GENE SYNTHESIS FROM OLIGONUCLEOTIDE MIXTURES BY SOLID PHASE PCR AND ASSEMBLY PCR IN A MICROFLUIDIC CHIP SYSTEM

by

Woong Hee Lee

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biomedical Engineering)
in The University of Michigan
2010

Dissertation Committee:

Professor Erodogan Gulari, Co-Chair
Associate Professor Shuichi Takayama, Co-Chair
Professor Charles A. Cain
Associate Professor Jinsang Kim
Assistant Professor Xiaoxia Lin

© Woong Hee Lee

All rights reserved
2010

To my Lord, Jesus Christ

And

To my beloved wife, Juhee

TABLE OF CONTENTS

LIST OF FIGURES

Figure

List of Tables

Table

ABSTRACT

The conventional gene synthesis methods, chemical or PCR, usually require over 2 weeks because of the separate executions of the different procedures. An integrated microfluidic chip system was designed to reduce this processing time to only 2 days with much less reaction volumes, and experimental reagent and solvent requirements. This fast high throughput gene synthesis method considerably minimizes contamination and simplifies material handling procedures. Our overall aim in this project is using the above-mentioned advantages of this system to synthesize long genes of arbitrary sequence with high purity, and cut the lead times and cost per base from the current values by at least one order of magnitude. In order to do this, four different steps are included in the microfluidic chip system: oligonucleotide synthesis and amplification on solid phase, on-chip purification, long DNA assembly, and gene transformation. The designed oligonucleotides to form the long DNAs were synthesized via light-directed phosphoramidite chemistry, and amplified on

solid phase. The amplified products were treated by on-surface hybridization using complementary probes to make single strands and purification. The purified oligonucleotides were assembled into long DNAs on chip, and amplified with polymerase chain reaction in a separate microfluidic chip chamber. Finally, the synthetic target gene was transformed on a chip for gene expression. Our results showed these individual steps in bringing the system capability to a simultaneous production level of tens of double stranded oligonucleotides of lengths ranging from 0.2 to 1.2 kb and the potential of microfluidic gene and protein synthesis system.

CHAPTER 1

INTRODUCTION

1.1 Motivation

After human genome project was completed in 2003 [1-4], the interest of human gene function was raised exponentially. Diagnosis of diseases has been developed at the DNA level [5-6], and gene therapy is researched to overcome various sicknesses, such as cancer [7-9]. For this DNA project, a novel gene synthesis approach was developed. Gene synthesis can be applied in many fields from combinatorial biology to generate target gene for diagnostic detection [10], De Novo gene synthesis, to produce new genes without template DNA, and gene modification to have interesting functions like over expression of proteins [11].

In gene generation method by PCR and cloning, there is a requirement for a template DNA, so there is the limitation of gene modification [12]. Gene synthesis from oligonucleotides overcomes this limitation because it does

not require template DNA and design for any DNA sequence. These days, hundreds of companies produce gene with oligonucleotides by chemical methods, such as β-phosphoramidite chemistry, and synthetic rate is around $0.5 per base pair [13]. Chemical oligonucleotide synthesis uses nucleotides with phosphoramidite group to prevent incorrect interaction of amine, hydroxyl groups, and phosphate groups.

One phosphoramidite is inserted on the column at a time and the desired oligonucleotide sequence can be synthesized on the same column. One small protein such as enhanced green fluorescence protein (EGFP) has 50 different oligonucleotides to be a full sequence by PCR [14]. To synthesize EGFP gene by chemical synthesis, 50 different columns should be prepared. It requires high cost and many steps. During oligonucleotide synthesis, several incorrect interactions could lead to mismatch sequences in oligonucleotide and reduce the amount of desired products. As the size of synthetic oligonucleotide is increased, the number of mismatch DNA has more opportunities of incorrect interaction. In assembly PCR, oligonucleotide mixture is the template DNA, so defects in oligonucleotides makes a critical effect for the purity of gene synthesis. After

oligonucleotide synthesis, they should be purified to remove mismatched DNAs.

In our laboratory, oligonucleotides could be synthesized on a microfluidic chip with photogenerated acid as a protecting group [15]. This technique can expose multi spots with 5 μm of diameter and remove protecting groups selectively. This means thousands of oligonucleotides could be synthesized on the same chip simultaneously. To synthesize EGFP gene, all oligonucleotides could be synthesized on one chip and therefore, reduce cost and time. Especially, all process of gene synthesize can be performed on an integrated microfluidic chip reducing contamination and cost below $0.05 per base pair.

This project includes oligonucleotide design by Gene2Oligo software program [14], light-directed synthesis with photogenerated acid, purification with DNA purification, and assembly by PCR. It also has the transformation step on a chip, and more importantly, it suggests the potential from DNA design to the production of target protein in an integrated microfluidic chip system.

1.2. On-chip Gene synthesis

On-chip gene synthesis system suggests the potential of automated and integrated protein synthesis from individual DNA monomers. In Figure 1.1, oligonucleotide set designed by Gene2Oligo software [14] is synthesized with multi-sequences on a microfluidic chip with photogenerated acid [15]. After phosphoramidite group of synthetic oligonucleotides is eliminated, synthetic oligonucleotides are recovered by solid phase PCR [16-18]. They are transferred into DNA purification chamber with capture probes on a chip surface and mismatched synthetic oligonucleotides are removed by DNA hybridization between them and capture probes.

Purified oligonucleotides are suspended again in solution by heating and moved to assembly PCR chamber to be assembled by polymerase chain reaction. In first PCR chamber, PCR produces gene fragments with below 350 bp. There is the limitation of the number of oligonucleotide set in assembly PCR because of the specificity of PCR. Thus, target gene should be combined from assembled fragments. In second PCR chamber, target gene is combined by fusion PCR.

Synthetic target gene is treated by restriction enzymes to have plasmid connecting sites at both ends and

transferred to ligation chamber. It is ligased with plasmid on a chip and transformed into competent cells by heat shock [19-20]. They are grown in nutrient liquid media and desired proteins will be expressed.

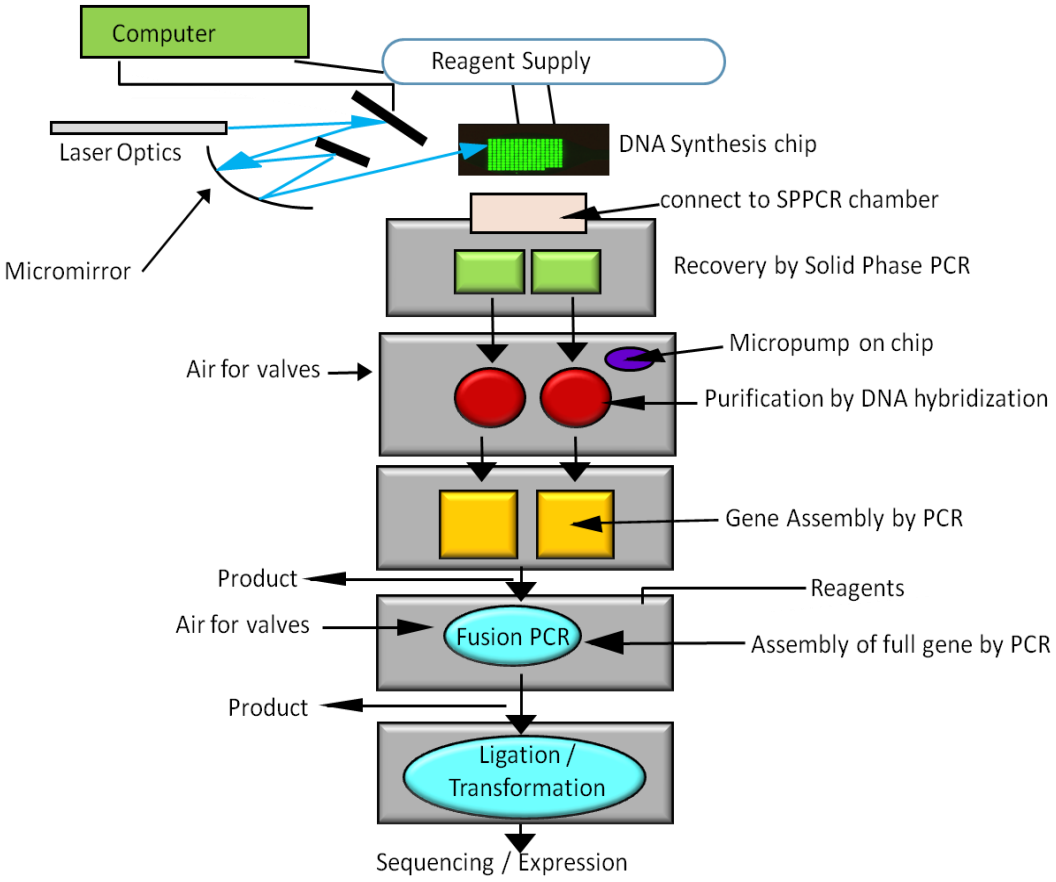


Figure 1.1 The overall scheme of on-chip gene synthesis system. Oligonucleotides are synthesized with phosphorgenerated acid, purified by hybridization, assembled by PCR, ligation with a plasmid, and transformed into competent cells. All processes are performed on microfluidic chips.

1.3. Research scope

In Chapter 2, the process is presented from the design of target gene to the production of synthetic oligonucleotides. Target gene is divided into oligonucleotide sets by Gene2Oligo to have overlapping sequence with desired melting temperature or hybridization unit size. Following designed oligonucleotide sequences, oligonucleotides are synthesized on a microfluidic chip. Solid phase PCR (SPPCR) is designed to recover synthetic oligonucleotides from the chip surface. To increase the yield of SPPCR, the density effect of synthetic oligonucleotides on a chip is investigated through manipulation by probe density on the surface.

In Chapter 3, DNA purification is set up and the critical factor is tested to determine the purification yield. DNA purification system is based on the binding force difference between perfect match DNA and mismatch in DNA hybridization. Capture probes are synthesized on a microfluidic chip to have selective mismatch DNAs and hybridized with Cy5-labeled perfect match DNA. The yield of DNA purification is measured by the sequence or the location of mismatch DNA. This is also investigated in assembly PCR. After assembly PCR with or without

purification, their sequences are compared and the purification yield was analyzed.

In Chapter 4, assembly PCR with synthetic oligonucleotides is performed on a chip. The efficiency of passivation step is tested by Bovine Serum Albumin (BSA), Polyethylene glycol (PEG), and formamide. The limit of assembly PCR on a chip is also measured by the analysis of product band intensities. To prevent competition between complementary DNA and the template in assembly PCR, the conversion of double stranded PCR product to single stranded one is presented. Finally, they are assembled to be target fragments and target gene is combined by fusion PCR.

In Chapter 5, on-chip transformation is tested to suggest the potential of protein expression in integrated chip. The ligation between assembled gene and plasmid is performed on a chip. Ligation product is transformed into competent cells on a chip and expressed in nutrient agar plate.

1.4. Bibliography

1. DeLisi, Charles (2001). "Genomes: 15 Years Later A Perspective by Charles DeLisi, HGP Pioneer". Human Genome News 11: 3–4. Retrieved 2005-02-03

2. Venter, J. C., M. D. Adams, et al. (2001). "The sequence of the human genome." Science 291(5507): 1304-51

3. Lander, E. S., L. M. Linton, et al. (2001). "Initial sequencing and analysis of the human genome." Nature 409(6822): 860-921

4. Osoegawa, K., A. G. Mammoser, et al. (2001). "A bacterial artificial chromosome library for sequencing the complete human genome." Genome Res 11(3): 483-96.

5. Holtzman, N. A., P. D. Murphy, et al. (1997). "Predictive genetic testing: from basic research to clinical practice." Science 278(5338): 602-5

6. Allingham-Hawkins, Diane (2008-08-01). "Successful Genetic Tests Are Predicated on Clinical Utility". Genetic Engineering & Biotechnology News 28 (14): pp. 6, 9

7. Morgan, R. A., M. E. Dudley, et al. (2006). "Cancer regression in patients after transfer of genetically engineered lymphocytes." Science 314(5796): 126-9.

