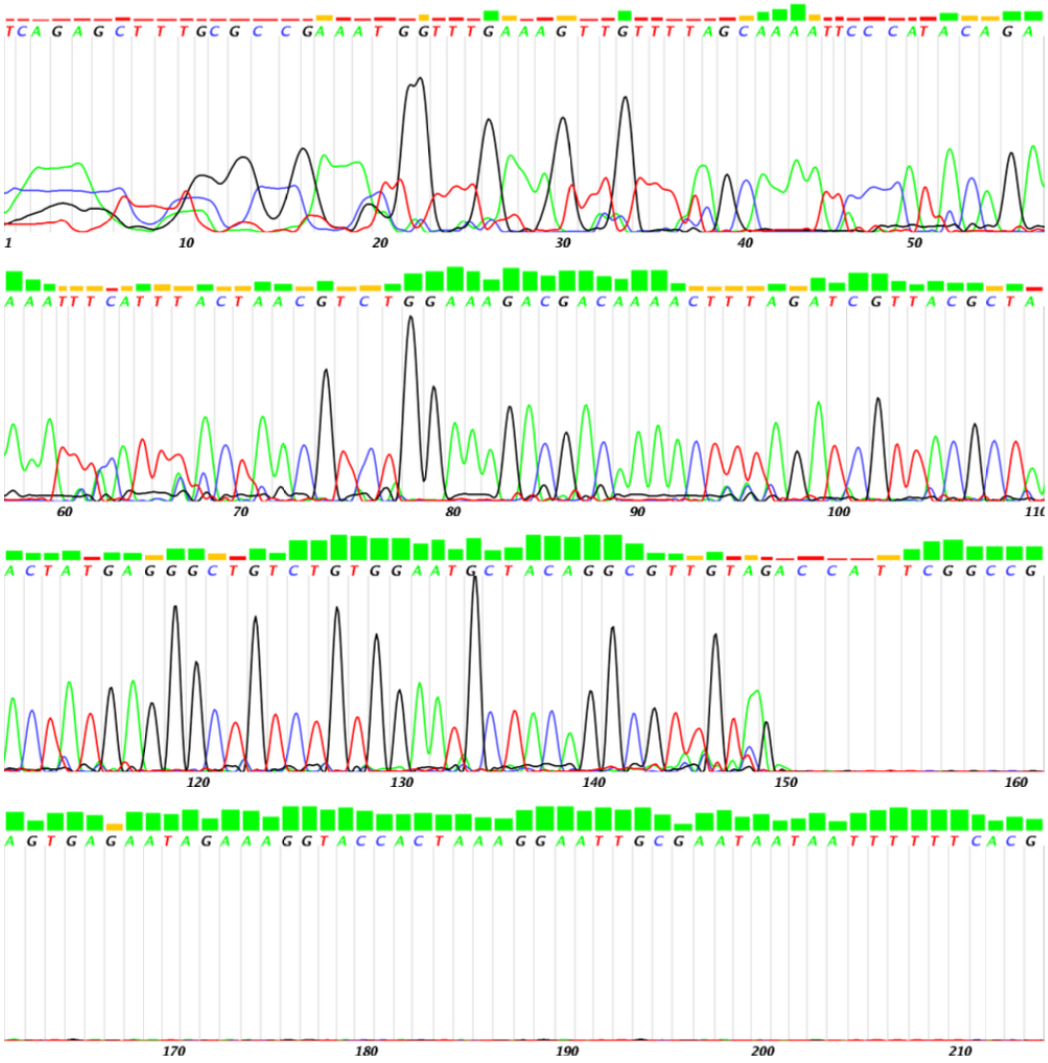
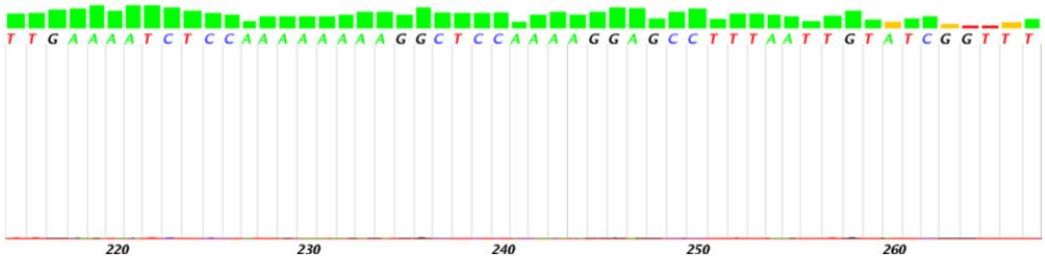
Quality Values 0 ... 10 11 ... 16 17 ... 99

