Ruveyda BENK CLONING OF 32-MER MASP1 GENE INTO pBbB6c PLASMID VECTOR and TRANSFORMATION TO *ESCHERIA COLI* NEB 10-beta AGU 2023

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A THESIS
SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND
SCIENCE OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

By
Ruveyda BENK
FEBRUARY 2023

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February 2023

SCIENTIFIC ETHICS COMPLIANCE

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M.Sc. thesis titled “Cloning of 32-mer Masp1 Gene Into pBbB6c Plasmid Vector and Transformation to *Escheria Coli* Neb10-beta” has been prepared in accordance with the Thesis Writing Guidelines of the Abdullah Gül University, Graduate School of Engineering & Science.

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ACCEPTANCE AND APPROVAL

M.Sc. thesis titled “Cloning of 32-mer Masp1 Gene Into pBbB6c Plasmid Vector and Transformation to *Escheria Coli* Neb10-beta” and prepared by Ruveyda BENK has been accepted by the jury in the Bioengineering Graduate Program at Abdullah Gül University, Graduate School of Engineering & Science.

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(Thesis Defense Exam Date)

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APPROVAL:

The acceptance of this M.Sc thesis has been approved by the decision of the Abdullah Gül University, Graduate School of Engineering & Science, Executive Board dated ….. /….. / …….. and numbered ……..……. .

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(Date)

Graduate School Dean
Prof. Dr. İrfan ALAN

ABSTRACT

CLONING OF 32-MER MASP1 GENE INTO pBbB6c PLASMID VECTOR and TRANSFORMATION TO *ESCHERIA COLI* NEB10-Beta

Ruveyda BENK

MSc in Bioengineering

Advisor: Dr. Fatih ORTAKCI

February 2023

The main purpose of my thesis was to clone Masp1 spider silk protein encoding gene from dragline type spider into *E.coli* NEB 10-beta organism. The recombinant microbial production of spider silk protein and converting it into a fiber format would ultimately produce a biomaterial also called as biosteel with high toughness and elasticity whereas low density compared to Kevlar, steel and carbon fiber. For this purpose, the gene encoding the dragline spider protein (MaSP1) was cloned into *E. coli* NEB 10-beta using recombinant molecular methods. First, 8-mer MaSP1 was synthesized and cloned via pGSI high copy cloning vector by sticky end cutting with restriction enzymes of KpnI,Kpn2I followed by heat-shock transformation into *E.coli*. Second, we performed restriction of the 8-mer plasmid by NheI and Kpn2I to extract the 8-mer. Later, restriction was performed by SpeI and Kpn2I to obtain linearized pGSI containing 8-mer Masp1. A ligation was applied to merge 8-mer and pGSI plasmid carrying 8-mer Masp1 to achieve 16-mer Masp1 containing pGSI. Again, this plasmid was heat-shock transformed into *E.coli*. Following the same restriction 32-mer Masp1 containing pGSI plasmid was achieved. Finally, 32-mer Masp1 fragment was cut from pGSI cloning vector and ligated to pBbB6c low copy expression plasmid followed by electroporation into *E.coli*. The band size of 32-mer Masp1 gene was aligned between 3 kb and 5 kb which is an agreement with the calculated size of 32-mer Masp1 gene. Future studies should focus on expression of Masp1 and efficient production of this valuable recombinant protein under bioreactor conditions with cutting edge bioprocessing techniques.

Keywords: Masp1, recombinant DNA technology, cloning, spider silk protein, Escherichia Coli

ÖZET

32-MER MASP1 GENİNİN pBbB6c PLAZMID VEKTÖRÜNE KLONLANMASI VE *ESCHERIA COLI* NEB10-Beta'YA TRANSFORMASYONU

Ruveyda BENK
Biyomühendislik Anabilim Dalı Yüksek Lisans
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Şubat 2023

Tezimin temel amacı, dragline tipi örümcekten Masp1 örümcek ipek proteini kodlayan geni E.coli NEB 10-beta organizmasına klonlamaktı. Örümcek ipeği proteininin rekombinant mikrobiyal üretimi ve bunun bir elyaf formatına dönüştürülmesi, sonuçta Kevlar, çelik ve karbon elyafına kıyasla düşük yoğunluklu, yüksek tokluğa ve esnekliğe sahip biyoçelik olarak da adlandırılan bir biyomateryal üretecektir. Bu amaçla, dragline örümcek proteinini (MaSP1) kodlayan gen, rekombinant moleküler yöntemler kullanılarak *E. coli* NEB 10-beta'ya klonlanmıştır. İlk olarak, 8-mer MaSP1 sentezlendi ve pGSI yüksek kopya klonlama vektörü yoluyla, KpnI,Kpn2I kesim enzimleri ile yapışkan uç kesimi ve ardından E.coli'ye ısı şoku dönüşümü yoluyla klonlandı. İkinci olarak, 8-mer'i çıkarmak için 8-mer plazmitinin Nhel ve Kpn2I ile kesimini gerçekleştirdik. Daha sonra, 8-mer Masp1 içeren doğrusallaştırılmış pGSI için SpeI ve Kpn2I ile kesim gerçekleştirildi. pGSI içeren 16-mer Masp1 elde etmek için 8-mer ve 8-mer Masp1 taşıyan pGSI plazmitini birleştirmek için bir ligasyon uygulandı. Yine bu plazmit ısı şoku ile *E.coli*'ye dönüştürüldü. Aynı kısıtlamanın ardından, pGSI plazmidi içeren 32-mer Masp1 elde edildi. Son olarak, 32-mer Masp1 fragmanı, pGSI klonlama vektöründen kesildi ve pBbB6c düşük kopya ekspresyon plazmitine bağlandı, ardından *E.coli*'ye elektroporasyon yapıldı. 32-mer Masp1 geninin bant boyutu, bir anlaşma olan 3 kb ile 5 kb arasında hizalandı. 32-mer Masp1 geninin hesaplanan boyutu ile. Gelecekteki çalışmalar, Masp1'in ekspresyonuna ve bu rekombinant proteinin biyoreaktör koşulları altında en son biyoişleme teknikleriyle verimli üretimine odaklanmalıdır.

Anahtar kelimeler: Masp1 Geni, Örümcek Ağı, Escherichia Coli, Rekombinant Klonlama

Acknowledgements

Firstly, I would like to start with expressing a deep appreciation to my advisor Dr. Fatih Ortakcı, who guided me through whole this process, taught everything patiently.

I would like to thank all members of Ortakcı lab for their huge helps in my studies and especially Ismail Gümüştop and Zehra Erkol, because she really makes my experiment more enjoyable.

I would like to thank to TUBITAK for their supporting me by 2210-D BIDEB Scholarship during my master process.

I would like to thank my parents Emine &Muzaffer Küçük, Müfide &Ahmet Benk, and my whole family for their support, encouragement, and love in my academic journey.

I would like to express biggest thanks to my husband Burak Benk, he deserves the best of thanks for his endless love and support me.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

MaSp1	Major Ampullate Spidroin 1
AOX1	Aldehyde Oxidase 1
tRNA	Transfer ribonucleic acid
DNA	Deoxyribonucleic acid
MaSp2	Major Ampullate Spidroin 2
SOB	Super Optimal Broth (SOB medium)

To my husband and my family,

Chapter 1
Introduction

Spider silks show remarkable physical characteristics as elasticity, toughness, and strength. When the density of spider silk protein (MaSP1) is considered, it is an alternative material to commonly used materials with its better strength than steel, higher toughness than carbon fiber and Kevlar, its elastic structure and low density. In addition, spider silk is a good biodegradable material at the same time, it is very biocompatible that makes it stand out in terms of health and environment [1].With these features, it has a structure that can have serious usage areas in the aerospace and defence industry. This protein can be used for surface coating as well as composite materials. It is a material that can be used in structures that require high toughness, such as bulletproof materials, by tapping appropriately[2].

1.1 Spider Silk Protein, MaSP1

Spider silk has amazing mechanical features such as great strength, toughness, and high elasticity. Also, this natural substance is one of the strong materials known. Given its high density, biodegradability, and biocompatibility, this material is both sturdier than steel and more flexible than nylon.[1-5]. If spider silk is compared with other fiber types and natural materials, it is obvious that its toughness and strength are extraordinary as demonstrated in Table 1. It is capable of withstanding forces three times greater than Kevlar, and five times higher than steel, despite having a lower density than both of these materials. Moreover, spider silk protein is a water-soluble, biocompatible, and biodegradable natural material [6-10]. These properties make spider silk protein suitable for some biomedical applications such as surgical threads [11-12], tendon/connective tissue [13-14], drug carriers [15-17] and surface coating of probes [18]. Spider web thread can be used in textiles and even in battery components [19-20]. However, it draws attention because it can be used in industrial products such as ropes in parachute systems, protective suits and composite materials in aircraft [21].

Table 1.1 Mechanical Features of Synthetic and Natural Fibers [22]

Material	Density[gcm^{-3}]	Strength [GPa]	Elasticity [%]	Toughness [MJm^{-3}]
MaSp1 Dragline Spider Silk	1.3	1.1	27	180
Flagella Spider Silk	1.3	0.5	270	150
Insect Silk	1.3	0.6	18	70
Nylon 6.6	1.1	0.95	18	80
Kevlar 49	1.4	3.6	2.7	50
Carbon fiber	1.8	4	1.3	25
Steel	7.8	1.5	0.8	6

Spider silk proteins may be existed in a wide various structure. Literature studies have focused on web-weaving spiders that can secrete many different fibers especially the *Nephila clavipes* spider species from the *Araneidae* family [1]. *N. clavipes* has six different cobweb threads and secretes an adhesive. Each yarn has different properties and has a different function in the spider silk structure [2,22]. Dragline silk is among them as major ampullate silk and has the strongest structure among these threads. Spider web has a skeletal function in its structure and there are two different types as MaSP1 and MaSP2 [23,24]. This dragline silk has a comparable strength to Kevlar [1,2,25]. Spider silk is another type of yarn, flagella form, which can expand to twice its own length [4-5]. The properties of each silk structure come from their genetic codes and by modifying the nucleotide patterns, synthetic fiber in protein structure can be obtained with the desired properties. The properties of spider silk proteins come from the various motifs in it. Repeating the motif that supplies the strength of the protein molecule increases its molecular weight. Thus, the stronger the fiber will be. Table 1 is taken from Bowen et al

(2018) and shows the relationship between the number of multimeric structures, toughness and strength [26].

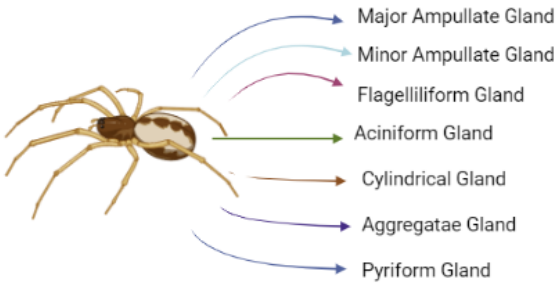

Figure 1.1 7 different spider glands for spidroin production

In spider silk proteins, repeating amino acid groups form three striking motifs. While the GPGXX/GPGGX is one of these motifs provides elasticity, [(GA)n/(A)n and GGX] increases the strength [27]. Since the amount of these motifs in the silk structure affects the mechanical properties of silk, fibers with diverse mechanical characteristics can be obtained by changing their genetic structures [5,28]. In addition, the GPGXX/GPGGX motif encodes the amino acid structure of the β-spiral structure, and these motifs allow the fiber to stretch and shrink without any persistent damage. [29]. Dragline fiber consists of two proteins, major ampullate 1 and 2 [30-31]. These fibers have a modular structure, and each consists of long and repeating strands [32]. MaSP1 is the major component of dragline fiber, and since the strength and toughness of the spider web comes from this part, studies to obtain spider web have focused on the MaSP1 protein [30].