8. Ott, M. G., M. Schmidt, et al. (2006). "Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1." Nat Med **12**(4): 401-9

9. Brown, B. D., M. A. Venneri, et al. (2006). "Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer." Nat Med 12(5): 585-91

10. Pelletier J, Sidhu S (August 2001). "Mapping protein-protein interactions with combinatorial biology methods". Curr. Opin. Biotechnol. 12 (4): 340–7

11. Riccardo, S., G. Tortoriello, et al. (2007). "The coding/non-coding overlapping architecture of the gene encoding the Drosophila pseudouridine synthase." BMC Mol Biol 8: 15

12. Kimi Yamakoshi, Nobuyoshi Shimoda (2003).”PCR-based cloning of an intronless zebrafish no tail gene.” Biochemical and Biophysical Research Communications, Volume 306, Issue 2, 27, Pages 598-602

13. Caruthers MH, Barone AD, Beaucage SL, Dodds DR, Fisher EF, McBride LJ, Matteucci M, Stabinsky Z, Tang JY, Chemical synthesis of deoxyoligonucleotides by the phosphoramidite method. Methods Enzymol, 1987. 154: 287-313

14. Rouillard, J. M., W. Lee, et al. (2004). "Gene2Oligo: oligonucleotide design for in vitro gene synthesis." Nucleic Acids Res 32(Web Server issue): W176-80

15. Gao X, Gulari E, Zhou X, In situ synthesis of oligonucleotide microarrays. Biopolymers, 2004. 73: 579-596

16. Adessi, C., G. Matton, et al. (2000). "Solid phase DNA amplification: characterisation of primer attachment and amplification mechanisms." Nucleic Acids Res 28(20): E87

17. Mitterer, G., M. Huber, et al. (2004). "Microarray-based identification of bacteria in clinical samples by solid-phase PCR amplification of 23S ribosomal DNA sequences." J Clin Microbiol *42*(3): 1048-57

18. Mercier, J. F. and G. W. Slater (2005). "Solid phase DNA amplification: a Brownian dynamics study of crowding effects." Biophys J 89(1): 32-42

19. Nagamine, K., S. Onodera, et al. (2005). "On-chip transformation of bacteria." Anal Chem 77(13): 4278-81

20. Ingham, C. J., A. Sprenkels, et al. (2007). "The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms." Proc Natl Acad Sci U S A 104(46): 18217-22

CHAPTER 2

DESIGN AND SYNTHESIS OF OLIGONUCLEOTIDES AND RECOVER THEM BY SOLID PHASE PCR (SPPCR)

2.1 Introduction

In the process of gene assembly by ligation chain reaction (LCR) [1] or polymerase chain reaction (PCR) [2-4], the first step is a design of DNA layout for synthesis which divides the target gene into oligonucleotides. DNA layout is designed following the desired conditions, such as specific length or similar melting temperature in hybridization units. At this step, the properties of divided oligonucleotides are key factors of successful gene assembly because they will determine the specificity of PCR. This division process of target gene is performed by the software, Gene2Oligo [5] which was developed in our laboratory.

In traditional oligonucleotide synthesis, divided oligonucleotides with different DNA sequences are synthesized on columns separately with phosphoramidite

chemistry [6-7]. However, this method requires the same number of columns with oligonucleotides and more time to collect synthetic oligonucleotides. It also brings in more cost and need for equipment to recover them. Thus, we developed oligonucleotide synthesis system on a microfluidic chip with light-directed synthesis using photogenerated acid [8], which can synthesize target oligonucleotides with different sequences on a chip simultaneously and recover synthetic oligonucleotides from chip surface by SPPCR efficiently.

In this chapter, the synthesis of target oligonucleotides on a chip and the development of SPPCR to recover them from the chip surface were discussed [9-13]. In addition, optimization of the yield of SPPCR through probe density as a variable for synthetic oligonucleotides synthesis was also performed by changing the linker ratio of linker chemicals to competitors in the derivatization of chip surface [12-13].

2.2 Materials and Methods

2.2.1 Gene2Oligo

Target gene, enhanced green fluorescent protein (EGFP), was divided into oligonucleotides for chip synthesis by Gene2Oligo which can be assessed on http://berry.engin.umich.edu/gene2oligo. The first step in Gene2Oligo is to enter the name of gene and paste DNA sequence of EGFP in Figure 2.1. In design mode, the desired conditions were set up to have specific hybridization unit size or melting temperature. The key factor of similar melting temperature among oligonucleotides was DNA sequence. Depending on it, the hybridization unit size of oligonucleotides was adjusted. Final step is the addition of DNA concentration and sodium concentration for calculations, and hit submit button. The sodium concentration is also important factor to design oligonucleotide sequence because it can reduce the electrostatic repulsion among DNA bases by binding on negative charge ion of DNA. Thus, it is also a factor in determining the specificity of DNA hybridization.

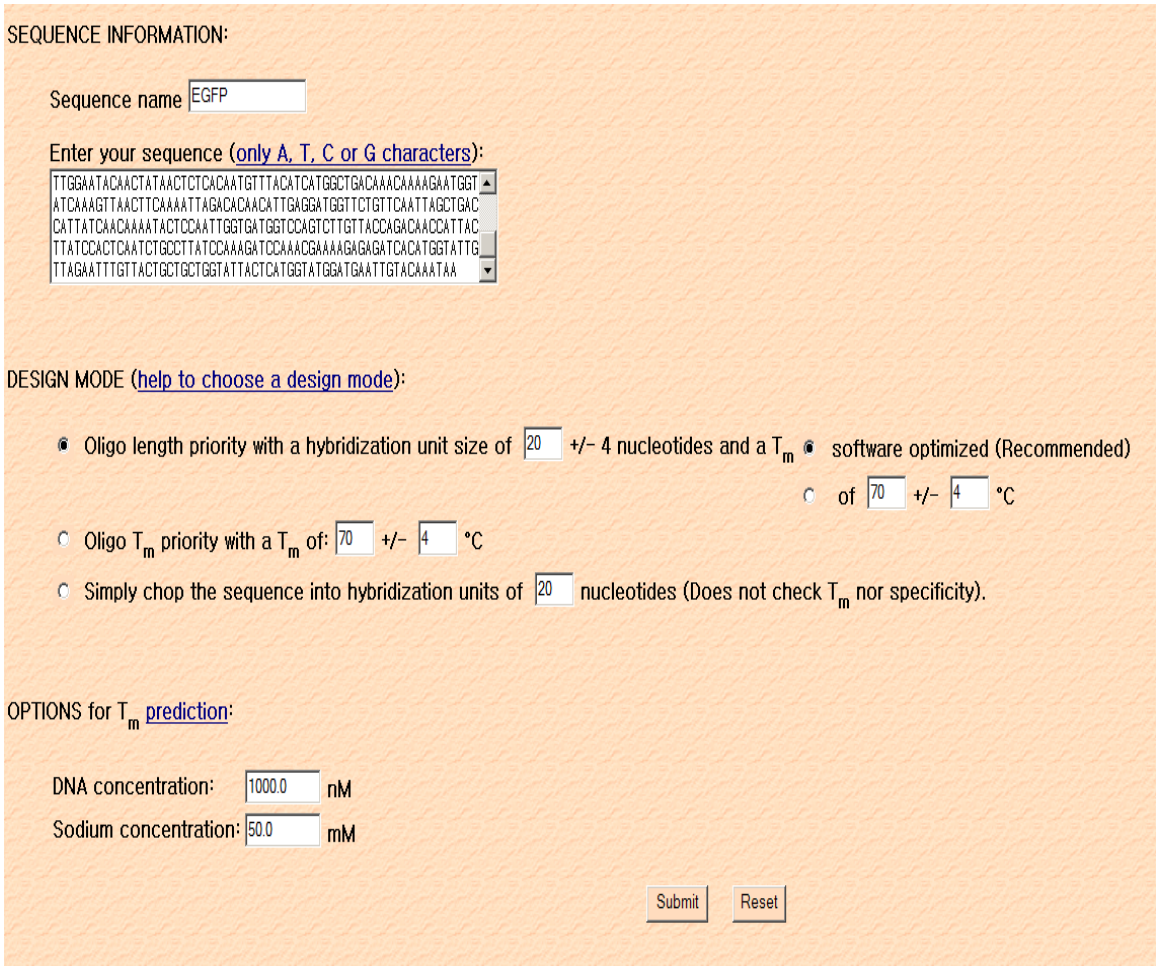


Figure 2.1 The main screen of Gene2Oligo software. It can be assessed on http://berry.engin.umich.edu/gene2oligo. The name of target gene, sequence, desired condition, and the concentration of DNA and sodium are typed.

```
ATGTCTAAAGGTGAAGA-ATTATTCACTGGTGTT GTCCCAATTTTGGT-TGAATTAGATGGTGAT GTTAATGGTCACA
TACAGATTTCCACTTCT TAATAAGTGACCACAA-CAGGGTTAAAACCA ACTTAATCTACCACTA-CAATTACCAGTGT
41.49             41.50            41.30          40.38            40.844

AAT-TTTCTGTCTCCGG TGAAGGTGAAGGTG-ATGCTACTTACGGT AAATTGACCTTAAAATTTA-TTTGTACTACTG
TTA AAAGACAGAGGCC-ACTTCCACTTCCAC TACGATGAATGCCA-TTTAACTGGAATTTTAAAT AAACATGATGAC
    41.76         43.15          41.532         40.418              41.43

GTAAA TTGCCAGTTCCAT-GGCCAACCTTAGT CACTACTTTCGGTT-ATGGTGTTCAATGTT TTGCTAGATACCCA-
CATTT-AACGGTCAAGGTA CCGGTTGGAATCA-GTGATGAAAGCCAA TACCACAAGTTACAA-AACGATCTATGGGT
      41.52         41.84         40.54          41.54           40.170

GATCATATGAAACAACA TGACTTTTTCAAGTCT-GCCATGCCAGAAG GTTATGTTCAAGAAAGA-ACTATTTTTTTCA
CTAGTATACTTTGTTGT-ACTGAAAAAGTTCAGA CGGTACGGTCTTC-CAATACAAGTTCTTTCT TGATAAAAAAAGT
41.42             41.87            45.04          41.26             41.88

AAGATG ACGGTAACTACAAGA-CCAGAGCTGAAGT CAAGTTTGAAGGTGA-TACCTTAGTTAATAGAATC GAATTAA
TTCTAC-TGCCATTGATGTTCT GGTCTCGACTTCA-GTTCAAACTTCCACT ATGGAATCAATTATCTTAG-CTTAATT
       42.19           41.61         42.31           40.526              40.87

AAGGTATTGATT-TTAAAGAAGATGGTAAC ATTTTAGGTCACAAATT-GGAATACAACTATAACTC TCACAATGTTTA
TTCCATAACTAA AATTTCTTCTACCATTG-TAAAATCCAGTGTTTAA CCTTATGTTGATATTGAG-AGTGTTACAAAT
             40.41            40.70            41.62              40.63

CATC-ATGGCTGACAAAC AAAAGAATGGTATCAAA-GTTAACTTCAAAATTAGAC ACAACATTGAAGATGG-TTCTGT
GTAG TACCGACTGTTTG-TTTTCTTACCATAGTTT CAATTGAAGTTTTAATCTG-TGTTGTAACTTCTACC AAGACA
     40.36         40.353           42.274             43.511           41.65

TCAATTAGCT GACCATTATCAACAAA-ATACTCCAATTGGTG ATGGTCCAGTCTTG-TTACCAGACAACCA TTACTT
AGTTAATCGA-CTGGTAATAGTTGTTT TATGAGGTTAACCAC-TACCAGGTCAGAAC AATGGTCTGTTGGT-AATGAA
           40.47           41.06           42.75          41.369         41.11

ATCCACTCAAT-CTGCCTTATCCAAA GATCCAAACGAAAAG-AGAGACCACATGG TCTTGTTAGAATTTGTT-ACTGC
TAGGTGAGTTA GACGGAATAGGTTT-CTAGGTTTGCTTTTC TCTCTGGTGTACC-AGAACAATCTTAAACAA TGACG
            40.22          41.52          40.980        40.42             44.14

TGCTGGTA TTACCCATGGTATGG-ATGAATTGTACAAATAAtg gacgggacactac
ACGACCAT-AATGGGTACCATACC TACTTAACATGTTTATTac-ctgccctgtgatg
         42.96          41.39              42.84
```