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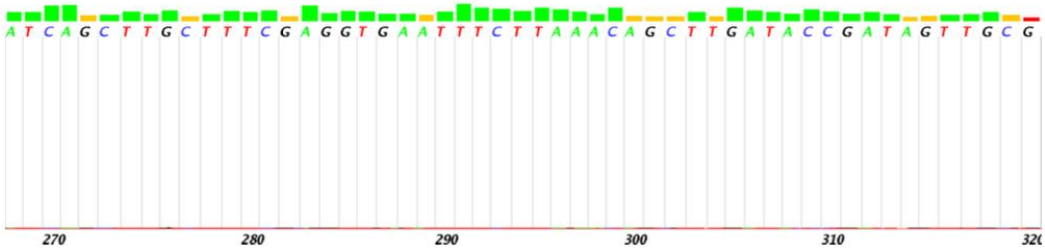
230
240
250
260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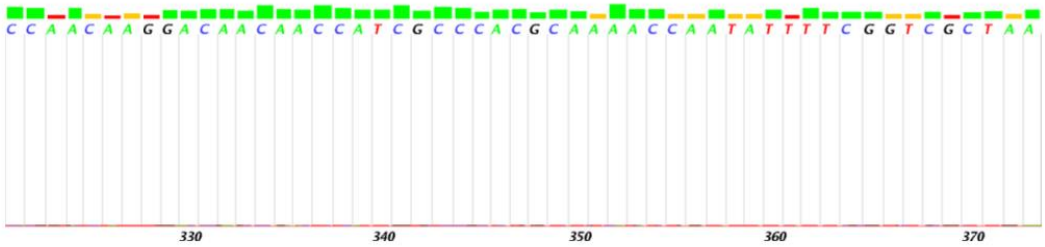
270
280
290
300
310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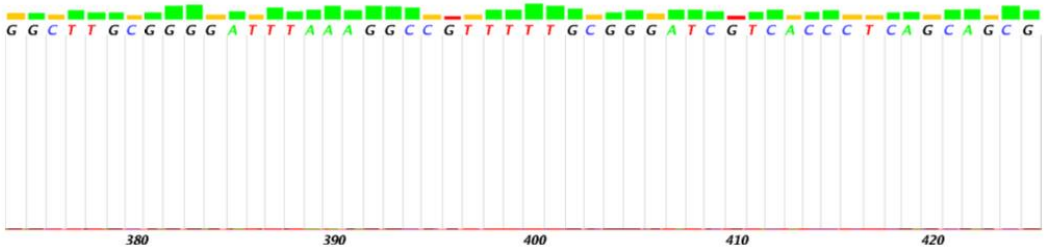
330
340
350
360
370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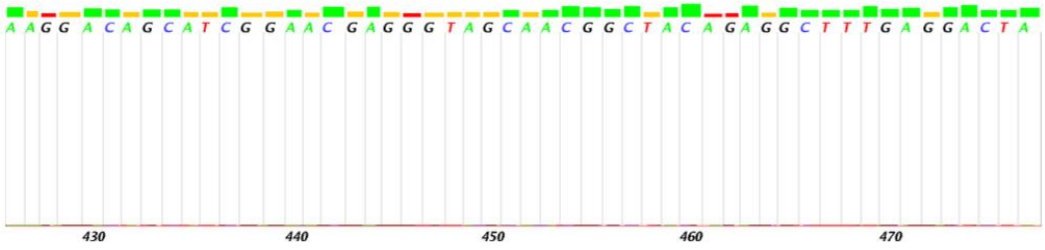
380
390
400
410
420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430
440
450
460
470