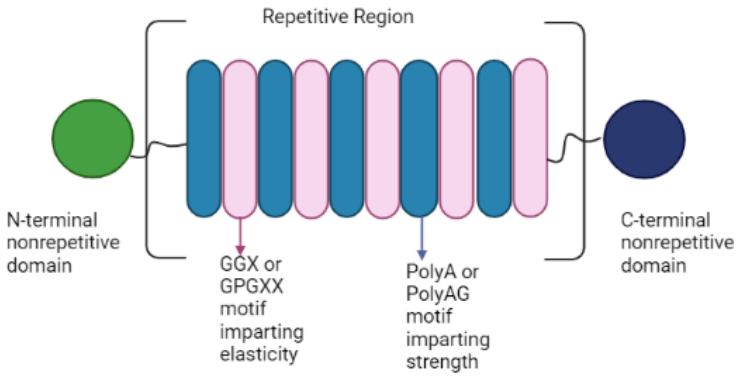

Figure 1.2 Demonstration of MaSP1 motif

1.2 Host Cells for Spider Silk Protein production

Since spiders are cannibalistic, it is not feasible to obtain desired amounts of spider silk directly from spiders [33]. In addition, 1.2 million spiders are required to knit just one dress [34]. Therefore, a recombinant expression system is required for the industrial production of high-efficiency and cost-effective spider silk. In this technology, the gene encoding the spider silk protein was expressed in bacteria, yeast, insect, plant and mammalian cells as well as in some other platforms. The most commonly used host cell for recombinant spider silk protein is *E. coli* organism [35]. However, a 65-kDa spider silk protein was expressed in *Pichia pastoris* yeast cell under the control of the AOX1 promoter [36]. In addition, a 94 kDa spider silk protein was obtained in *Saccharomyces cerevisiae* yeast cell [37]. However, since plant expression systems are more genetically stable than microorganisms, they have been used in spider silk protein production. In addition, recombinant proteins produced in plant cells are more stable and easier to purify. However, it is problematic due to low expression yields [38-40].

N. clavipes dragline fiber has been expressed in transgenic tobacco, mouse ear cress, clover and potato plants, and dragline protein at different molecular weights has been detected in the leaves and seeds of the plants [41-45]. On the other hand, spider silk proteins have also been expressed in genetically modified insects [46-48]. Silkworm, which is a silk producer, stands out in these cells. The most important advantage of the expression system in silkworm compared to other host cells is that the produced proteins can be obtained as fibers. Proteins obtained in other host cells must be spin-processed to obtain fiber [33]. However, since the fibers obtained are a small amount of the total fiber, they showed lower mechanical properties than natural dragline MaSP1. Another host cell type used for recombinant spider silk protein expression is mammalian cells. Expression of eukaryotic genes such as spider silk protein occurs more efficiently in mammalian cells. In addition, since mammalian cells can secrete proteins out of the cell, the purification process is also facilitated [49]. MaSP1 and MaSP2 proteins were expressed at different sizes in infant hamster kidney and bovine, goat and mouse mammary epithelial alveolar cells. However, it was observed that the production of spider silk proteins secreted out of the cell decreased significantly as their size increased [50-51]. In

another study, it was stated that the production and secretion of spider silk proteins in the mammary glands of goats is feasible. *N. clavipes* 65-kDa weight MaSP1 was obtained from the milk of goats. However, although there is no statement about the amount of production in the study, the product obtained could not keep up with the mechanical properties of natural dragline fiber [52].

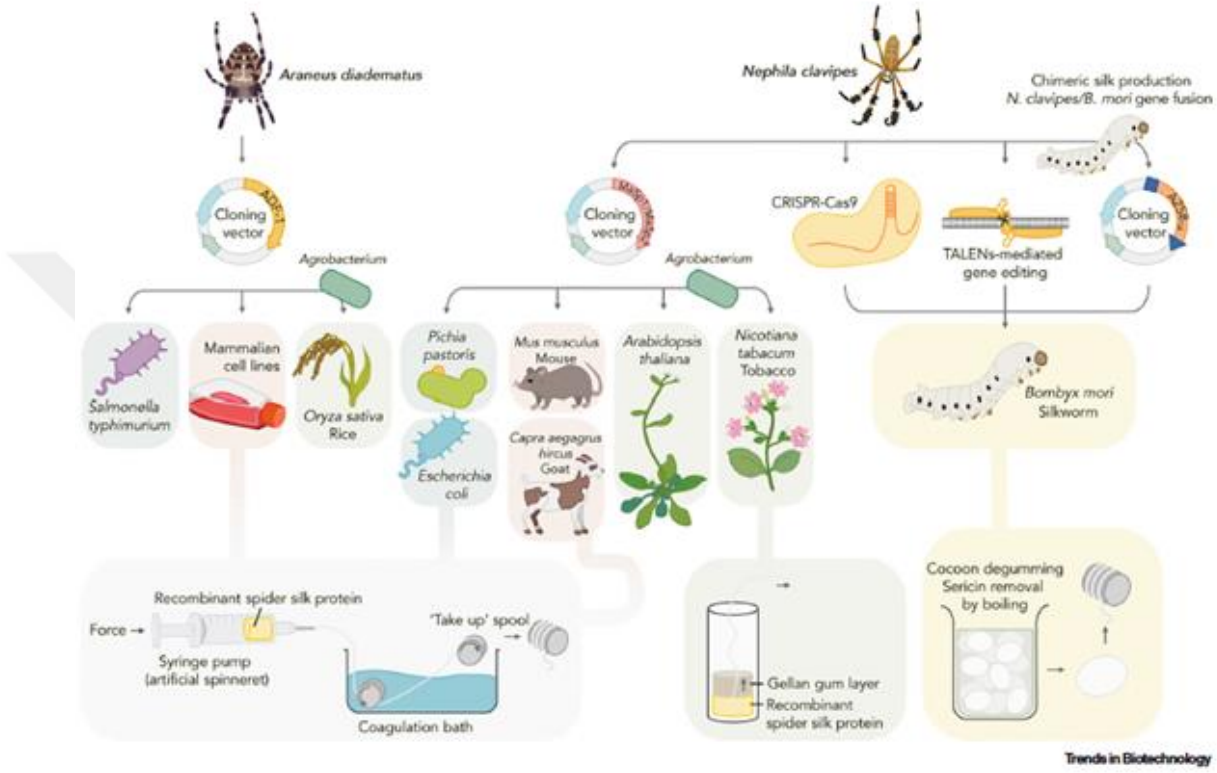

Figure 1.3 Spider silk production in different expression systems [24]

1.2.1 *Escherichia coli* (*E. coli*)

E. coli is a gram-negative bacterial strain most used in the producing of recombinant spider silk protein [35]. *E. coli* cells are one of the most preferred species in heterologous protein expression systems. Some of the reasons for this are that it can be genetically modified easily, reproduce rapidly, survive, and reproduce in relatively low-cost environments, and can be easily scaled industrially [53]. In addition, since it is one of the most frequently used microorganisms in synthetic biology, its gene structure and metabolic activities are among the best-known microorganisms. This has enabled it to be used as a host cell in recombinant spider silk protein studies, from the earliest studies to the most recent ones.

In the early stages of spider silk protein production in *E. coli* host cells, low production and unstable proteins were obtained. Transcriptional problems and deletion of some nucleotides have been reported as the reason for this [36]. Spider silk proteins are composed of repeating motifs and these motifs contain large amounts of alanine and glycine amino acids. It is clear that spider silk protein could not be produced in desired amounts because alanine and glycine tRNA pools were depleted during expression [24]. However, when the silk-producing glands of spiders were examined, it was understood that alanine and glycine tRNA genes were upregulated before translation [54]. Since this alanine and glycine upregulation is not present in *E. coli* cells, spider silk expression could not occur in sufficient amounts. Adding essential amino acids as glycine, alanine and proline to the medium did not increase spider silk protein production either [36].

When the amount of glycine tRNA was increased *in E. coli* cells by metabolic engineering, approximately 285 kDa spider silk protein production was achieved, comparable to its natural state, and these proteins were turned into fibers by spinning. It has been suggested that the mechanical traits of these silks are similar to those of dragline fibers. [21]. In a subsequent study, a protein with a total molecular weight of 556 kDa was obtained by ligating the N- and C- regions of spider silk proteins with a size of approximately 278 kDa, which were produced separately by metabolically engineered *E. coli* cells by overexpressing glycine tRNA. This protein had 192 repeating regions and showed the same mechanical properties as native dragline fiber [26].

In this study, *E. coli* was chosen as the host cell. Because the MaSP1 spider silk protein to be produced recombinantly in the *E. coli* cell can provide the mechanical features of the natural dragline fiber. In addition, systems designed using *E. coli* cells can be easily scaled up for high volumes and thus spider silk protein can manufacture industrially in this way. The necessary metabolic engineering can be done easily, as it allows for easy genetic modification. The *E. coli* cell can multiply rapidly and thus allows for a rapid production. High production efficiency can be achieved with its growth in a cheap and easily accessible environment. On the other hand, there are some disadvantages of *E. coli* cell. The first of these is the inability to perform post-translation processes, which are effective in gaining the three-dimensional structure of proteins. In addition, acetate produced during the process has a toxic effect on cells. *E. coli* does not secrete the proteins it produces out of the cell like some other cells, making protein purification processes difficult. However, *E. coli* was preferred as the host cell because its advantages

outweigh its disadvantages. *E. coli* is a type of gram-negative bacteria and not a mold cell. In addition, after the protein is produced and the cell membrane is destroyed, the protein produced will be purified and no living microorganism will remain in the product. Therefore, the product will not have negative effects such as corrosion.

Chapter 2

Material And Methods

2.1 Material

2.1.1 Plasmid and Strain

The pGSI plasmid was modified by adding target restriction sites which are KpnI, NheI, SpeI, BspEI, EcorRI, EcoRV. This plasmid was ordered from FICUS Biotechnology company. The target fragment Masp1 inserted into this plasmid. The pBbB6c plasmid was ordered from AddGene and sent to Gene Script for modifying it by silencing 2 restriction sites on plasmid. *E.coli* NEB 10-beta(C3020K) which is chemically competent cell was ordered from New England Biolabs. All plasmids and their steps were shown in figures below.

Table 2.1 Amino acid sequence of MaSP1 1-mer.