Statistics:
Min Tm is 40.17 °C
Avg Tm is 41.51 °C
Max Tm is 45.04 °C
Min hybridization unit size is 13 nucleotides
Avg hybridization unit size is 15.5 nucleotides
Max hybridization unit size is 19 nucleotides

Figure 2.2 The statistical result of fragmented DNAs of Enhanced Green Fluorescent Protein (EGFP).

```
F0      ATGTCTAAAGGTGAAGAATTATTCACTGGTGTT
R17     ACCAAAATTGGGACAACACCAGTGAATAAT
F33     GTCCCAATTTTGGTTGAATTAGATGGTGAT
R47     ATTTGTGACCATTAACATCACCATCTAATTCA
F63     GTTAATGGTCACAAATTTTCTGTCTCCGG
R79     CACCTTCACCTTCACCGGAGACAGAAA
F92     TGAAGGTGAAGGTGATGCTACTTACGGT
R106    TAAATTTTAAGGTCAATTTACCGTAAGTAGCAT
F120    AAATTGACCTTAAAATTTATTTGTACTACTGGTAAA
R139    ATGGAACTGGCAATTTACCAGTAGTACAAA
F156    TTGCCAGTTCCATGGCCAACCTTAGT
R169    AACCGAAAGTAGTGACTAAGGTTGGCC
F182    CACTACTTTCGGTTATGGTGTTCAATGTT
R196    TGGGTATCTAGCAAAACATTGAACACCAT
F211    TTGCTAGATACCCAGATCATATGAAACAACA
R225    AGACTTGAAAAAGTCATGTTGTTTCATATGATC
F242    TGACTTTTTCAAGTCTGCCATGCCAGAAG
R258    TCTTTCTTGAACATAACCTTCTGGCATGGC
F271    GTTATGTTCAAGAAAGAACTATTTTTTTCAAAGATG
R288    TCTTGTAGTTACCGTCATCTTTGAAAAAAATAGT
F307    ACGGTAACTACAAGACCAGAGCTGAAGT
R322    TCACCTTCAAACTTGACTTCAGCTCTGG
F335    CAAGTTTGAAGGTGATACCTTAGTTAATAGAATC
R350    AATCAATACCTTTTAATTCGATTCTATTAACTAAGGTA
F369    GAATTAAAAGGTATTGATTTTAAAGAAGATGGTAAC
R388    AATTTGTGACCTAAAATGTTACCATCTTCTTTAA
F405    ATTTTAGGTCACAAATTGGAATACAACTATAACTC
R422    GATGTAAACATTGTGAGAGTTATAGTTGTATTCC
F440    TCACAATGTTTACATCATGGCTGACAAAC
R456    TTTGATACCATTCTTTTGTTTGTCAGCCAT
F469    AAAAGAATGGTATCAAAGTTAACTTCAAAATTAGAC
R486    CCATCTTCAATGTTGTGTCTAATTTTGAAGTTAAC
F505    ACAACATTGAAGATGGTTCTGTTCAATTAGCT
R521    TTTGTTGATAATGGTCAGCTAATTGAACAGAA
F537    GACCATTATCAACAAAATACTCCAATTGGTG
R553    CAAGACTGGACCATCACCAATTGGAGTAT
F568    ATGGTCCAGTCTTGTTACCAGACAACCA
R582    ATTGAGTGGATAAGTAATGGTTGTCTGGTAA
F596    TTACTTATCCACTCAATCTGCCTTATCCAAA
R613    CTTTTCGTTTGGATCTTTGGATAAGGCAG
F627    GATCCAAACGAAAAGAGAGACCACATGG
R642    AACAAATTCTAACAAGACCATGTGGTCTCT
F655    TCTTGTTAGAATTTGTTACTGCTGCTGGTA
R672    CCATACCATGGGTAATACCAGCAGCAGT
F685    TTACCCATGGTATGGATGAATTGTACAAATAAtg
R700    gtagtgtcccgtccaTTATTTGTACAATTCAT
```

Figure 2.3 Divided oligonucleotide sequence of EGFP by Gene2Oligo.

The result of Gene2Oligo in Figure 2.2 shows the minimum, average, and maximum values of melting temperature and hybridization unit size in synthetic oligonucleotides. In Figure 2.3, the oligonucleotide sequences are displayed from 5'end and 3'end. F and R in oligonucleotide name means forward and reverse, respectively, and the number represents the end-cut position of oligonucleotide in full target gene. Based on these sequences, DNA layout of oligonucleotide synthesis was designed and prepared in Microsoft Excel software.

When the size or melting temperature of oligonucleotides was designed in Gene2Oligo, another potential factor, secondary structure, should also be considered. The secondary structure of oligonucleotide is the self hybridization when it is in single stranded form and could not be stretched. It can lead to the failure of assembly PCR if melting temperature of secondary structure is similar or higher than one of oligonucleotides, and template DNA cannot hybridize. In order to figure out this problem, first method is to control the hybridization unit size or melting temperature to minimize the effect of secondary structure. In this case, the desired hybridization unit size or melting temperature could be

changed. If it should be a fixed number, the second method is to find DNA bases forming secondary structure in the oligonucleotide, and modify the related DNA bases. When DNA monomer is substituted by another one, it should be based on amino acid table to keep the same peptide sequence after DNA translation.

2.2.2. Oligonucleotide Synthesis

Derivatization of chip surface

The oligonucleotide synthesis uses the phosphoramidite chemistry to have optimal properties such as efficient and rapid coupling, and the stability. The starting material is the solid support, silicon chip, derivatized with a linker chemical, aminopropyltriethoxysilane (SIA0610.0, Gelest Inc, Pennsylvania, USA). First, the chip surface was washed by hydrogen peroxide, ammonium hydroxide, and water solution which had a volume ratio of 1:1:5 (H_2O_2: NH_4OH:H_2O). Next step was the introduction of amino group using 0.4 mM of aminopropyltriethoxysilane [14]. This incubation was repeated for 100 mins and washed by ethanol. Overall process requires 150 mins.

Oligonucleotide synthesis

Traditional oligonucleotide synthesis used phosphoramidite chemistry on a column or chip. Derivatized chip with amino group was transferred to oligonucleotide synthesis machine using phosphoramidite chemistry in Figure 2.4. Phosphoramdite process had three steps which were deblocking, coupling, and oxidation.

The first step of phosphoramide process was deblocking which eliminates protecting group, Dimethoxytritl (DMT) group in DNA monomer. The detritylation was induced by the addition of a trichloroacetic acid solution (TCA) and exposed a hydroxy group at 5’end of monomer. Next step was the coupling between DNA monomer on a chip surface and new desired DNA monomer with DMT at 5’ end and amidite group at 3’ end. In this step, 3’end with amidite group of DNA monomer bound to 5’ hydroxyl group of one on a chip surface by nucleophilic substitution reaction. Next, the capping step was followed because unreacted DNA chain should be blocked in synthesis and completed by acetic anhydride and 1-methylimidazole. This step could enhance the purity of oligonucleotide synthesis. The last step was oxidation which phosphate group was converted to phosphotriester. By this step, phosphate group must be more stable. This

synthetic cycle with 3 steps was repeated until oligonucleotide sequence was produced. We used this method to synthesize just T-monomer spacer on the chip.

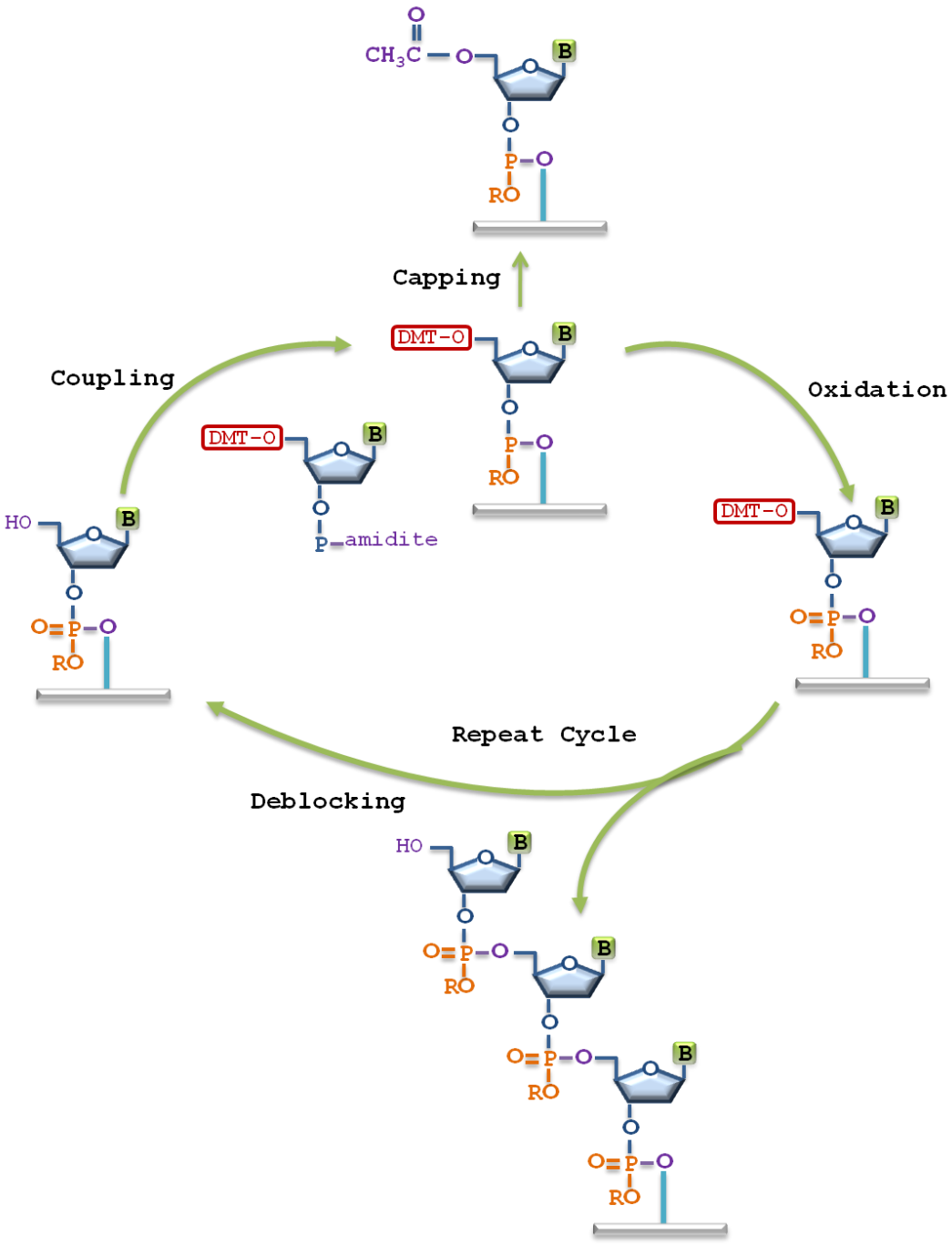


Figure 2.4 DNA synthesis by phosphoramidite chemistry

This DNA synthesis method was well defined and stable, but only one DNA sequence could be synthesized on a column or a chip surface. In our lab, new oligonucleotide synthesis method was developed using photogenerated acid (PCA) as a deprotection group, as in Figure 2.5. Following designed light pattern, light was exposed to selective spots on a chip for each cycle and protecting group was released. The number of selective spots was over 10,000 at one chip and meant over 10,000 different oligonucleotides could be synthesized at one chip. One cycle takes 4 minutes and over 98% of coupling yields.