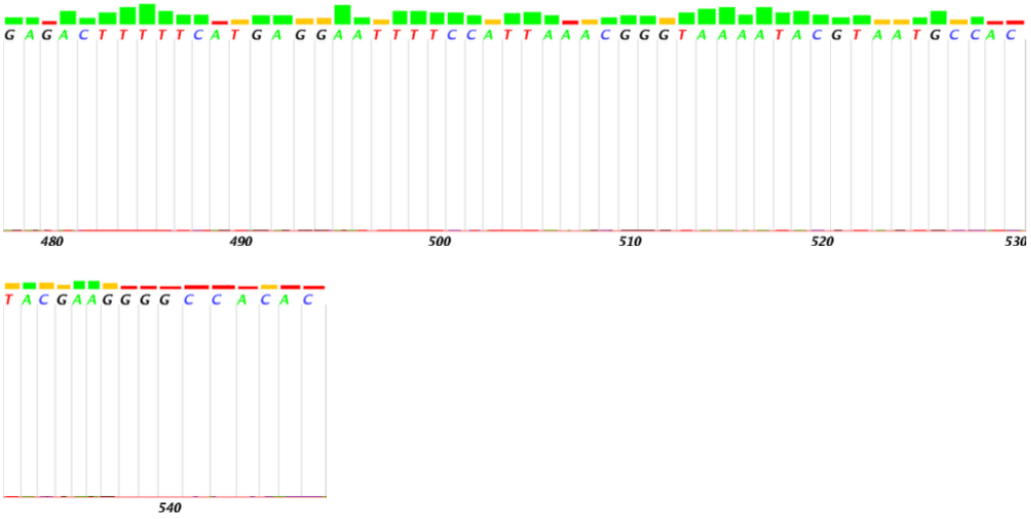

Table 1: ABI Information

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GATC Template Reference:	-
Primer:	SF
GATC Primer Reference:	048575
Run Start:	04.Sep.2020 02:24:18 AM
Run Stop:	04.Sep.2020 04:38:43 AM
Basecaller Version:	KB 1.4.1.8
Dye Primer Set:	KB_3730_POP7_BDTv3.mob
Read Length	79
Average Signal:	G(540) A(568) T(595) C(615)

8. CURRICULUM VITAE

PERSONAL INFORMATION

Name	Esma	**Surname**	AYBAKAN
City	Istanbul	**Date of Birth**	04.01.1995
Nationality	T.C.	**Gsm**	+90 545 456 19 00
E-mail	esmaaybakan@hotmail.com		

EDUCATION

	INSTITUTION	**Graduation Year**
University	Istanbul University, Faculty of Science, Molecular Biology and Genetic (with high distinction- Magna Cum Laude)	2017
High School	Beşiktaş Anatolian High School	2013

INTERNSHIPS

INSTITUTION	**Duration Time**
Istanbul Kultur University - Mobigen Model Organisms Laboratory - C. Elegans Laboratory	1 month
IU Çapa Faculty of Medicine - Asdetae Molecular Medicine Laboratory	2 weeks
IU Cerrahpasa Faculty of Medicine – Department Of Medical Genetics- Clinical, Molecular Genetic And Cytogenetic Laboratory	1 month
Acibadem Mehmet Ali Aydinlar University- Research Laboratory and Medical Microbiology Department	1 month

Foreign Language	**Reading**	**Listening**	**Writing**
English	Intermediate	Intermediate	Intermediate

Exams	Notes
ALES	85.29875
YÖKDİL	66.25

Computer Skills	Level
Microsoft Office	Well