MaSp1 1-mer	SGRGGLGGQGAGMAAAAAMGGAGQGGYGGLGSQGT

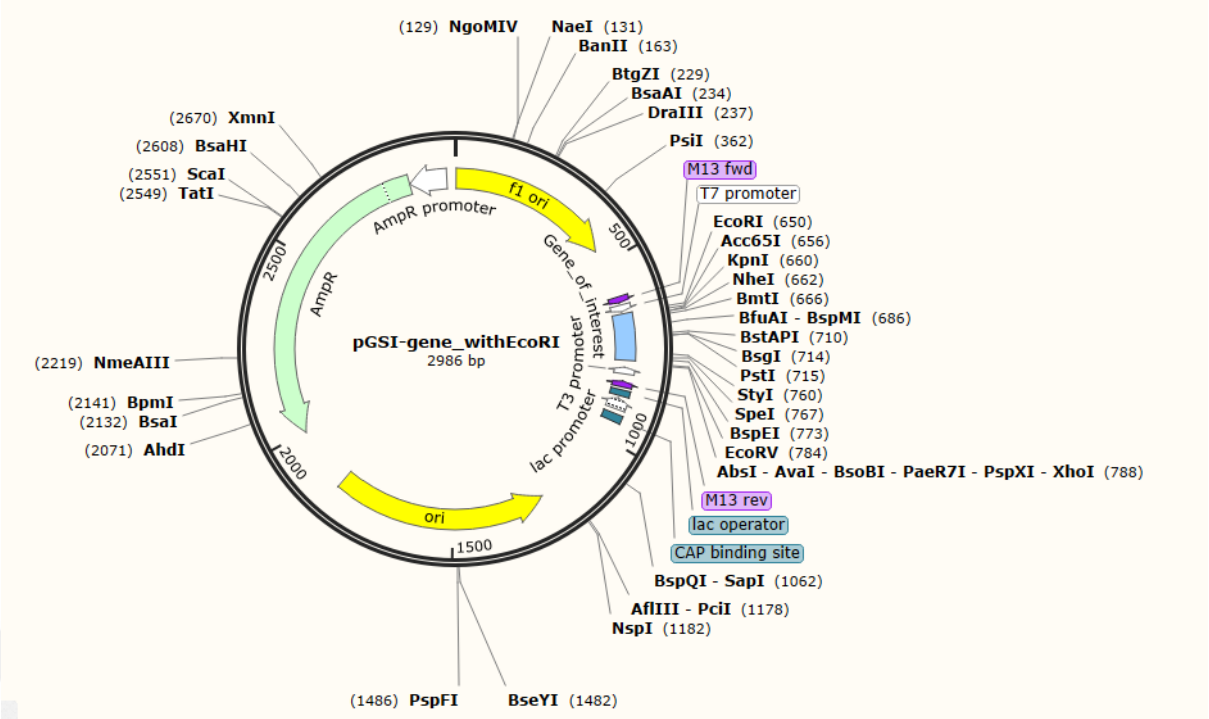

Figure 2.1 The first pGSI plasmid backbone for cloning of Masp1

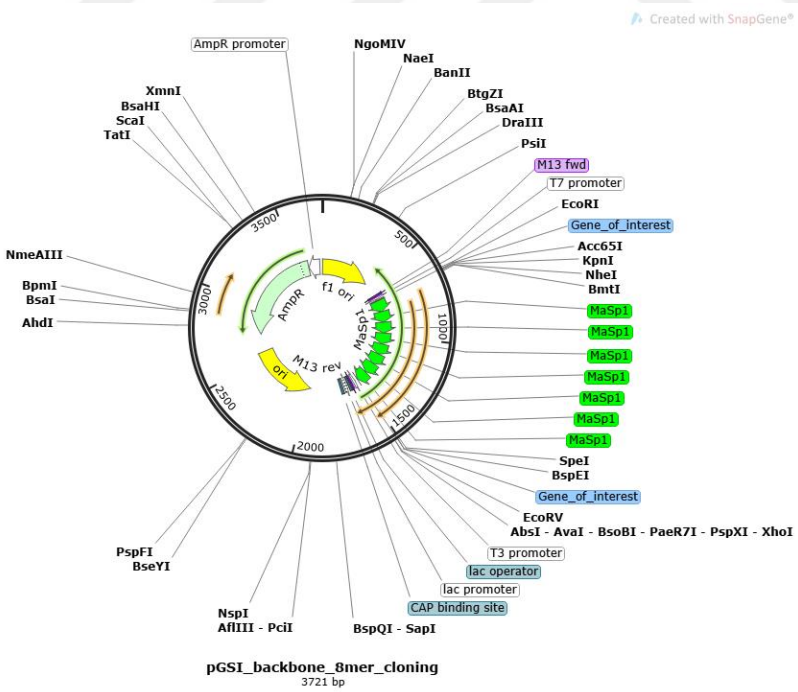

Figure 2.2 Snap gene image of pGSI 8-mer plasmid

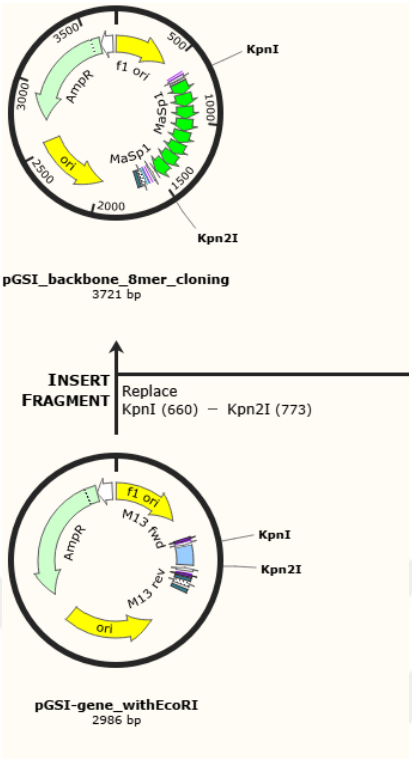

Figure 2.3 Schematic representation of 8-mer fragment cloning into pGSI backbone

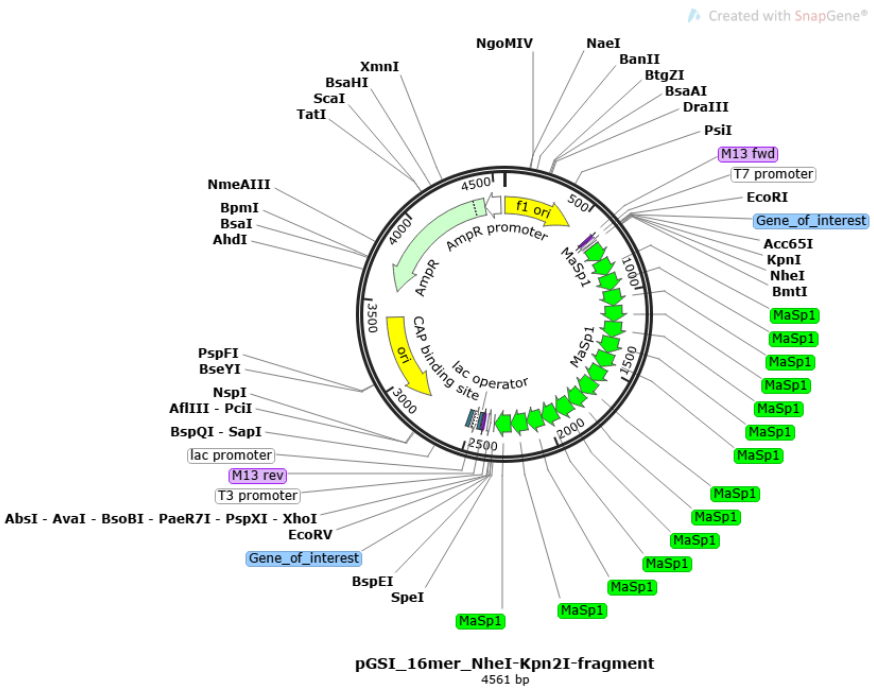

Figure 2.3 Snap gene image of pGSI 16-mer plasmid

Figure 2.4 Schematic representation of where the plasmid was restricted and linearized for ligation process. Image was extracted from Snap Gene (www.snapgene.com)