Deprotection

After oligonucleotide synthesis, each monomer still had a protection group in base group because Adenine, Guanine, and Cytosine had an active amino group (NH_2). Cytosine and Adenine were protected by a benzoyl group and Guanine by isobutyryl group. These protecting groups were removed by ethylenediamine-ethanol solution [15].

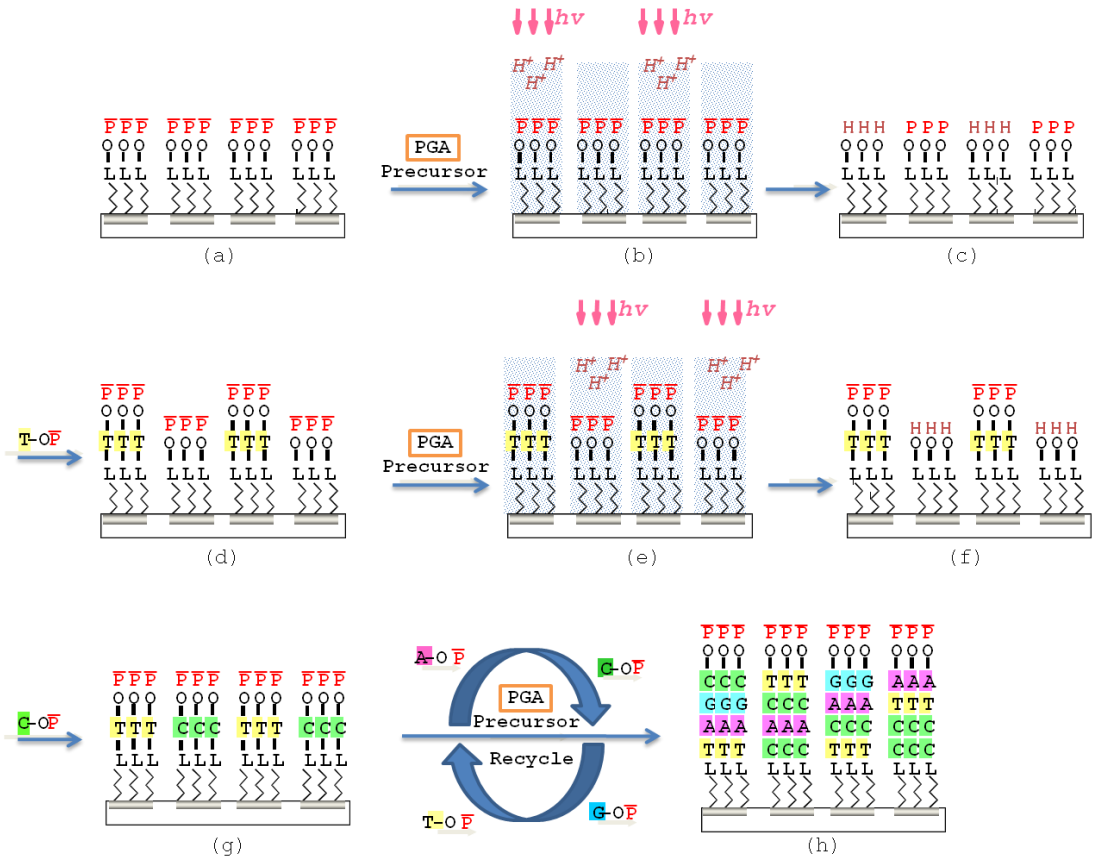


Figure 2.5 Muti-sequenced DNA synthesis by photogenerated acid on a chip. (a) A chip with spacer was ready to synthesis. (b) and (e) Light was exposed following DNA layout. (c) and (f) Protecting groups on light exposed spots were released. (d) and (g) Desired DNA monomer were coupled. (h) Desired target oligonucleotides were synthesized on a chip after repeating synthesis steps.

2.2.3. Solid Phase Polymerase Chain Reaction (SPPCR)

After oligonucleotide synthesis, the chip was treated with ammonium hydroxide (NH_4OH) to recover synthetic oligonucleotide in traditional method. However, this method requires 16 hours and several steps to remove chemicals and recover synthetic DNAs completely. In order to reduce time and steps of synthetic oligonucleotide recovery, Solid Phase PCR was designed and developed.

PCR is a DNA amplification technique which was developed in 1984 by Kary Mullis [16]. Using *Taq* polymerase, which has activity at high temperature, DNA could be elongated and duplicated. PCR solution consists of template DNA, primer set, deoxynucleotide with Adenine, Thymine, Guanine, or Cytosine base (dNTPs), *Taq* DNA polymerase, buffer, and water. PCR has 3 different thermal steps, as shown in Figure 2.6. First step is denaturation that double stranded template DNA is made single stranded DNA by heating. In this step, all template DNAs are ready to bind primers. Next, primers bind to template DNAs following their complementary sequences in annealing step. During the annealing step, primers could have non-specific binding at low temperature and no binding at high temperature. Thus, annealing temperature should be optimized by primer

sequence before PCR. Final step of PCR is extension. *Taq* DNA polymerase recognizes 3’end of primers on a template DNA and makes double stranded DNAs with dNTPs.

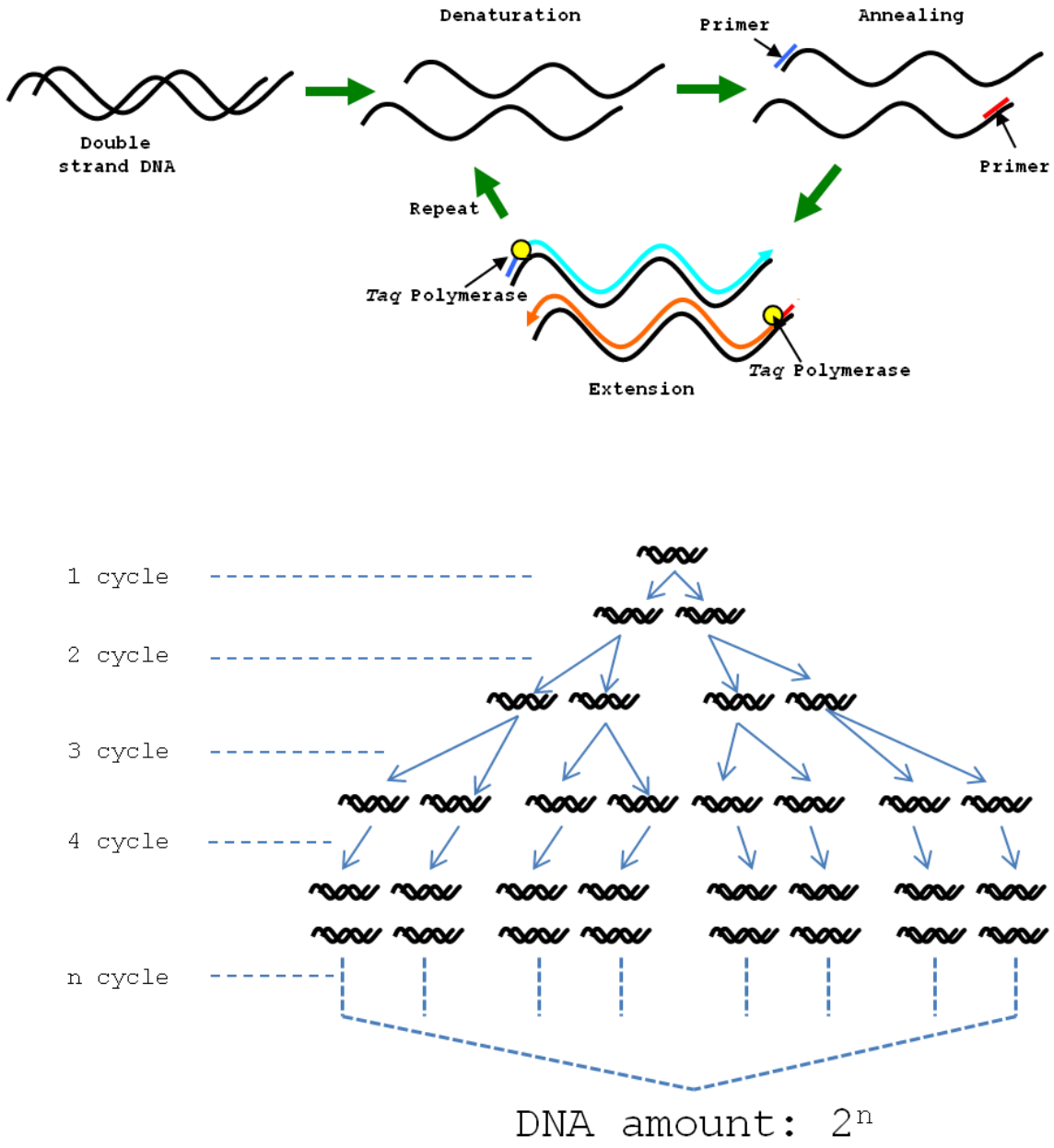


Figure 2.6 PCR is consisted of 3 steps; denaturation for single stranded form of template DNA, annealing for primer binding on specific template sequence, and extension for DNA elongation following template DNA. The amount of DNA increases exponentially by the number of cycle.

After oligonucleotide synthesis, one chip has oligonucleotides with over 20 different DNA sequences. In order to amplify different oligonucleotides simultaneously, universal primer sites were inserted at both 5′ and 3′end of target oligonucleotide sequences on DNA layout in Figure 2.7. Forward and reverse universal primer sites had Mly I and Bsa I restriction enzyme recognition sequences. PCR mixture had 0.5 U of Vent DNA polymerase, 50 pmol of forward and reverser primers, 10 mM dNTPs, buffer, and water in 50 uL. 15 uL of PCR mixture was inserted into chip chamber with synthetic oligonucleotides and mineral oil was dropped on backside of chip and slide glass was put to prevent evaporation during PCR. Solid Phase PCR was performed under 95°C, 30 sec/35°C, 4 min,/ 35°C(+3°C/cycle), 4 min for 11 cycles; 95°C, 30 sec/45°C, 30 sec, / 68°C, 30 sec for 30 cycles, 68°C, 5 min for 1 cycle.

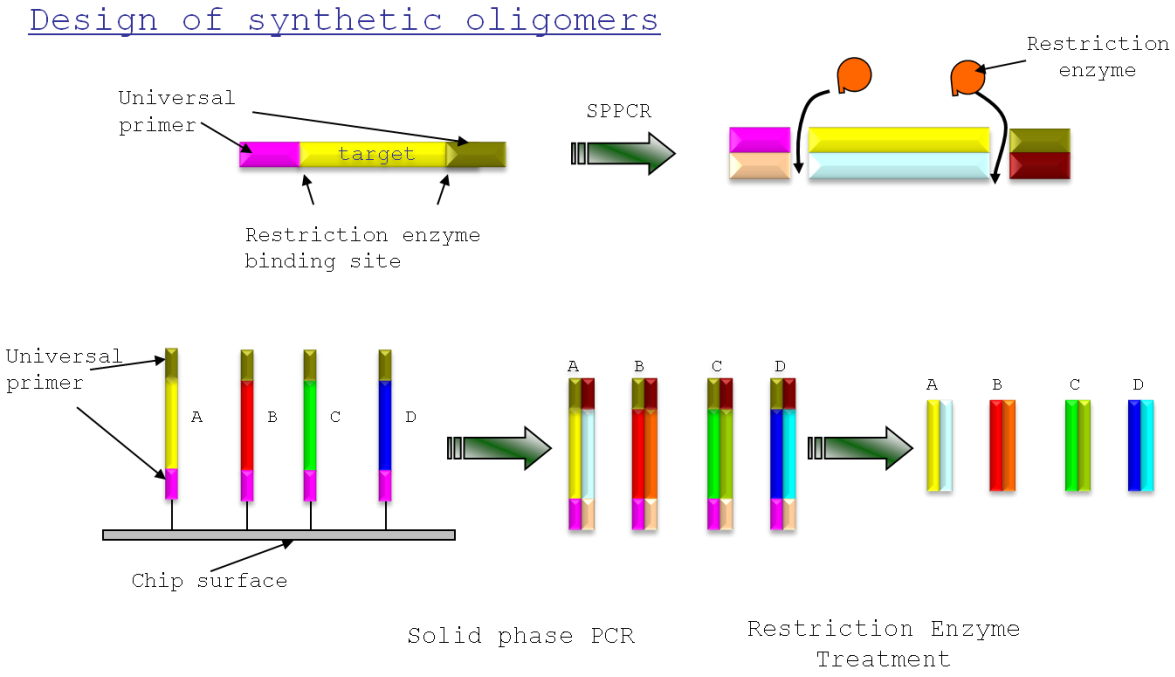


Figure 2.7 Design of synthetic oligonucleotides for SPPCR.