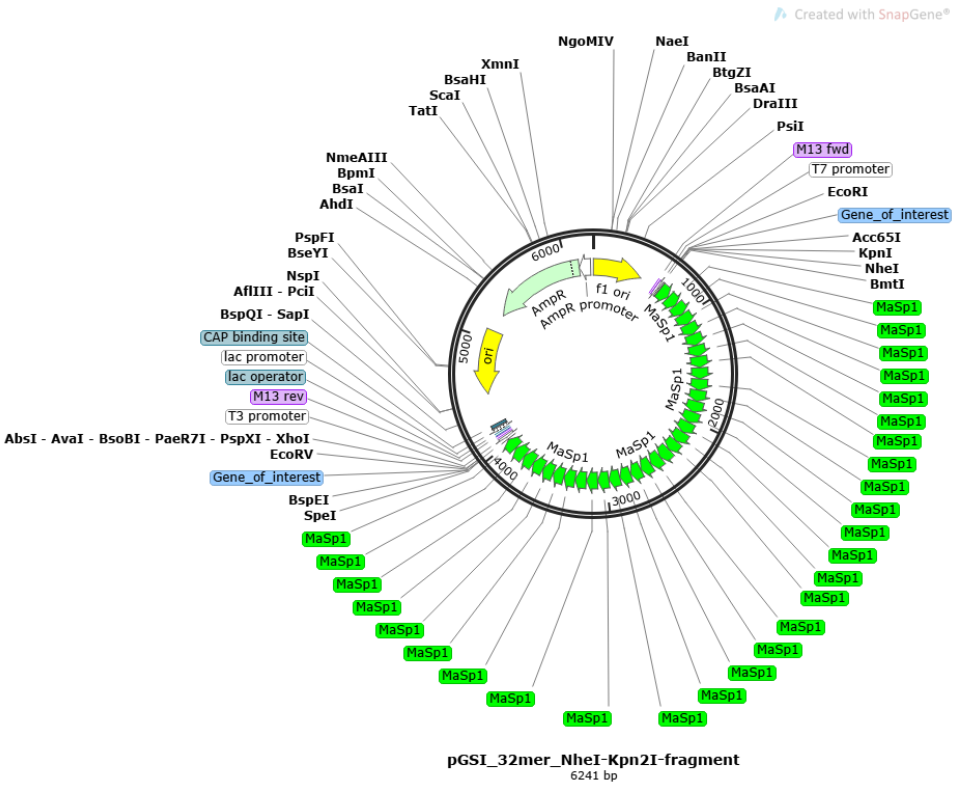

Figure 2.4 Snap gene image of pGSI 32-mer plasmid

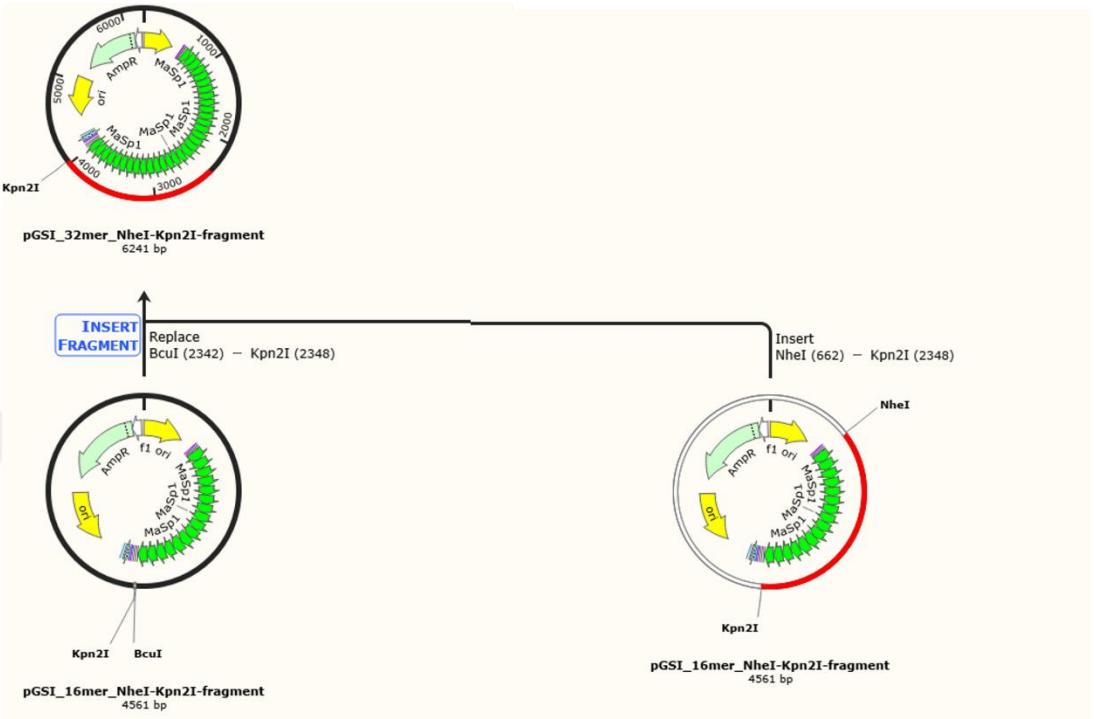

Figure 2.5 Demonstration of method for conducting pGSI 32-mer plasmid

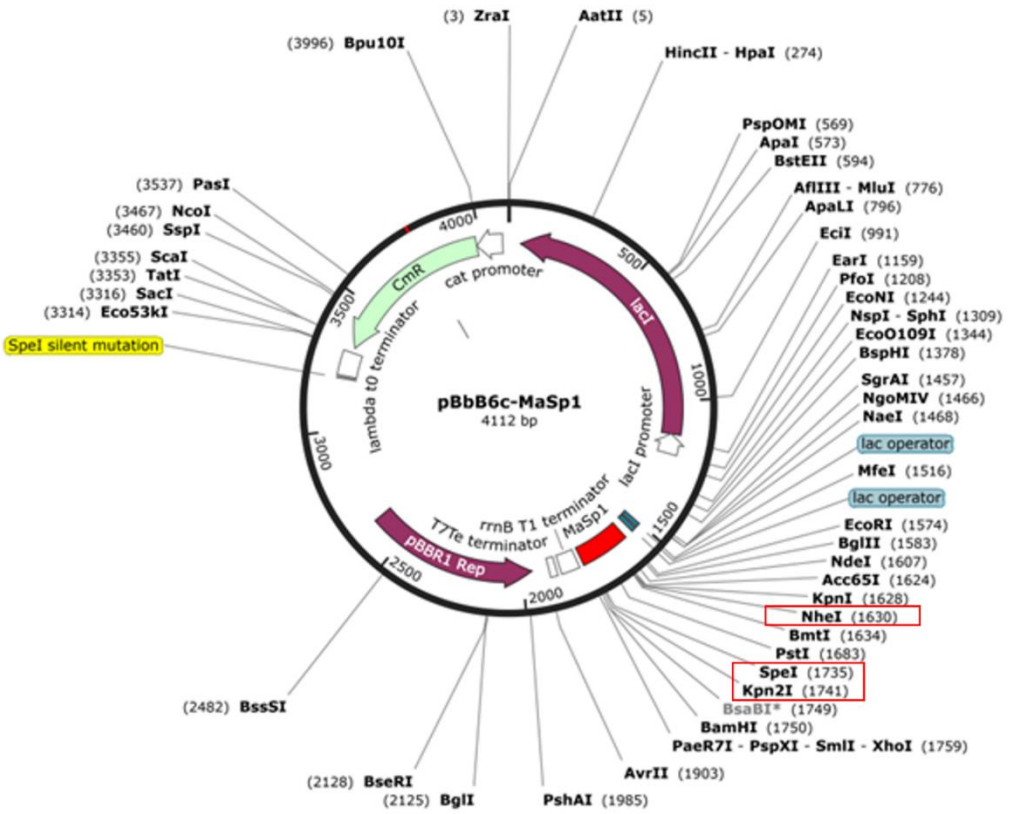

Figure 2.5 Snap gene image of pBbB6c 1-mer plasmid

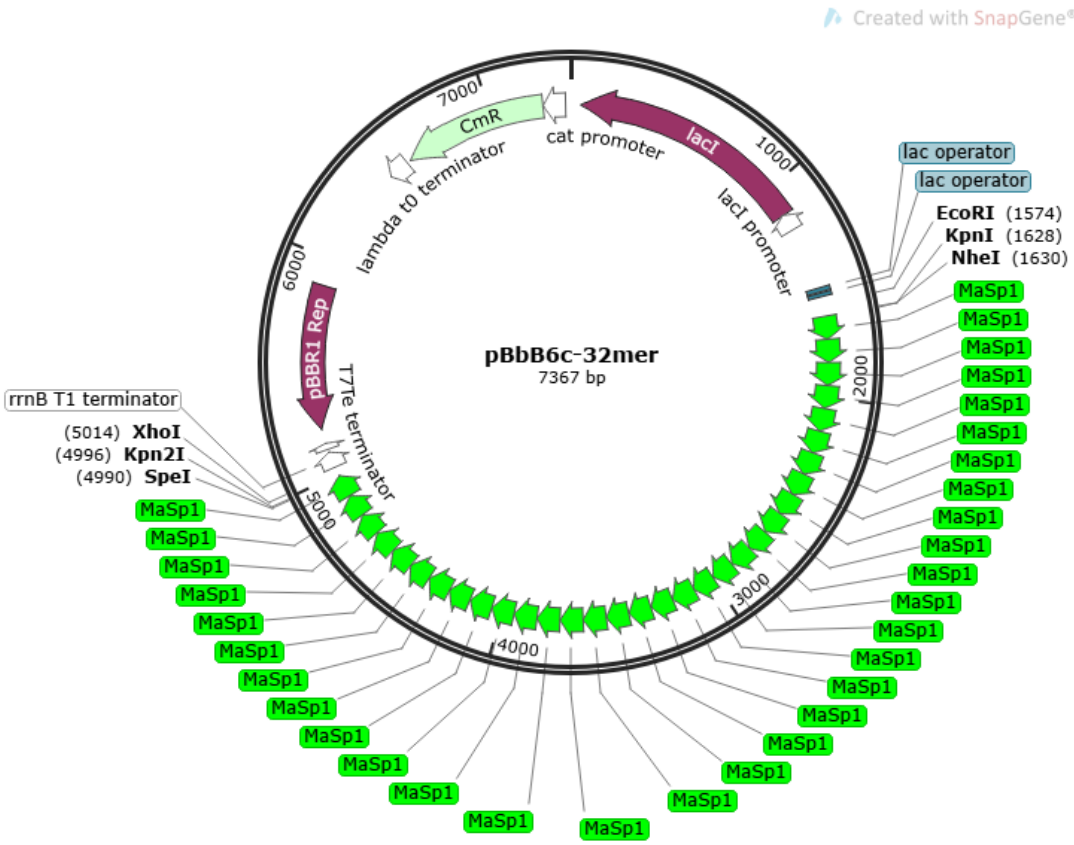

Figure 2.6 Snap gene image of pBbB6c 32-mer plasmid

2.1.2 Restriction Enzyme

KpnI,Kpn2I,SpeI,NheI,EcoRI, enzymes were tracked as Slow digest and Fast Digest from Scientific Thermo Fisher. Also, KpnI, SpeI, NheI were ordered from Fly Cut as fast digest enzymes.

2.1.3 Purification Kits

Purification kits were ordered from Thermo Scientific. Thermo Scientific™ GeneJET, Gel Extraction Kit (K0691) and Thermo Scientific™GeneJET Plasmid Miniprep Kit(K0502). The Hipure Plasmid Mini Prep Kit(EM111) was tracked from Transgen.

2.2 Methods

2.2.1 Preparation of Media and Medium

For preparing 1 liter of terrific agar and broth, tryptone, yeast extract, glycerol, agar, potassium dihydrogen phosphate, dipotassium hydrogen orthophosphate were used.

For phosphate buffers, the ingredients were mixed with 100 mL distilled water and autoclaved. Other ingredients were mixed in a 1 litre bottle by help of a magnetic fish and 900 mL water was added and autoclaved. Then when the temperature of media came to 45-50 °C degrees, antibiotics and phosphate buffer could be mixed.

Table 2.1 Composition of terrific agar and terrific broth

Terrific Agar		**Terrific Broth**	
Tryptone	12 g	Tryptone	12 g
Yeast Extract	24 g	Yeast Extract	24 g
Glycerol	5 mL	Glycerol	5 mL
Agar	15 g		
Potassium Phosphate Buffer			

potassium dihydrogen phosphate	2.3 g	potassium dihydrogen phosphate	2.3 g
dipotassium hydrogen orthophosphate	12.5 g	dipotassium hydrogen orthophosphate	12.5 g

2.2.2 Cloning Vector Design

pGSI cloning vector was ordered from Ficus Biotechnology by adding our target restriction sites into plasmid.

2.2.3 Cloning Masp1 into pGSI High Copy Plasmid

2.2.3.1 Restriction

Restrictions were applied by KpnI2 and NheI enzymes to get fragment from the plasmid. The DNA amount was calculated according to its concentration and mixed with water and buffer. NheI enzymes also added into eppendorf and spin down for 5 seconds. Then tube was wrapped by parafilm and incubated for 4 hours in water bath which was 37 °C degree. After that Fast Digest Kpn2I enzyme was added and mixed and continued incubation for 15 minutes more at 37 °C degree. Finally, it was placed on heat block to inactivate the enzyme for 20 minutes at 80 °C degree.

Table 2.2 Amount of material used in restriction of plasmids

	Cohesive Ends
10 X Tango Buffer	2 µl
Kpn2I	2 µl
NheI	1 µl
Plasmid DNA	Up to 1 ng
Autoclaved Ultra-Pure Water	Up to 20 µl

2.2.3.2 Ligation

Concentration of plasmid was measured by nanodrop, and according to the result, their fmol values were calculated. The ligation mixture was prepared in an Eppendorf tube as shown in table 2.3. This ligation product was incubated in 23-26 °C degree for 1 hour. The calculations were done according to protocol of Invitrogen (051502).

Table 2.3 Materials and amounts used in ligation protocol

Materials	Cohesive Ends
Ligase Reaction Buffer (5X)	4 µl
T4 DNA Ligase	0.5 µl
Vector DNA	3-30 fmol
Insert DNA	9-90 fmol
Autoclaved Ultra-Pure Water	Up to 20 µl

2.2.3.3 Transformation

A vial of NEB 10-beta competent cell was taken from -80 C freezer and was thawed on the ice for 10 minutes. The plasmid also was taken and after it was thawed, it was resuspended with 20 µl autoclaved ultra-pure water.1 minute centrifuge was applied to resuspend effectively.

2.2.3.4 Heat-Shock Transformation

40 µl E. coli cells were mixed with 100 ng plasmid in a 15 ml falcon tube. They were mixed gently by pipetting and by tapping to the tube and incubated in ice box 30 minutes. Later, the mixture put on the water bath which at 42 °C degrees, for 30 seconds. Later they were incubated on ice again for 5 minutes.