2.3 Results and Discussion

2.3.1 Recovery of synthetic oligonucleotides by Solid Phase PCR

After Solid Phase PCR, synthetic DNAs were amplified by tube PCR under 95°C, 2min for 1 cycle, 95°C, 30 sec/45°C, 30 sec, / 68°C, 30 sec for 35 cycles, 68°C, 5 min for 1 cycle to increase the amount of target DNAs. Amplified products were treated with 5 U of Mly I and Bsa I at 37°C for 1 hour to check the purity of product. They were analyzed on 2% agarose gel.

Expected product size was from 70 to 95 bp. In Figure 2.8, all product bands were between 70 and 100 bp. After restriction enzyme treatments, their sizes dropped below 55 bp. Thus, SPPCR could amplify and recover synthetic oligonucleotides on a chip properly. Next, amplified products were verified whether all different synthetic oligonucleotides could be recovered by DNA hybridization on a microfluidic chip. Most of oligonucleotides had similar intensities (data not shown), so recovery process of synthetic oligonucleotides was efficient.

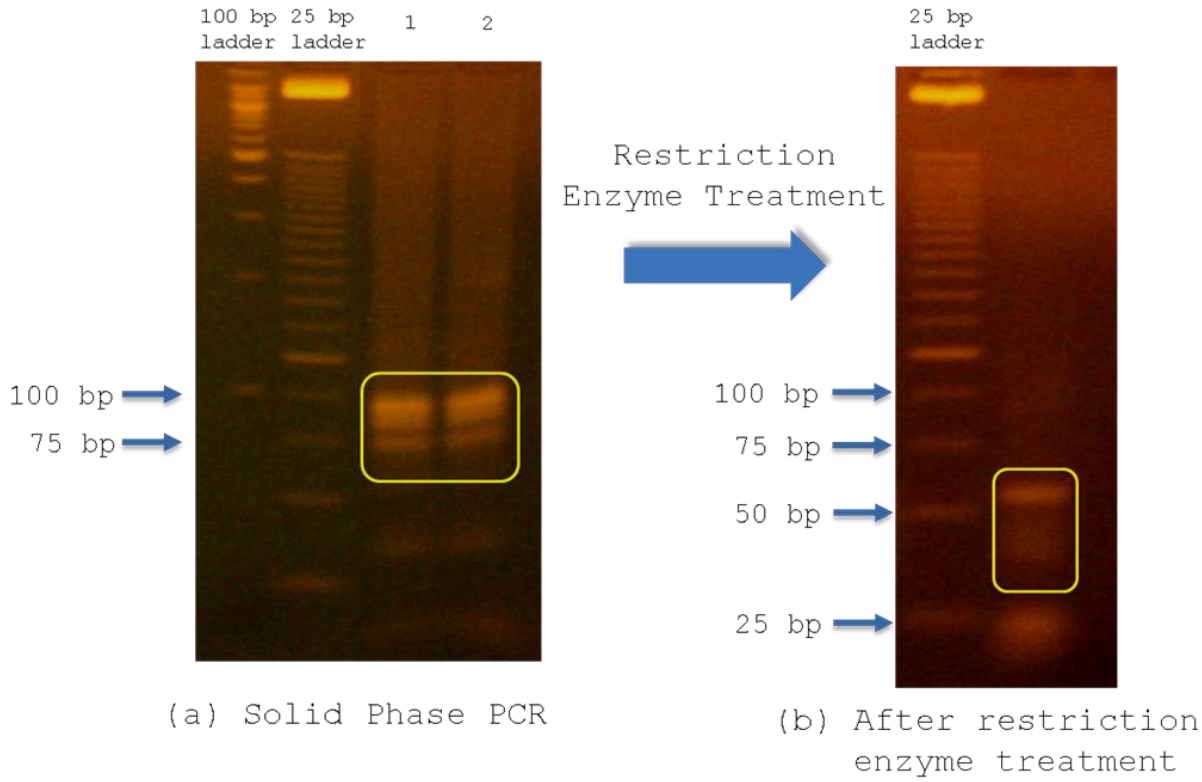


Figure 2.8 (a) The result of SPPCR on a chip. (b) The product of SPPCR was treated by Mly I and Bsa I to remove universal primer sites. Product band shifted down below 55 bp.

2.3.2 Residual synthetic oligonucleotides after repeated solid phase PCR

Solid phase PCR had over 55°C of temperature difference continuously during PCR steps, so synthetic oligonucleotides immobilized on a chip surface could have damage or loss after SPPCR. Residual amount of synthetic oligonucleotides on a chip after repeated SPPCR was investigated. First, SPPCR was performed and hybridized by complementary DNA probes with Cy5-fluorescence. Their intensities were scanned and compared in GenePix Axon 4000B (Molecular Devices Inc, California, USA). SPPCR was repeated 3 times.

Synthetic oligonucleotides were lost over 75% after first SPPCR in Figure 2.9. 5% of them were remained after third SPPCR and could be still amplified and produce double stranded DNAs.

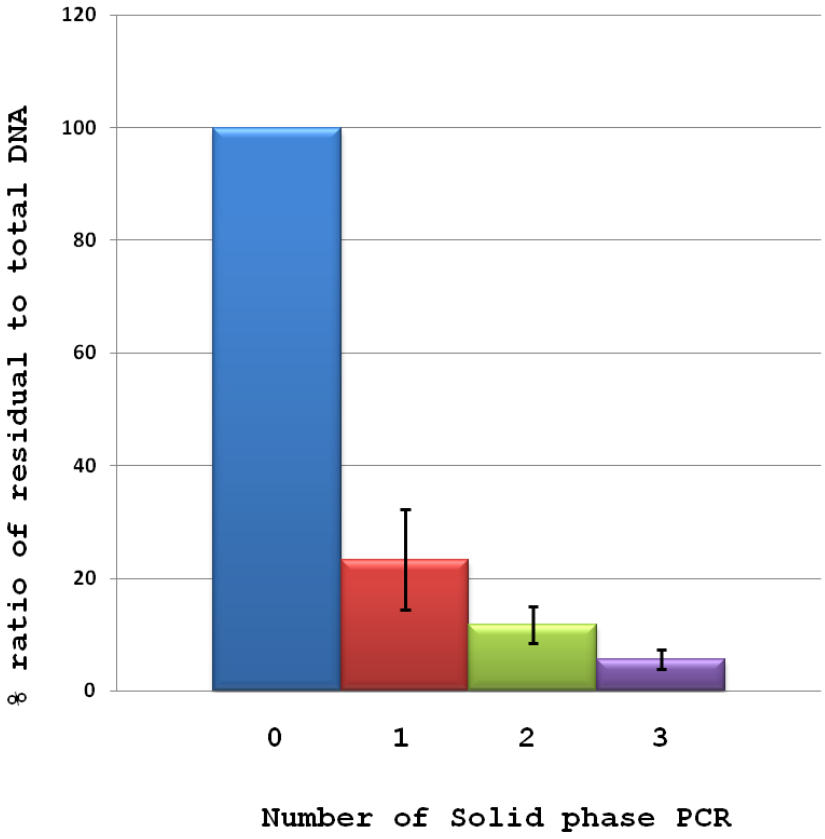


Figure 2.9 The residual amount of synthetic oligonucleotides immobilized on a chip surface after repeated SPPCR. Ratio was investigated by the intensity analysis of Cy5-labeled capture probe binding.

2.3.3 The optimization of SPPCR with the density of synthetic.

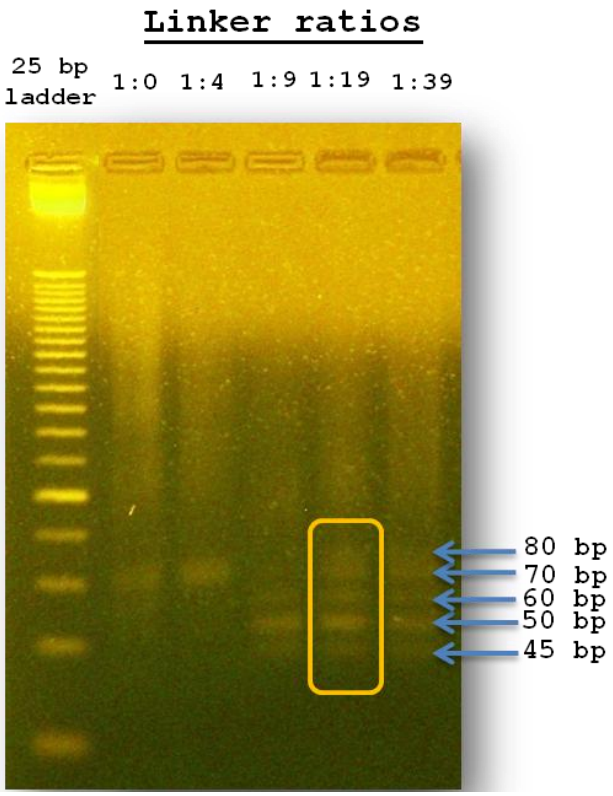
3’ends of Template DNAs in SPPCR were immobilized on solid phase and this means that primer and DNA polymerase should have binding reaction close to chip surface area. High density of synthetic DNA could inhibit the bind of them because of steric hindrance [17-20]. In order to overcome this problem, surface synthetic DNA density was controlled by chip derivatization step. When chip surface was derivatized by linker chemical, aminopropyltriethoxysilane, linker solutions was mixed with propyltriethoxysilane (SIP6917.0, Gelest Inc, Pennsylvania, USA) as a competitor. Propyltriethoxysilane included methyl group ($-CH_3$) instead of amino group (NH_2) in aminopropyltriethoxysilane, so DNA could not be synthesized on competitor and allow more space for binding reaction on the chip surface [21].

In this experiment, 5 different linker ratios were designed, such as 1:0, 1:4, 1:9, 1:19, and 1:39 (Aminopropyltriethoxysilane: Propyltriethoxysilane) and 5 different chips were derivatized with these linker solutions. Target DNAs have 45, 50, 60, 70, and 80 bp in length. They were synthesized on the chip, which has 107 of

separated rectangular synthesis spots. Each target DNA had different number of spots to determine SPPCR yield. 45, 50, 60, 70, and 80 bp of target DNAs had 7, 10, 20, 30, and 40 spots. The small number of spot must be a key factor of SPPCR yield. For example, if 45 bp could be amplified in specific linker ratio, it means this linker ratio makes an effect for steric hindrance of chip surface during SPPCR. All products were analyzed on 2% agarose gel and stained by ethidium bromide. The intensity of each product band was scanned by Image J software (Wayne Rasband, National Institutes of Health, MD, USA).

DNA Size	Number of Spots
45	7
50	10
60	20
70	30
80	40

(a)



(b)

Figure 2.10 (a) The number of synthetic spots by target DNA size. (b) The result of SPCPR on 5 chips with different linker ratios.

In Figure 2.10, 45, 50, and 70 bp of target in 1:0 and 1:4 ratio could not be amplified properly and only 70 bp of target DNA was observed clearly. All target DNAs in 1:19 and 1:39 ratio were amplified and detected. When the intensities among product bands in 1:19 and 1:39 were scanned by Image J, 1:19 ratio had the best uniformity among bands. Thus, the optimal linker ratio for SPPCR was determined to be 1:19.

2.4 Conclusions

In this chapter, Target gene, Enhanced Green Fluorescent Protein was divided into 47 oligonucleotides with 41.51°C of average melting temperature and 15.5 nucleotides of average hybridization unit size by Gene2Oligo software.

Oligonucleotides were synthesized on a chip by designed DNA layout and SPPCR was performed to recover synthetic oligonucleotides from a chip surface. SPPCR showed that it could recover synthetic oligonucleotides successfully without chemical cleavage step in short time, and amplify their amounts.

In order to solve the steric hindrance problem between DNA polymerase and synthetic DNAs on a chip surface [8], linker chemical was mixed with competitor when the chip surface was derivatized with amino group. Optimal linker ratio of amoniumpropyltriethoxysilane to propyltriethoxysilane was decided to be 1:19, which has the best SPPCR yield by product band analysis. We found the most important factor of SPPCR to be the spacing and steric hindrance problem for primer and DNA polymerase binding.

2.5 Bibliography

1. Barany, F. (1991). "Genetic disease detection and DNA amplification using cloned thermostable ligase." Proc Natl Acad Sci U S A 88(1): 189-93

2. Dillon, P. J. and C. A. Rosen (1990). "A rapid method for the construction of synthetic genes using the polymerase chain reaction." Biotechniques 9(3): 298, 300

3. Stemmer, W. P., A. Crameri, et al. (1995). "Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides." Gene 164(1): 49-53

4. Young, L. and Q. Dong (2004). "Two-step total gene synthesis method." Nucleic Acids Res 32(7): e59

5. Rouillard, J. M., W. Lee, et al. (2004). "Gene2Oligo: oligonucleotide design for in vitro gene synthesis." Nucleic Acids Res 32(Web Server issue): W176-80

6. Caruthers MH, Barone AD, Beaucage SL, Dodds DR, Fisher EF, McBride LJ, Matteucci M, Stabinsky Z, Tang JY, Chemical synthesis of deoxyoligonucleotides by the phosphoramidite method. Methods Enzymol, 1987. 154: 287-313

7. Tanaka, Toshiki; Letsinger, R. L. (1982). "Syringe method for stepwise chemical synthesis of oligonucleotides". Nucl. Acids Res. 10 (10): 3249–3259

8. Gao X, Gulari E, Zhou X, In situ synthesis of oligonucleotide microarrays. Biopolymers, 2004. 73: 579-596

9. Adessi, C., G. Matton, et al. (2000). "Solid phase DNA amplification: characterisation of primer attachment and amplification mechanisms." Nucleic Acids Res 28(20): E87

10. Mitterer, G., M. Huber, et al. (2004). "Microarray-based identification of bacteria in clinical samples

by solid-phase PCR amplification of 23S ribosomal DNA sequences." J Clin Microbiol *42*(3): 1048-57

11. Mercier, J. F. and G. W. Slater (2005). "Solid phase DNA amplification: a Brownian dynamics study of crowding effects." Biophys J 89(1): 32-42

12. Carmon, A., T. J. Vision, et al. (2002). "Solid-phase PCR in microwells: effects of linker length and composition on tethering, hybridization, and extension." Biotechniques 32(2): 410, 412, 414-8, 420

13. Khan, Z., K. Poetter, et al. (2008). "Enhanced solid phase PCR: mechanisms to increase priming by solid support primers." Anal Biochem 375(2): 391-3

14. A. Subramanian, S.J. Kennel, P.I. Oden, K.B. Jacobson, J. Woodward and M.J. Doktycz (1999). "Comparison of techniques for enzyme immobilization on silicon supports." Enzyme Microb Technol 24(1):26–34

15. Hogrefe, R. I., M. M. Vaghefi, et al. (1993). "Deprotection of methylphosphonate oligonucleotides using a novel one-pot procedure." Nucleic Acids Res 21(9): 2031-8

16. Mullis, K., F. Faloona, et al. (1986). "Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction." Cold Spring Harb Symp Quant Biol 51 Pt 1: 263-73

17. Peterson, A. W., R. J. Heaton, et al. (2001). "The effect of surface probe density on DNA hybridization." Nucleic Acids Res. 29(24): 5163-8

18. Watterson JH, Paul A. E. Piunno, Christopher C. Wust, Ulrich J. Krull (2000). "Effects of Oligonucleotide Immobilization Density on Selectivity of Quantitative Transduction of Hybridization of Immobilized DNA." Langmuir 16: 4984-4992

19. Steel AB, Levicky RL, Herne TM, Tarlov MJ (2000). "Immobilization of nucleic acids at solid surfaces: effect of oligonucleotide length on layer assembly." Biophys J 79: 975-981

20. Fei Xu, August M. Pellino, Wolfgang Knoll (2008). "Electrostatic repulsion and steric hindrance effects of surface probe density on deoxyribonucleic acid (DNA)/peptide nucleic acid (PNA) hybridization." Thin Solid Films 516 (23): 8634-8639

21. Peterson AW, Wolf LK, Georgiadis RM (2002). "Hybridization of mismatched or partially matched DNA at surfaces." J Am Chem Soc124: 14601-14607

Chapter 3

THE PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES ON A MICROFUDIC CHIP

3.1 Introduction

During oligonucleotide synthesis on a chip, unexpected DNA monomers could be coupled or missed by uncompleted gene synthesis process. These monomers will be mismatch DNAs in synthetic DNAs and become the error sequences of template DNAs in assembly PCR. In traditional purification of synthetic DNAs, they were purified on polyacrylamide gel by DNA size or High-performance liquid chromatography (HPLC) [1-2]. However, these purification systems require high cost and time to select desired DNAs [3-6].

In our project, synthetic oligonucleotides were purified by capture probes on a microfluidic chip which had many applications, such as gene expression profiling [7-8] and mutation detection [9-11]. This method based on the binding force difference between perfect match DNA and mismatch DNA, as shown in Figure 3.1. All double stranded DNAs made hydrogen bondings between DNA bases. For example,

adenine binds to thymine by two hydrogen bonding and cytosine does guanine by three ones [12-13].

Non-specific binding of DNA means there are no or weak bindings among some mismatch bases and makes unstable hydrogen bonding at specific temperature [14], so the binding force of mismatch DNA is lower than perfect match DNA. The point of maximum binding force difference can be controlled by hybridization temperature. Thus, mismatch DNA can be eliminated on a chip with the hybridization of capture probes at an optimal temperature.

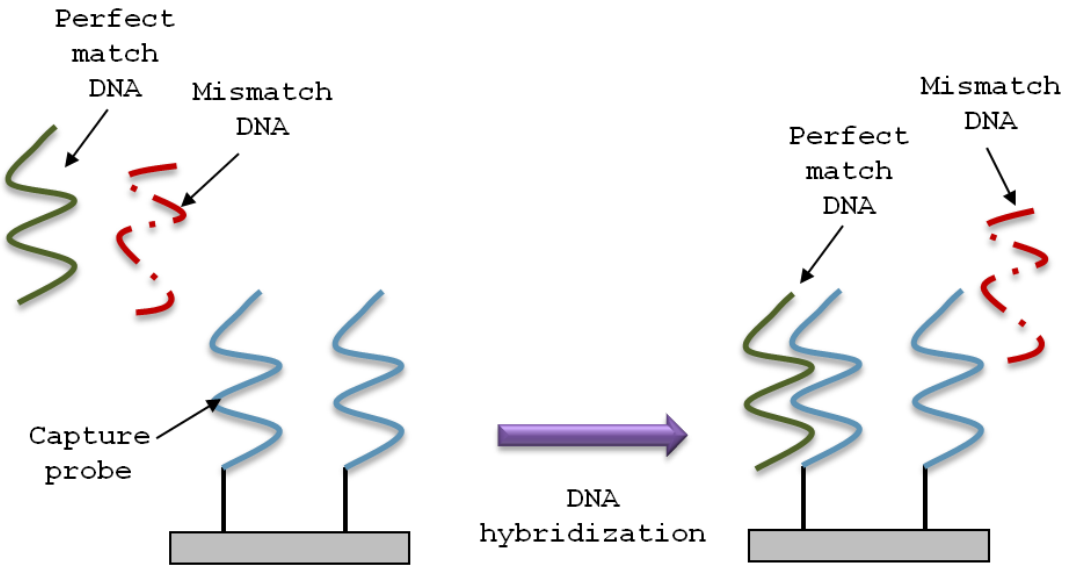


Figure 3.1 The scheme of DNA hybridization.

When hybridization is performed in a microfluidic chip, temperature should be optimized to have the highest binding force difference between perfect match DNA and mismatch one. Generally, hybridization temperature is set less than 10 degree of melting temperature in solution, but in solid phase it should be done less than 20 degrees because the environment of hybridization is changed like steric hindrance [15-19]. This is caused by the electrostatic repulsion of capture probes on chip surface because of negative charge of dense synthetic DNAs on the surface [20-21]. Hybridization temperature in DNA purification is the most important factor to determine the specificity and the purity of DNA.

In this chapter, DNA purification system on a chip was developed and analyzed by the location and sequence of mismatch DNAs. Its yield is also investigated in assembly PCR.

3.2 Materials and Methods

3.2.1 The investigation of the efficiency of DNA purification with single mismatch DNA

DNA capture probe preparation

Two DNA capture probes were prepared with or without single mismatch DNAs from IDT DNA Corporation (Iowa, USA). Perfect match oligonucleotide had 'GAGCTC' oligonucleotide sequence, which is also SacI restriction enzyme cutting site. Single mismatch oligonucleotide had SacI site and 'CTCGAG' for XhoI cutting site in Figure 3.2. When single mismatch matches DNA sequence compared with perfect one, cytosine monomer sequence was inserted in the middle of mismatched DNA and it made XhoI cutting site. Thus, single mismatched DNA could be digested by Xho I. Target DNA size had 50 bp in length and universal primer sites at both ends.

DNA purification in hybridization station

DNA purification test was performed in hybridization station, Invitrogen, Inc, Carlsbad, CA). DNA purification process consists of 3 elements: hybridization station, purification chip, and hybridization solution. DNA probes were synthesized on 7 channel serpentine chip using

photogenerated acid and deprotected by ethylenediamine-ethanol solution (50% v/v) solution.

Synthetic DNA chip was fixed on a metal chip holder by a double-sides tape and put into the hybridization station. Hybridization station was washed with prime solution (6X Saline-Sodium phosphate-EDTA, 1M NaCl) to clean and fill up connecting tubes. It also removed air bubbles in the chip channel. Perfect match DNA and mismatch one were mixed by 1:1 ratio (mole:mole). They were connected with tubes in hybridization station and performed hybridization reaction with 500 μL/min flow rate at 40 or 50°C for overnight.