2.2.3.5 Transformant Propagation

960 µl stable outgrowth medium was mixed with transformant cells and incubated in shaking incubator,37 °C degrees at 250 rpm for 1 hour. Then the 5-fold serial dilution performed as 15 µl cells with 135 µl cell-free media. 100 µl was taken from each dilution and spreading to the selective media which is terrific agar with ampicillin was prewarmed at 37 °C degrees. As a negative control group,10 µl NEB 10-beta competent cell was spread into plate. The culture was incubated one night at 37 °C degrees. Single colonies were chosen and cultured into 5 ml terrific broth which had ampicillin.

2.2.3.6 Plasmid Purification

The bacteria cells were harvested by ultra-centrifugation for 2 minutes at 6800 g(8000 rpm). The supernatant was discharged. 10 ml culture can be used for low copy plasmid, while 1-5 ml can be enough for high copy plasmids. Cell pellets were resuspended with resuspension solution. Pipetting and vortex were applied until there is no pellet in bottom. Lysis solution was used to destroy cell walls. The eppendorf was mixed by flipping 5-6 times until the mixture became clear and viscous. Neutralization solution was mixed immediately until cloudy structure became. Then whole mixture was centrifuges for 5 minutes and supernatant was transferred into spin column.1 minute centrifuge was conducted to remove unwanted molecules. The flow-through was removed and column was put into same tube. Wash solution was added and 1minute centrifuge was repeated. Flow-through was discarded and was placed into same tube and this step was repeated. Additional 1 minute centrifuge was performed to remove any residual ethanol in column. The spin column was transferred into new 1.5 mL tube and elution buffer was added into the centre of column and was incubated for 2minutes in room temperature. The last centrifuge was done for 2 minutes to elute plasmid DNA from membrane to the buffer. The purified plasmid DNA was stored at -20 °C degrees.

Table 2.4 Solutions and volume in plasmid purification

Solutions	Amounts
Resuspension Solution	250 µL
Lysis Solution	250 µL
Neutralization Solution	350 µL
Wash Solution	500 µL
Wash Solution	500 µL
Elution Buffer	20-50 µL

2.2.3.7 Linearization of Plasmid

Plasmid, nuclease free water and fast digest buffer were mixed in a tube on an ice box. Then enzymes were added and mixed by pipetting. The tube was spined down and wrapped by parafilm. It was incubated at 37 °C degree water bath for 15 minutes and put

on 80 °C degree heat block for inactivation of enzymes. Finally, they were stored at -20 °C degree.

Table 2.5 Contents of linearization process and amounts

	Amounts
Fast Digest SpeI Enzyme	1 µl
Fast Digest Kpn2I Enzyme	2 µl
10X Fast Digest Buffer	2 µl
Plasmid DNA	Up to 1000 µg
Nuclease Free Water	Up to 20 µl

2.2.3.8 Gel Electrophoresis

Different gels were prepared at different concentration. Mostly 1.0 % TAE agarose gel was used in electrophoresis. Agarose and 1X TAE buffer were mixed and boiled for 1 minute in microwave. They were waited until it cooled, and ethidium bromide was added and mixed gently. The gel mix was poured into gel tank and the comb was placed on it carefully. The gel dried in 10 minutes. Then samples were loaded to gel wells as 7 µL with 2 µL loading buffer or 10 µL sample with 3 µL loading buffer.

Table 2.6 Ingredients and amounts in gel preparation step

	Amounts
1X TAE Buffer	60 mL
Agarose	0.6 g
Ethidium bromide	2.4 µL

2.2.3.9 Gel Extraction

Gel was imaged under UV light and the target piece was cut by a sterile surgical blade and put into an eppendorf tube which was weighted before. After gel transfer, weight of tube measured again. The binding buffer amount was decided according to

weight of gel size and added 1:1 ratio. Then the gel mixture incubated at 50-60 °C degrees for 10 minutes till all gel was dissolved in buffer. After vortex and spinning the column, mixture was loaded into purification column. The centrifuge was applied for 1 minute at 12000×g and flow-through was discarded. As an additional step, binding buffer was added into column and centrifuged 1 minute more. Then wash buffer was added and centrifuged 1 minutes. Flow-through was removed and one more centrifuge was done to remove residual wash buffer in column. Finally, elution buffer was put as one droplet into centre of the column, and it was centrifuged 1 minute the elute the DNA in column. The DNA that had been purified was kept at -20°C degrees.

Table 2.7 Buffers and their volume used in gel extraction

	Amounts
Binding Buffer(1:1 volume)	Gel piece weight: buffer
Binding Buffer	100 µL
Wash Buffer	700 µL
Elution Buffer	20-50 µL

2.2.4 Designing Expression Plasmid pBbB6c

Amplification of cells containing plasmid pBbB6c-GFP ordered from Addgene, extraction and purification of the corresponding plasmid was performed in our laboratory. The propagated pBbB6c-GFP plasmid was multiplied up to the mass desired by GenScript, 5 µg, and lyophilized was carried out and sent to the GenScript Singapore laboratory. In parallel, the in-silico design of the pBbB6c-MaSp1 plasmid, in which the 32-mer MaSp1 gene will be cloned, was created using SnapGene software and transferred to GenScript. GenScript has gene silenced Kpn2 and SpeI regions with point mutation.

2.2.4.1 Electrocompetent Cell Preparation

Firstly, SOB medium was prepared. One colony was incubated into 10 mL SOB medium and incubated at 250 rpm, 30 °C degrees for 16 hours. Two drops from this medium were incubated into 250 SOB medium which was pre-warmed and shaked at 250 till OD600 was reached to 0.5-0.7.Then the culture was put down on ice for 15 minutes

and poured into centrifuge bottles. Culture was centrifuged at 4 °C degree,5000 rpm for 15 minutes. The supernatant was poured off and residual broth was aspirated. 250 mL cold glycerol added into bottle and pellets were suspended by pipetting up and down. This step was repeated 2 more times. Finally, cells were suspended with residual glycerol by pipetting up and down. 100 µL of culture was transferred to microcentrifuge tube and they were stored at -80 °C degrees.

Table 2.8 Composition and amounts in electrocompetent cell preparation

	Amounts
SOB Medium(First seed)	10 mL
SOB Medium(Second seed)	250 mL
Glycerol (First wash)	250 mL
Glycerol (Second wash)	250 mL
Glycerol (Third wash)	250 mL
Suspended Cell	100 µL

2.2.4.2 Cloning 32-mer Masp1 into pBbB6c

32-mer fragment was purified according to purification method. Then ligated into pBbB6c plasmid depend on ligation protocol. The ligation product was transferred to *E.coli* by described below:

2.2.4.3 Electroporation

Transformation was applied as electroporation method to increase the yield f transformant number. The electroporator was set as 1.8 kv,200 ohms, 25 µF. SOC medium was prepared as adding 20 mM glucose to the SOB medium. SOC medium and terrific agar plates with chloramphenicol were pre-warmed at 37 °C degrees for providing optimum growth condition for cells while 1mm-electroporation cuvette was placed on ice. The electrocompetent cells were thawing on ice for 10 minutes and 25 µL electrocompetent cells and 1 µL DNA solution were mixed in a centrifuge tube and transferred to cuvette and incubated on ice for 10 minutes. Later the cuvette was placed on electroporation module and pulse was pressed. SOC media was added into cuvette immediately and mixed by pipetting up and down, the mixture was put into 15 ml falcon tube and incubated on 37 °C degrees for 1 hour at 250 rpm. 3 serial dilutions were

performed to observed single colonies and 100 µL culture was spread to terrific agar. They were growth overnight at 37 °C degrees.

2.2.4.4 Propagation of Transformants

The plates were checked, and single and clean colonies were chosen and cultivated to 5 ml terrific broth with chloramphenicol (33 ng/uL).They growth at 250 rpm at 37 °C degrees for overnight.

2.2.4.5 Plasmid Purification

The amounts that used was decided according to terrific broth media. The overnight culture was centrifuges at 10,000x g for 1 minutes. The supernatant was removed, and appropriate volume of resuspension buffer(mixed with RNase) was added. Pellet was resuspended by vortex and pipetting up and down. Then lysis buffer was added and inverted 4-6 times quickly till colour became bright blue. Neutralization buffer was added and was mixed by inverting 6 times immediately, so the colour became yellowish and cloudy. It was incubated for 3 minutes in room temperature and centrifuged at 12,000 g for 5 minutes. The supernatant was transferred to spin column gently and centrifuged for minute. The flow-through was discarded , toxin out buffer was added and incubated at room temperature for 10 minutes. Then it was centrifuged, and wash buffer (ethanol added) added and centrifuged for 1 minute. Flow through removed and empty column was centrifuged to remove residual wash buffer. Finally, 30-50 µL elution buffer was added into the centre of membrane to elute DNA in membrane and stored at -20 °C degrees. Also, plasmid concentrations were measured by nanodrop machine.

Table 2.9 Volume guideline of plasmid purification kit

Culture Media	Resuspension Buffer	Lysis Buffer	Neutralization Buffer	Wash Buffer	Elution Buffer
≤5 mL	250 µL	250 µL	350 µL	650 µL	30-50 µL
5-10 mL	500 µL	500 µL	700 µL	650 µL	30-50 µL
10-15 mL	750 µL	750 µL	1050 µL	650 µL	30-50 µL
15-20 mL	1000 µL	1000 µL	1400 µL	650 µL	30-50 µL

2.2.4.6 Linearization

For linearization of plasmid was important to check and see DNA size. Flycut SpeI and KpnI enzymes were used. The amounts were calculated according to plasmid concentration and mixture was prepared. Mixture was incubated in 37 degrees water bath for 15 minutes and inactivated in heat block at 80 °C degrees for 20 minutes. It was stored at -20 degrees for uploading to the gel.

Table 2.10 Substances and their amount used in linearization of plasmid

	Amount
DNA	1-2 µg
10 X Flycut Buffer	5 µL
Flycut SpeI	1 µL
Flycut KpnI	1 µL
Autoclaved Ultrapure Water	Variable(up to 50 µL)

2.2.4.7 Gel Electroporation

0.7 % TAE agarose gel was used in electrophoresis. Agarose and 1X TAE buffer were mixed and boiled for 1 minute in microwave. They were waited until it cooled, and ethidium bromide was added, mixed gently. The gel mix was poured into gel tank and the comb was placed on it carefully. After gel dried, samples were loaded to gel wells as 7 µL with 2 µL loading buffer. 400 A, 65V was applied for 60 or 90 minutes. Then gel was imaged under UV light.

Table 2.11 Material amount used during gel preparation

	Amounts
1X TAE Buffer	60 mL
Agarose	0.42 g
Ethidium bromide	2.4 µL

Chapter 3

Results And Discussion

3.1 Cloning of Masp1 gene to pGSI plasmid

3.1.1 8-mer Masp1 containing plasmid

8-mer Masp1 gene was synthesized by GenScript Inc. (Singapore). pGSI backbone was linearized by KpnI and Kpn2I enzymes and imaged in gel electrophoresis. The pGSI backbone purified from the gel and ligated with 8-mer fragment. The ligation product was transferred to the *E.coli* Neb 10-beta chemically competent cells by heat shock method. The positive and single colonies were chosen, and plasmid purification was applied. The concentration of pGSI 8-mer plasmid was measured by nanodrop, as shown in Table 3.1. DNA concentration was 329 ± 26.15 ng/µl which was a good concentration. The DNA qualities were checked by A260/280 ratio which yielded ~ 1.9 indicating high quality DNA isolation.

Table 3.1 DNA concentration of pGSI 8-mer in nanodrop

	DNA Concentration	**A260**	**A280**	**A260/280**
pGSI 8-mer 1	355.4 ng/µl	7.107	3.715	1.91
pGSI 8-mer 2	303.7 ng/µl	6.075	3.195	1.90

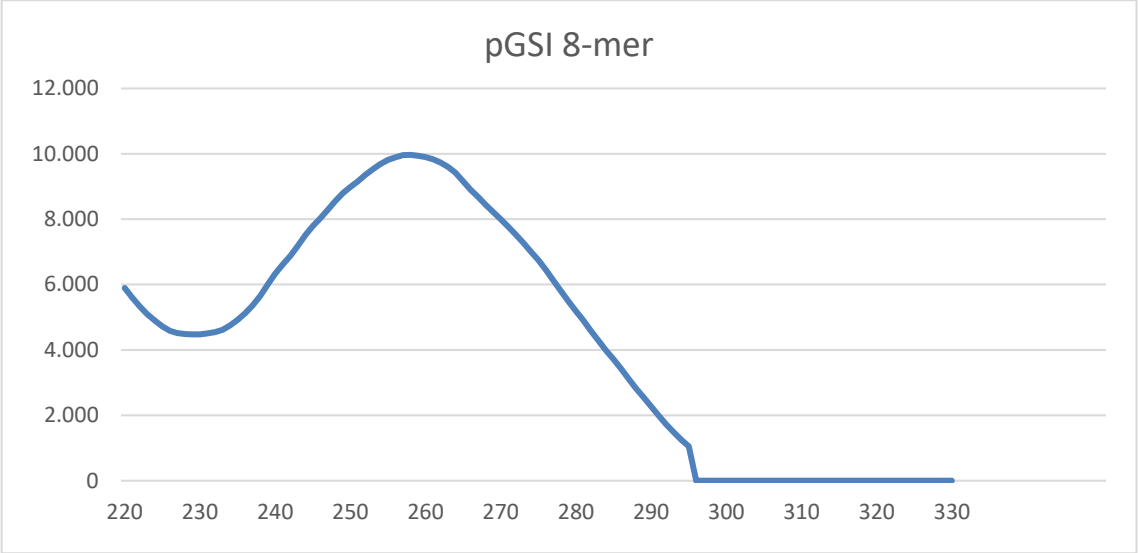

Figure 3.1 Graph of pGSI 8-mer DNA concentration

3.1.2 16-mer Masp1 Containing Plasmid

pGSI 8-mer was cut with NheI and Kpn2I restriction enzymes to get 8-mer fragment in the gel and pGSI 8-mer was linearized with SpeI and Kpn2I. These samples were loaded into 1% agarose gel and run for 1.5 hour at 65 V followed by visualizing under UV gel imaging system (Bio-Rad)(Figure 3.2). The approximate size of 8-mer Masp1 fragment was appeared to be in between 0.8 kb and 1 kb. This is in alignment with the expected size of 8-mer Masp1 as 1-mer Masp1 is at 105 bp (105*8=840 bp).When we looked at the pGSI plasmid containing 8-mer Masp1 in the gel image, it appeared that the band resides in between 3 kb and 5 kb; although closer to 3 kb reference size. Therefore, we confirmed the accurate fragment and backbone according to the sizes achieved in gel electrophoresis images and we moved to the next step of gel extraction. The target DNA fragments of 8-mer Masp1 and pGSI plasmid backbone containing 8-mer Masp1 were manually cut. The extracted gels were purified using Gene Jet Gel Purification Kit after which DNA concentrations of fragments and fragment+ plasmid were quality checked in nanodrop. The 8-mer Masp1 fragment was ligated into 8-mer Masp1 containing pGSI plasmid using T4 DNA ligase enzyme. The ligation product was transformed into *E.coli* through heat-shock procedure (NEB) followed by incubating in terrific agar including 100 µg/ml ampicillin at 37 Cº, overnight. Positive *E.coli* cells (i.e., the cells that harbor 16-mer Masp1 containing pGSI) that formed colonies were randomly picked and propagated in TB that had 100 µg/ml ampicillin. The positive *E.coli* cells that were

propagated in ampicillin containing TB broth were further processed for plasmid isolation using Mini Prep Plasmid Purification Kit according to protocol described by Thermo Scientific™

The plasmid purification was also quality checked in Nanodrop (Thermo Fisher). DNA concentration and A260/280 results were shown in Table 3.2. The plasmid DNA concentrations achieved were 321.5± 33.37 ng/µl. A260/280 ratio was calculated at 1.92±0.01 implying high quality plasmid DNA isolation.

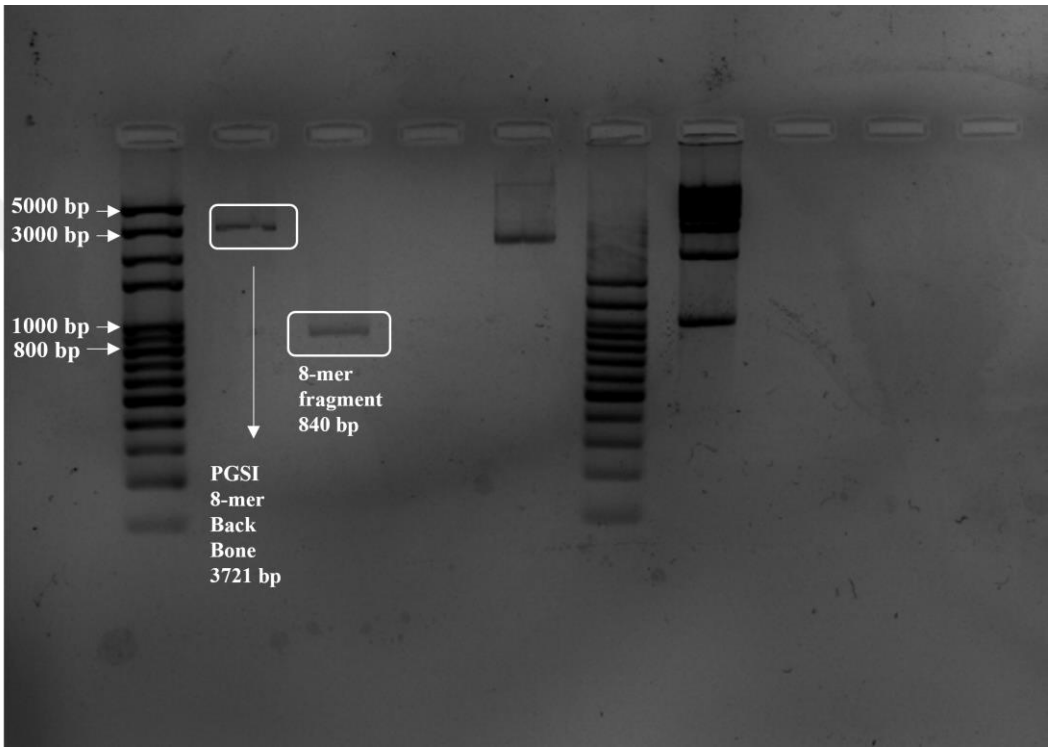

Figure 3.2 Gel image of pGSI 8-mer backbone and 8-mer fragment with 100 bp+II ladder

Table 3.2 DNA concentration of pGSI 16-mer in nanodrop

	DNA Concentration	**A260**	**A280**	**A260/280**
pGSI 16-mer 1	345.4 ng/µl	6.908	3.573	1.93
pGSI 16-mer 2	298.2 ng/µl	5.964	3.127	1.91

3.1.3 32-mer Masp1 Containing pGSI

Purified pGSI 16-mer plasmids were restricted and linearized by NheI,Kpn2I and SpeI enzymes. The 16-mer fragment and pGSI 16-mer backbone were loaded into 1% agarose gel and run for 90 minutes. The gel result was as shown in Figure 3.3, the approximate size of 16-mer Masp1 fragment was appeared to be in between 1000 bp and 2000 bp. This is in alignment with the expected size of 16-mer Masp1 as 1-mer Masp1 is at 105 bp (105*16=1680 bp). When we looked at the pGSI plasmid containing 16-mer Masp1 in the gel image, it appeared that the band resides in between 3 kb and 5 kb; although closer to 5 kb reference size. Thus, we confirmed the accurate fragment and backbone according to the sizes achieved in gel electrophoresis images and we moved to the next step of gel extraction. The target DNA fragments of 16-mer Masp1 and pGSI plasmid backbone containing 16-mer Masp1 were manually cut. The 16-mer Masp1 fragment was ligated into 16-mer Masp1 containing pGSI plasmid by 1:3 ratio using T4 DNA ligase enzyme. Ligation product was transformed to the *E.coli* NEB 10-beta by heat-shock transformation. The transformant cells were cultured into terrific agar and broth which has ampicillin. Thus, we obtained *E.coli* with pGSI 32-mer plasmid. After overnight culture of this bacteria, the plasmid purification was applied, and their concentration were measured. The plasmid purification was also quality checked in nanodrop and DNA concentration and A260/280 results are shown in Table 3.3. The plasmid DNA concentrations achieved were 306.9 ±1.5 ng/µl. A260/280 ratio was calculated at 1.99± 0.005 implying high quality plasmid DNA isolation.

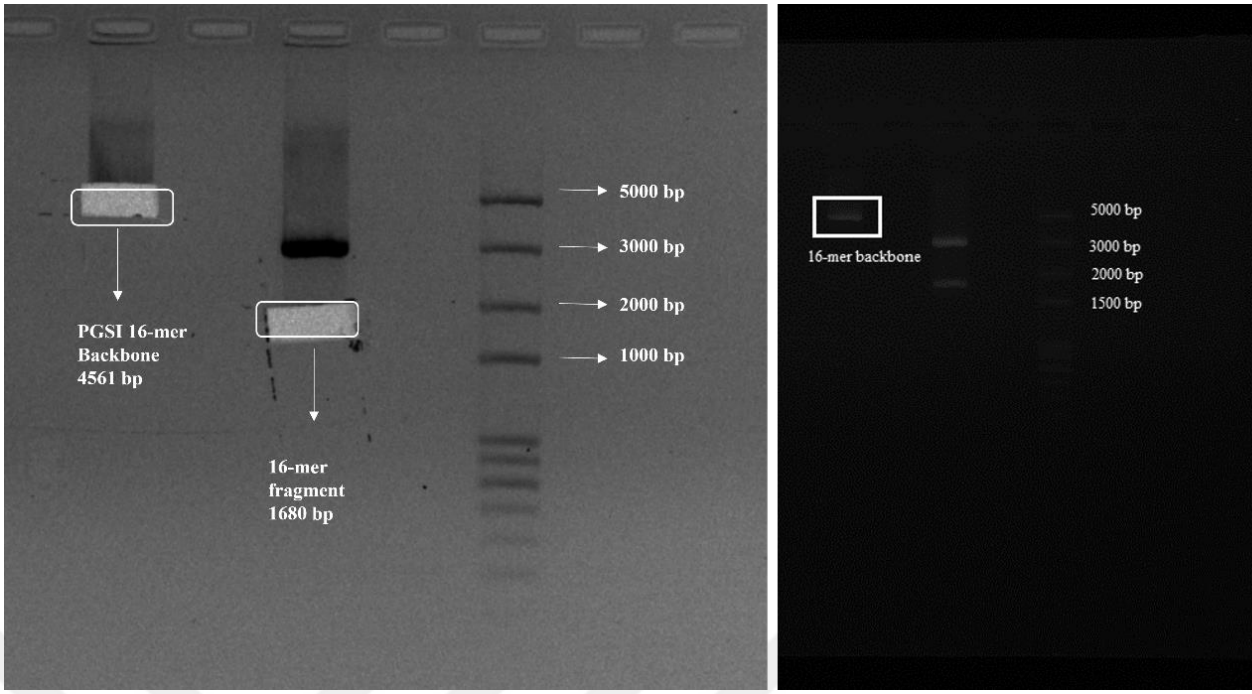

Figure 3.3 Gel image of gel extraction pGSI 16-mer backbone and 16-mer fragment with 100 bp+II ladder