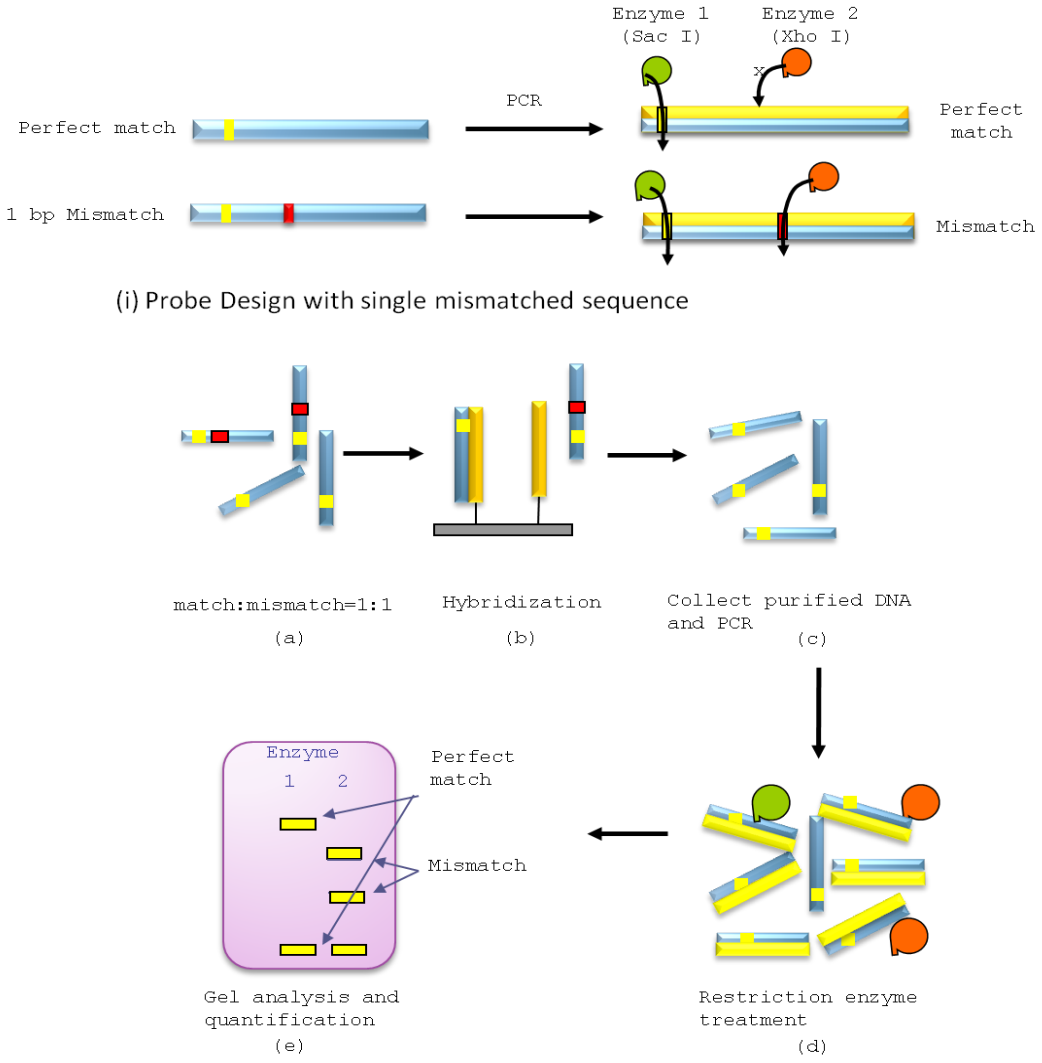


Figure 3.2 The design for testing the yield of DNA purification. (i) The restriction enzyme sites in perfect match and mismatch DNAs. (ii) Perfect matched DNA were mixed with mismatched DNA by 1:1 ratio (a) and hybridized with capture probes on a chip(b). Purified DNAs were collected and amplified (c), and treated by restriction enzyme (d). Finally, the pattern of digested bands was analyzed on agarose gel (e).

3.2.2 The analysis of DNA purification yield by sequences or location of mismatch DNA in capture probes.

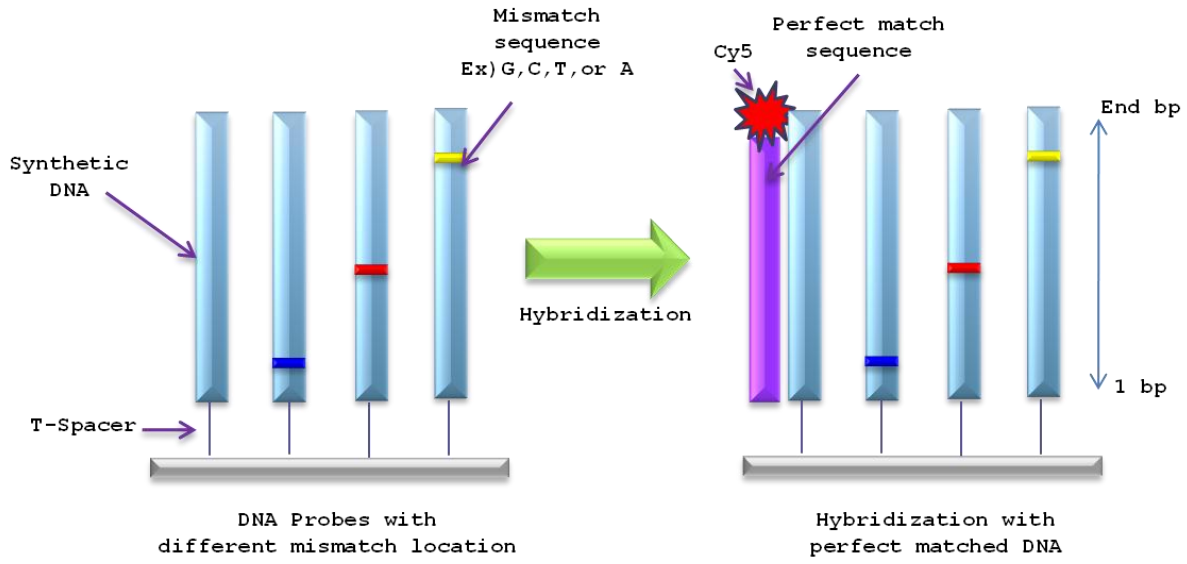
The experiment was tested in fluorescence laser scanner, GenePix Axon 4000B (Molecular Devices Inc., California, USA) with designed capture probes.

DNA capture probes design

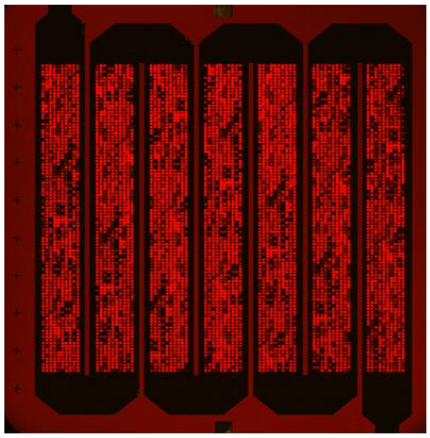
4 different sized capture probes were synthesized on a 7 channel serpentine chip. When capture probes were synthesized on a chip, synthetic chip had over 10,000 DNA spots (synthetic area). Capture probes of F31, F195, R265, and R297 are 22, 27, 31, and 28 bp in length, respectively. All synthetic capture probes had 20 nt of Thymine (T) nucleotide spacer to allow more space between the chip surface and target DNA.

Each probe was designed to have perfect match spot and mismatch DNA ones during DNA hybridization in Figure 3.3. For example, Thymine (T) in 1st base of F31 was a proper complementary sequence. One spot had T as a perfect match sequence and adjacent 3 spots had Guanine (G), Adenine (A), and Cytosine (C) instead of T as a mismatch one. Thus, one perfect match and 3 mismatch DNAs were one set in 1st base of F31 for the measurement of DNA purification yield. One

set was repeated 10 times on the same chip and each set was located randomly. It was prepared from 1st base to the end base in each probe DNA. Target DNAs were labeled by PCR with Cyanine-5 labeled primer and hybridized with synthetic capture probes on a chip and scanned by fluorescence laser scanner.



(a) The Design of capture probe to test the purification yield by monomer or size of mismatch DNA



(b) The synthesis and hybridization of capture probes on 7 channel serpentine microfluidic chip

Figure 3.3 The mismatched DNA was designed to have different location or sequences (a) and hybridized with cy5 labeled perfect matched DNA on microfluidic chip (b).

Experimental hybridization system

DNA hybridization was performed in an integrated experimental system which consisted of 3 major parts: a fluorescence laser scanner, a peristaltic pump, and temperature controller in Figure 3.4.

a. A temperature controller

A chip with synthetic capture probes was equipped in fluorescence laser scanner using chip holder which was connected with temperature controller. Temperature in microfluidic chip was controlled by an electric heater between chip holder and chip. Depend on melting temperature of capture probes, hybridization temperature was set up and found to have the best purification yield.

b. A peristaltic pump

500 μL of hybridization solution in 1.5 mL eppendorf tube was connected with a chip using a tube and circulated during the hybridization by using a peristaltic pump with a flow rate of 500 μL/min.

c. A fluorescence laser scanner

GenePix Axon 4000B (Molecular Devices Inc. California, USA) was connected with temperature controller and computer.

The scanner was controlled by GenePix 5.0 software in computer. In this software, the conditions of intensity measurement could be controlled, such as spot size and number. For the detection of Cy5 fluorescence, scanner used the laser exciting at 635 nm and emission at 650 nm (reference). All intensity values of spots were transferred to Excel program in Microsoft Inc. (Redmond, WA, USA) and background signal was subtracted. All mismatch intensities were compared with perfect match ones and the purification yield among mismatch DNA monomers were analyzed.

Double stranded DNA was denatured at 95°C for 5 minutes in 6X SSPE solution (1.0 M Sodium Chloride, 0.06 M Sodium Hydrogen Phosphate, 6 mM EDTA, pH 7.4) and cooled down on ice for 10 minutes to maintain their single stranded form. Chilled and denatured DNA target solution was connected with tubes in hybridization station and performs hybridization reaction with 500 μL/min flow rate at 50°C for overnight. Hybridized DNAs were washed with 1x SSPE and measured spot intensities.

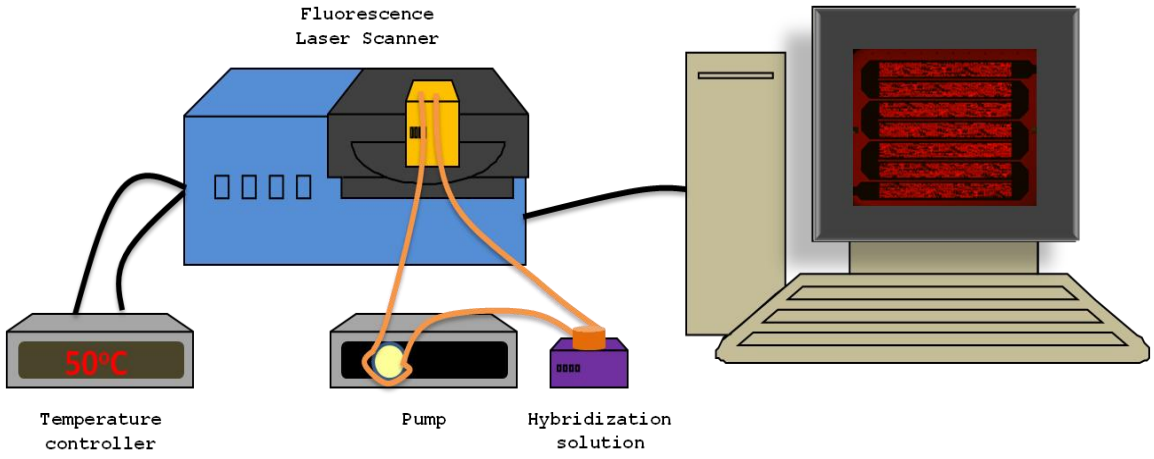


Figure 3.4 The experimental system of hybridization test between perfect match and mismatch DNAs. Fluorescence laser scanner is connected with temperature controller, pump, hybridization solution, and computer.

3.2.3 The analysis of purification yields in assembly PCR with synthetic oligonucleotides

Target gene, Enhanced Green Fluorescent Protein (EGFP), was divided into 3 small fragments with 250 bp in length and 20 different oligonucleotides. One fragment, EGFP1 was synthesized on a chip and recovered by Solid Phase PCR (SPPCR). The other two fragments were synthesized on 2 chips separately. After the elimination of universal primer sites with restriction enzyme treatment in SPPCR products, synthetic double stranded oligonucleotides were assembled by PCR with or without DNA purification in order to verify purification efficiency in gene assembly.