Table 3.3 DNA concentration of pGSI 32-mer in nanodrop

	DNA Concentration	A260	A280	A260/280
pGSI 32-mer 1	308.4 ng/µl	6.167	3.097	1.99
pGSI 32-mer 2	305.4 ng/µl	6.107	3.047	2.00

3.2 Cloning of 32-mer Masp1 gene to pBbB6c Expression plasmid

pBbB6c-GFP plasmid purchased from AddGene (MA,USA) was sent to GenScript for replacing GFP-region with 1-mer Masp1 gene sequence. Moreover, Kpn2I and SpeI multiple cutting sites in pBbB6c were reduced to single cutting regions via silent

mutations of respective nucleotide sequences. The 1-mer Masp1 containing pBbB6c was cut from KpnI and Kpn2I restriction sites and pBbB6c backbone was obtained without Masp1 gene. Later 32-mer Masp1 extracted from pGSI cloning plasmid was ligated into pBbB6c backbone. The ligation product was transferred into electrocompetent *E.coli* NEB 10-beta cells via electroporation under following conditions of 1.8 kv,200 ohms, 25 µF. Transformant cells were spread plated on terrific agar containing 33 µg/ml chloramphenicol and incubated overnight at 37 ºC aerobically. Randomly picked single colonies were propagated in TB broth containing 33 µg/ml chloramphenicol at 225 rpm shaker incubator at 37 ºC, overnight. The pBbB6c 32-mer Masp1 plasmid isolation was performed using Mini Prep Plasmid Purification Kit according to protocol described by Thermo Scientific™. The plasmid purification were also quality checked in nanodrop and DNA concentration and A260/280 results are shown in Table 3.5. The plasmid DNA concentrations achieved were 455.5±57.23 ng/µl. A260/280 ratio was calculated at 1.89± 0.004 implying high quality plasmid DNA isolation.

Amplification of cells containing pBbB6c-GFP plasmid ordered from Addgene, extraction and purification of the relevant plasmid were performed in our laboratory. The propagated pBbB6c-GFP plasmid was amplified and lyophilized was carried out and sent to the GenScript Singapore laboratory. In parallel, the in-silico design of the pBbB6c-MaSp1 plasmid, in which the 32-mer MaSp1 gene will be cloned, was created using Snap Gene software and transferred to GenScript. Propagation of the pBbA2k-glyV plasmid was performed in our laboratory. According to the figure 3.4, pBbB6c backbone size was 4688 bp, so it was placed between 4000 bp and 5000 bp ladder bands and it was closer to 5 kb band. Their plasmid concentration and purity ratios were shown in table 3.4. pBbB6c was shown a higher concentration while pBbA2k was 134.7 ng/µl. The pBbB6c 32-mer plasmid was linearized by EcoRI enzyme to check its size in gel electrophoresis. As shown in figure 3.6, its size had to be 7367 bp, and it was seen under 8000 bp band and above 6000 bp band and closer to 8 kb.

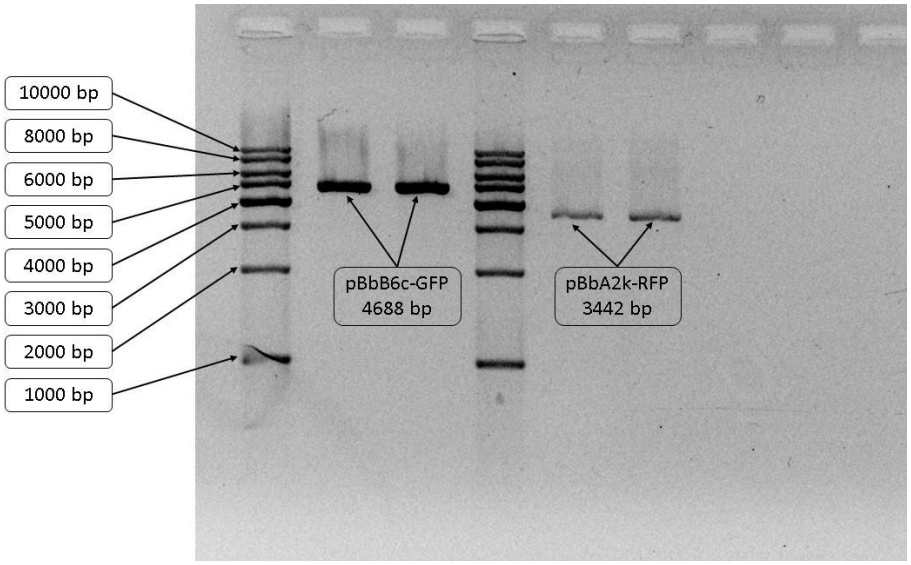

Figure 3.4 Gel image of pBbB6c backbone and pBbA2K backbone with 1 kb ladder

Table 3.4 DNA concentration of pBbB6c and pBbA2K in nanodrop

	DNA Concentration	**A260**	**A280**	**A260/280**
PBbB6C Backbone	302.5 ng/µl	6.051	3.102	1.95
PBbA2K	134.7 ng/µl	2.693	1.451	1.86

32-mer fragment was planned to clone into an expression vector which is pBbB6c. For this cloning, pGSI 32-mer was restricted by KpnI and Kpn2I enzymes and loaded into gel. 32-mer size was 3366 and it was located between 5000 bp and 3000 bp ladders and closer to the 3000 bp band as demonstrated in Figure 3.5. It was corrected our cloning. 32-mer fragment gel pieces were cut and mixed in an eppendorf to get higher yield of DNA. pBbB6c vector also cut by same enzymes and they ligated each other. Finally, we were conducted the pBbB6c 32-mer plasmid.

Ligation products were transformed to the *E.coli* NEB 10-beta electrically competent cells. They were cultured in terrific agar and broth with chloramphenicol. Then plasmid purification was applied to be sure about the plasmid ligated and cloned correctly.

The plasmid DNA concentrations achieved were 455.5±57.23 ng/µl. A260/280 ratio was calculated at 1.89± 0.004 implying high quality plasmid DNA isolation. Their graph and result showed us, we got a pure plasmid. The pBbB6c 32-mer plasmid was linearized by EcoRI enzyme to check its size in gel electrophoresis. As shown in figure 3.6, its size had to be 7367 bp, and it was seen under 8000 bp band and above 6000 bp band and closer to 8 kb.

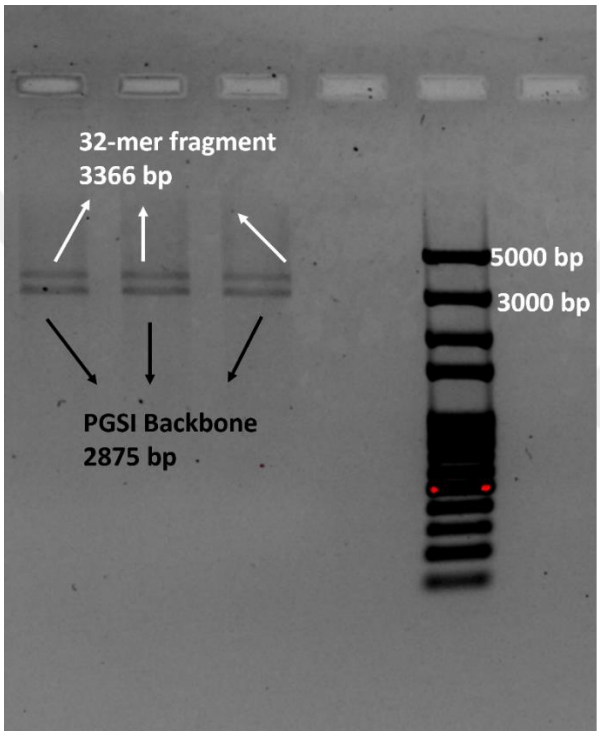

Figure 3.5 Gel image of pGSI backbone and 32-mer fragment with 100 bp+II ladder

Table 3.5 DNA concentration of pBbB6c 32-mer in nanodrop

	DNA Concentration	**A260**	**A280**	**A260/280**
PbB6c 32-mer 1	535.6 ng/µl	10.712	5.675	1.89
PbB6c 32-mer 2	426.0 ng/µl	8.520	4.490	1.90
PbB6c 32-mer 3	405.1 ng/µl	8.102	4.296	1.89

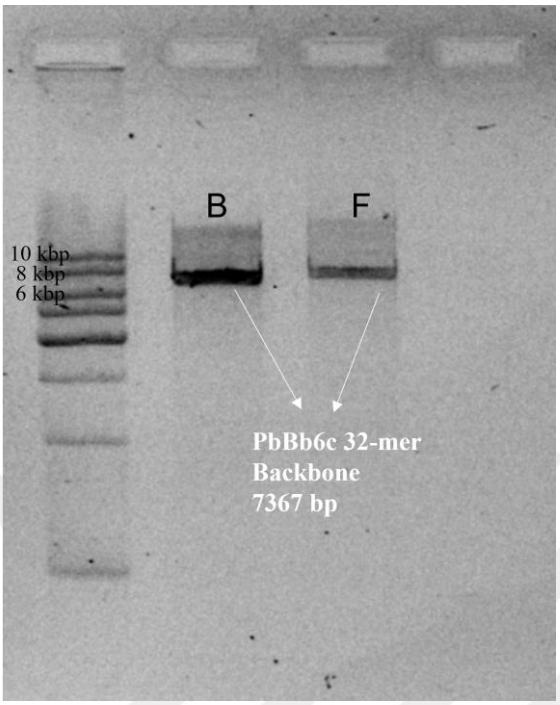

Figure 3.6 Gel image of pBbB6c 32- mer backbone with 1 kb ladder

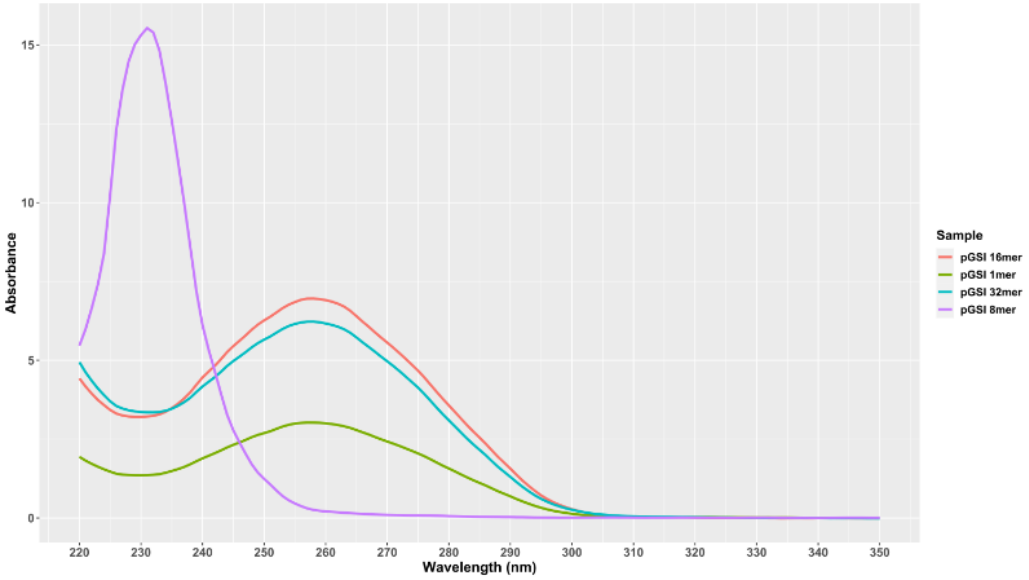

Figure 3.7 The absorbance of plasmids in nanodrop.

Zhang et al.,(2018) studied recombinant spider silk. Like us, they also used pB6c plasmid in their research to clone the Masp1 gene to *E.coli*. However, we developed a different strategy from them by using the NheI and Kpn2I enzymes instead of SpeI and NheI. Compared to them, we add a new gene in front of the already consisting of gene [26]. On the other hand, we started the cloning process from 8-mer Masp1 gene whereas they started from 1-mer to construct 96-mer Masp1.

One of the oldest studies in recombinant spider silk research was done by Prince et al.,(1995), they used pUC-LINK plasmid for their cloning process. In restriction digestion, they preferred to cut the pUC-LINK using NheI and SpeI enzymes. Also, their cells were *E.coli* NM522, a very economically competent cell in cloning [56]. We preferred the *E.coli* NEB 10-beta strain according to Zhang et al.,(2018).

An et al.(2011) performed a similar study on the Masp1 gene which they chose the *E. coli* GM2163 as the host organism for cloning and *E. coli* strain BL21 for protein production of the Masp1. We chose the *E.coli* NEB 10-beta as the host organism due to their high efficiency in the cloning process [58]. For plasmid construction, they started with pET-19b and modified it with a kanamycin selection marker instead of the ampicillin resistance gene. They continued with an expression vector pBluescript II SK (þ) in their study [57].

No patent was found in the search made by entering "spider silk protein" in the Turkish patent institute resources shows. In addition, these methods will also form a basis for biomaterials that can be developed and produced because the designed development and production platform will be suitable to produce many other biomaterials [55].

In this thesis, genetic engineering is aimed for the development of genetically modified microorganism. In the future steps of the project, there are various stages such as developing a bioprocess for spider web protein production, purification of the protein and turning it into fiber by electrospinning. Among these, special focus should be given to the bioprocess development of recombinant *E.coli* NEB 10-beta that is capable of expressing Masp1 gene to produce recombinant spider silk protein. There is a gap in the literature in terms of bioprocess optimization of spider silk producing *E.coli* using various fermentation techniques in modern bioreactor conditions. We think that microbially expressed spider silk protein can be produced in higher yields when the relevant bioprocess optimizations are performed such as pH, temperature, dissolved oxygen, different feeding regimens etc.

When we compare our study with the current or completed projects in literature; we applied a different method in designing SI-bricks. During addition of new fragment into backbone, by a different strategy we added from the front of the target place [26].

In the beginning of experiments, we used heat shock method for bacterial transformation. However, we obtained a smaller number of colonies after heat-shock, therefore we change our method to electroporation. Chemically competent cells which are *E.coli* NEB 10-beta cells were changes electrocompetent cells in our laboratory.

At first, we were using pGSI plasmid for cloning. When we came to 32-mer, we couldn't take up next step which is cloning of 64-mer. We understood that our plasmid capacity was up to 10 kbp, therefore it cannot take more than 10 kbp. So, we ordered a new plasmid which was also expression vector, pBbB6c. This plasmid could carry higher plasmids.

We got some difficulties during ligation because our plasmid concentration was so low after gel extraction. Thus, we repeated ligation process one or more times. DNA concentrations decreased about to 30 times in gel extraction process. We tried some ways to increase the yield such as heating the elution buffer to 40-60 °C degree or cutting 2-3 DNA bands and extracting them together in one eppendorf.

Chapter 4

Conclusions and future prospects

4.1 Conclusions

My thesis focused on cloning the Masp1 spider silk protein-encoding gene from a dragline spider into the E. coli NEB 10-beta organism. Spider silk endocing gene was transformed into the *E.coli* which is a microbial cell factory for us to produce the target protein in future. The spider silk is very extreme biomaterial that is elastic as nylon, very tough, strong as Kevlar and steel and not dense. Thus, it has a huge usage area in medicine, industry and defence industry.

To achieve this, the 8-mer MaSP1 gene was synthesized, then cloned into the pGSI high copy cloning vector via sticky end by using the KpnI and Kpn2I restriction enzymes. This was followed by heat-shock transformation into the E. coli NEB 10-beta. Restriction digestion was then employed using NheI and Kpn2I to extract the 8-mer, and SpeI and Kpn2I to obtain the linearized pGSI plasmid containing 8-mer Masp1. A ligation step fused the 8-mer fragment and pGSI plasmid to achieve 16-mer Masp1, which was then heat-shock transformed into the E. coli NEB 10-beta. This protocol was repeated to obtain the 32-mer Masp1. Later 32-mer plasmid were cut by Kpn2I and KpnI enzymes and ligated into pBbB6c expression vector. The final plasmid were transformed to the *E.coli* by electroporation to have the more efficient result. The estimated size of 8-mer, 16-mer and 32-mer Masp1 were correlated with the literature. In future plan of this study will be to continue with large scale production in bioreactor with optimization on temperature, pH and dissolved oxygen.

4.2 Societal Impact and Contribution to Global Sustainability

To produce 1 kg of spider silk, 1.3 million spiders are needed. This spider silk is very biocompatible and biodegradable material. Thus, using this silk instead of other toxic materials like plastic, some metals give us a more sustainable world. Recombinant

production of spider silk will be beneficial for reducing water amount, not playing with the nature of spiders, and efficient time. On the other hand, this material can be formed in different fields such as the defence industry. Due to the bulletproof property, it can save the life of warriors. Also considering economic issues, producing this silk laboratory will be more logical and practical. Also, spider silk is water-soluble and biodegradable material, so it won't destroy nature. It is very essential for a more sustainable world. In the future, it will be more popular on manufacturing clothes and some products. Thus, it will decrease the carbon fingerprint by using this biomaterial. The production of spider silk can also have a positive impact on global sustainability through its ability to reduce the amount of energy and resources required for production. Spider silk is produced through a process known as "spinning", which does not require any chemical or mechanical treatments. This makes it much more environmentally friendly than many synthetic materials. Additionally, spider silk production does not require the use of any toxic chemicals, meaning it is also much safer for the environment and human health. Finally, the production of spider silk also has the potential to reduce our dependence on fossil fuels. Spider silk is a renewable resource, meaning it can be produced without depleting natural resources and reducing our reliance on oil-based products. This could significantly reduce our carbon footprint, helping to protect the environment and promote global sustainability. In conclusion, spider silk production has the potential to revolutionize our approach to global sustainability. Its unique properties make it incredibly useful for a wide range of applications, while its production process is much more environmentally friendly than many synthetic materials. Additionally, its renewable nature means it could significantly reduce our dependence on fossil fuels, helping to protect the environment and promote global sustainability. As such, recombinant spider silk production could have a huge positive impact on global sustainability.

4.3 Future Prospects

4.3.1 Bioprocess Development for Production of Recombinant Spider Silk Protein

Recombinant protein is expressed by the work of the cell's enzymes and biological systems by giving inputs such as carbon, nitrogen, potassium, inorganic salts to

genetically modified host cells. With this method, recombinant products can be produced across numerous industries such as pharmaceutical, agriculture, chemical and food industries. In addition, since these products can be obtained at low pressure and temperature, operational costs are significantly reduced. At the same time, many protein-based products can be produced using this production platform.

Parameters affecting recombinant protein production in bioprocesses can be examined under three headings: host cell selection and genetic modification, bioreactor environment design and bioreactor operating parameters. Other media components will be used from previous studies. There are three types of bioreactor operation: batch, fed batch and continuous. Intermittent feed operation is the most used of these. With this mode of operation, cells can proliferate significantly more than with batch operation, and the production of recombinant protein is correspondingly increased. On the other hand, temperature, oxygen transfer, and hydrogen ion concentration (pH) in the environment are bioreactor operating parameters, and these parameters can significantly affect cell propagation and recombinant protein assembly. However, in these systems, the process-specific conditions developed for each recombinant protein and host cell need to be optimized, because each of these processes has its own specific conditions [59].

4.3.2 Protein Induction Strategies

The generally used method for inducing *E. coli* protein expression systems is described by Sambrook et al. al (1999) as described [60,61]. In this method, induction recombinant protein production is performed in the exponential phase in that bacterial growth rate is highest. Therefore, induction begins at low cell concentrations. As a result, cell proliferation cannot occur adequately since cell resources are utilized in the recombinant protein production and the cell concentration obtained because of the process remains in low amounts. In cases where the cell concentration is low, the yield decreases and the required fermentation volume increases [61]. Therefore, at the end of the bioprocess, it is necessary to optimize both the cell concentration and the amount of recombinant protein to a high level.

On the other hand, the inducer concentration and how long induction process will continue are optimized. The most efficient production of different recombinant proteins in the same expression systems at different inducer concentrations and induction times was observed. While 0.55 mM inducer and 6 hours is optimal for one protein, 0.55 mM inducer

concentration and 3 hours induction time were obtained for the construction of another protein in the same expression system [59].

4.3.3 Protein Purification

The fermentation-optimized OAP solution will be passed through a double ultrafiltration fiber cartridge. The first filter cartridge has 750 kDa pores and will ensure the separation of large molecules. The second filter cartridge has 50 kDa pores and will expel the unwanted proteins from the filter and the recombinant OAP will be concentrated. Proteins will be precipitated by adding ammonium sulfate to the concentrated OAP solution. The protein will then be concentrated by centrifugation. Later the supernatant will be poured off, and salty protein pellets will be diluted with ultra-pure water. The centrifugation process and wash repeats will be continued until the supernatant conductivity is less than 20 μS/cm. The protein pellet will be lyophilized, and the final product will be purified protein powder that used in fiber production.

4.3.4 Electrospinning of Purified MasP1 protein

Oap produced in powder form will be electro spined into solution using hexafluoro isopropanol (hfip) solution. Hfip and formic acid will be mixed at a ratio of 1:1 and used to dissolve oap, thus sustainable electrospinning process will be realized. The produced fibers will be obtained with a diameter of 60-80 nanometers [3].

4.3.5 Mechanical Test

Tensile strength and modulus, service temperature range (min-max), electrical conductivity, electromagnetic properties (dielectric constant and loss tangent) and electron microscope imaging tests will be tested and reported by outsourcing.

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