DNA purification probes of 20 oligonucleotides were synthesized on 7 channel serpentine chip and had full complementary sequences. Each probe had 274 repeated synthetic spots and was positioned randomly on chip channels. DNA purification was performed in hybridization station and under temperature of 50°C, a flow rate of 500 µL/min, and 16 hours of hybridization time.

Purified DNAs were recovered by heating chip surface at 95°C and collecting the supernatant. Their concentration was measured in Nanodrop (Thermo Scientific, Waltham, MA). Assembly PCR was carried out with 0.5 U of Vent DNA polymerase, 50 pmol of forward and reverser primers, 10 mM dNTPs, buffer, and water in 50 µL under 95°C, 2 min for 1 cycle, 95°C, 30 sec/35°C, 1 min,/ 35°C(+3°C/cycle), 1 min for 11 cycles; 95°C, 30 sec/45°C, 30 sec, / 68°C, 30 sec for 30 cycles, 68°C, 5 min for 1 cycle. After assembly PCR, PCR products were amplified again by secondary PCR to increase the amount of template. PCR product was analyzed on 1% agarose gel.

In order to verify purification yield, the DNA sequences of assembled gene with or without purification step should be compared. Their sequences were analyzed by gene cloning method in a plasmid. PCR product were cut and

purified by a gel purification kit (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI). They were ligated with pcrSMART vector and transformed into Electrocompetent cell (CloneSmart® Cloning Kits, Lucigen, Madison, WI). Transformation was carried out in a 0.1 cm gap cuvette. The settings for electroporation were 10 µF, 600 Ohms, and 1800 volts. Time constants were 3.5 to 4.5 msec. After transformation, competent cells were diluted in 975 µL of express media from Lucigen (Madison, WI, USA) and cultured at 250 rpm and 37°C for 1 hour. 100 µL of cultured media and concentrated one were spread on nutrient agar plates containing Kanamycin as an antibiotic and incubated at 37°C for overnight.

Grown colonies were picked up and inoculated in 2 mL of liquid nutrient media. They were cultured at 250 rpm and 37°C for 16 hours. Liquid media was concentrated by centrifuge machine at 5000 rpm for 3 minutes and mini-prepped by QIAprep Spin Mini-prepped Kit(QIAGEN Inc., Valencia,CA).

Mini-prepped samples were treated by EcoRV restriction enzyme (New England Biolab, Ipswich, MA, USA) which can cut at the both end of EGFP1 in the plasmid and analyzed on 1% agarose gel to identify whether GFP1 fragment were inserted

into the plasmid or not. Identified mini-prepped samples were sent to the DNA Sequencing Core at the University of Michigan and their sequences with purification and without purification were compared.

3.3 Results and discussion

3.3.1 The DNA purification yield with 1 mismatch DNA in an oligonucleotide

The DNA purification efficiency on a microfluidic chip was tested with perfect match DNA and 1 mismatch DNA. Perfect match DNA had 50 bp in length and 69.3°C of melting temperature. DNA hybridization system with capture probes tried to eliminate mismatch DNA in perfect match and mismatch DNAs complex on a chip at 45°C and 55°C. The purified DNAs were collected by heating from chip surface, and amplified by PCR to increase the amount of DNA and make double strand DNA for restriction enzyme binding. Purified PCR products were treated by restriction enzymes and analyzed on 2% agarose gel. The intensities of product bands were scanned and compared by Image J software (Wayne Rasband, National Institutes of Health, MD, USA).

Table 1. The comparison of purification yield at 45 and 55°C

Temperature (°C)	45	55
Purification ratio (perfect match/mismatch)	4	6

In Table 1, when DNA purification was performed at 45°C, perfect match DNA had 4 times higher intensity value than mismatch one. In DNA purification at 55°C, the intensities of perfect match one was higher than 6 times. With this result, DNA purification system was efficient to eliminate mismatched DNAs. In the next experiment, the factors to determine DNA purification efficiency were tested.

3.3.2 The determination of DNA purification efficiency by the location or sequences of mismatch DNA

The effect of mismatch DNA location in DNA purification

The DNA purification yield was compared depending on mismatch DNA location. Capture probes of 4 target DNAs with different lengths were synthesized on a chip with T-spacer at the 3'end and each probe had one mismatch DNA in each DNA location. They were hybridized with Cy5-labeled perfect match target DNA at 50°C for overnight and washed at 57°C

with 1XSSPE. The intensities of mismatch probe were scanned in GenePix 4000B fluorescence scanner and compared with one of perfect match probes. The purification efficiency in each location of probes was shown by the percentage ratio of mismatch DNA to perfect DNA. Location of mismatch DNA in capture probe was also converted to the percentage ratio of each position from 3’end to 5’end of probes to whole probe length in order to compare the purification yield among 4 target DNAs. The ratio of 4 target DNAs were combined in Figure 3.5.

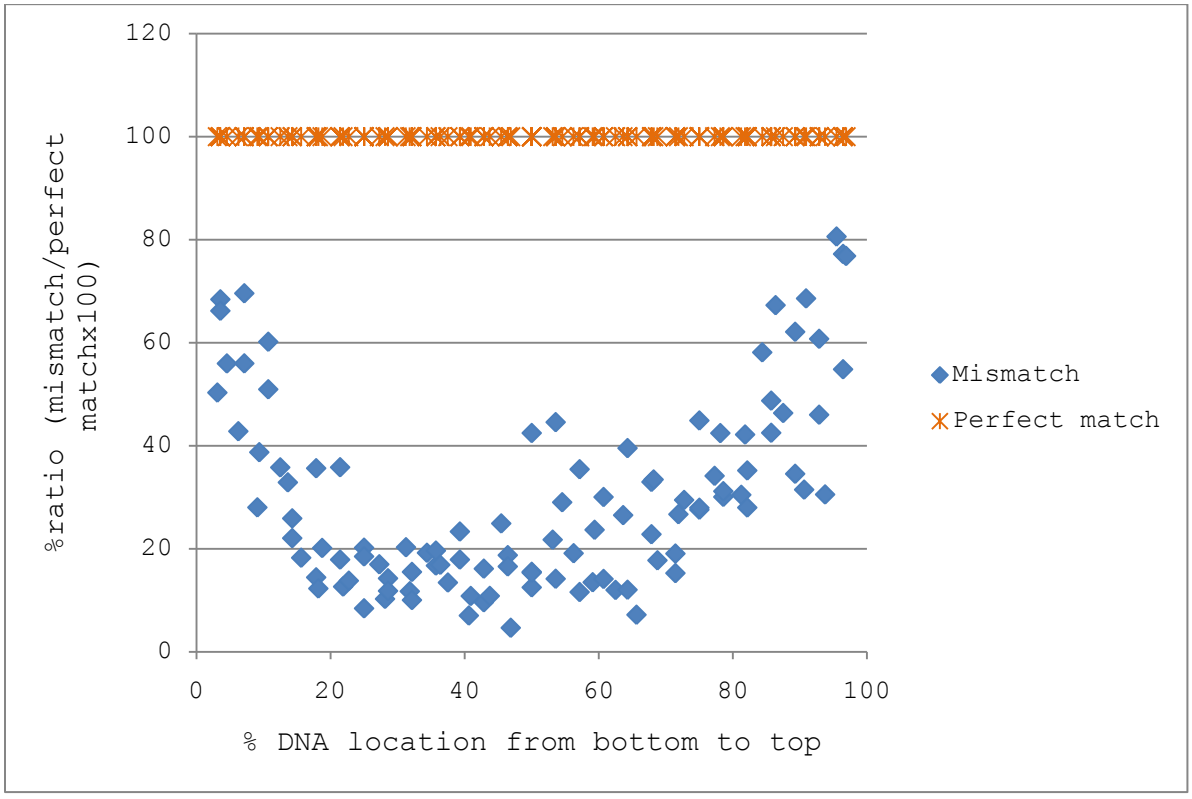


Figure 3.5 The analysis of DNA purification efficiency by the location and sequences of 4 probes.

In Figure 3.5, the intensity ratios (Y-axis) of mismatch DNA between 20 and 65% of target DNA location (x-axis) was below 20%. This means that when mismatch DNAs were located in the middle of capture probe, over 80% of mismatch DNA was removed. As the location of mismatch DNA was close to top of the capture probes, the amount of remained mismatch DNA was increased. When it was in the top of capture probe, the intensity was almost the same as one in perfect match DNA. When mismatch DNA was in 1st base of capture probe (bottom location), there was 30% of mismatch DNA elimination. The reason it had a different pattern compared with the top location is that 1st base was connected with 20 nt of T-spacer on the chip surface.

In this experiment, we found mismatch location to be an important factor for the determination of DNA purification efficiency. Even though there is the same mismatch DNA sequence, DNA purification yield must be different depending on location. For the next experiment, the effect of mismatch DNA sequence in DNA purification was tested.

The effect of mismatch DNA sequence in DNA purification

Even though synthetic DNAs had a mismatch DNA in the same location, purification yield could be different depending on DNA sequence because each DNA base had different hydrogen bonding structures. In this experiment, the purification yield was tested by the difference in mismatch DNA sequence. Each perfect match DNA can have 3 different substitutions of mismatch DNAs, so one base of synthetic capture probe was designed to have 3 different capture probes with mismatch sequences and one with perfect match.

Synthetic probes of 4 different targets such as F31, F195, R265, and R297 were synthesized and hybridized with Cy5-labeled perfect match DNA on microfluidic chip at 50°C for overnight and washed at 57°C with 1XSSPE. The intensities of mismatch and perfect match probe were scanned in GenePix 4000B and compared to convert the ratio of mismatch to perfect match.

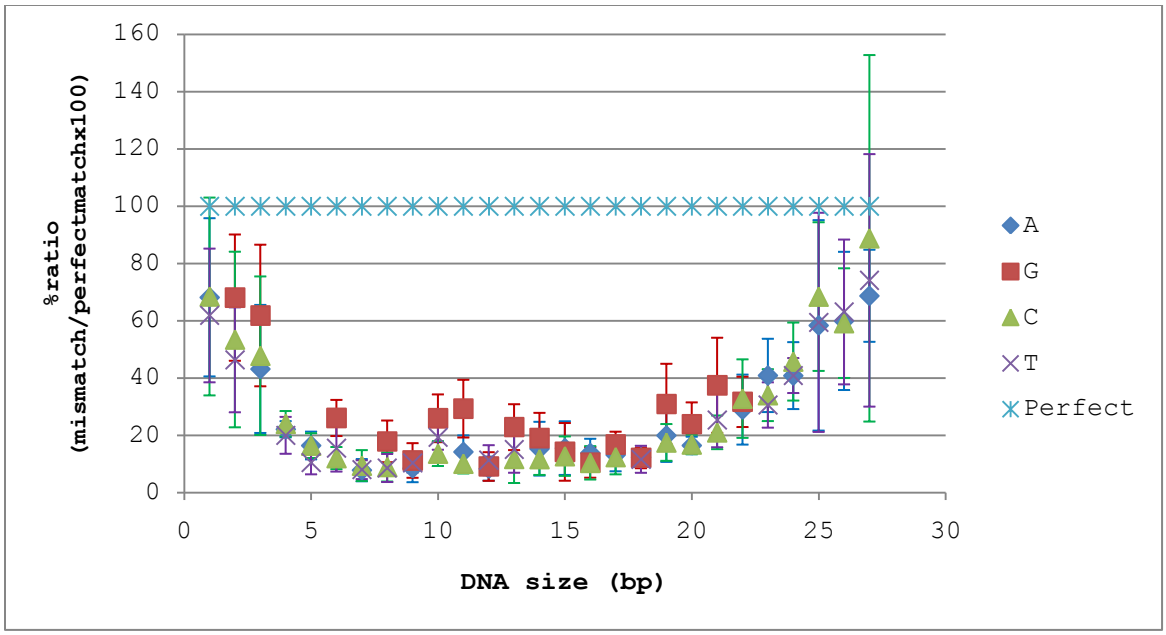


Figure 3.6 The analysis of DNA purification efficiency by the location and sequences of F31.

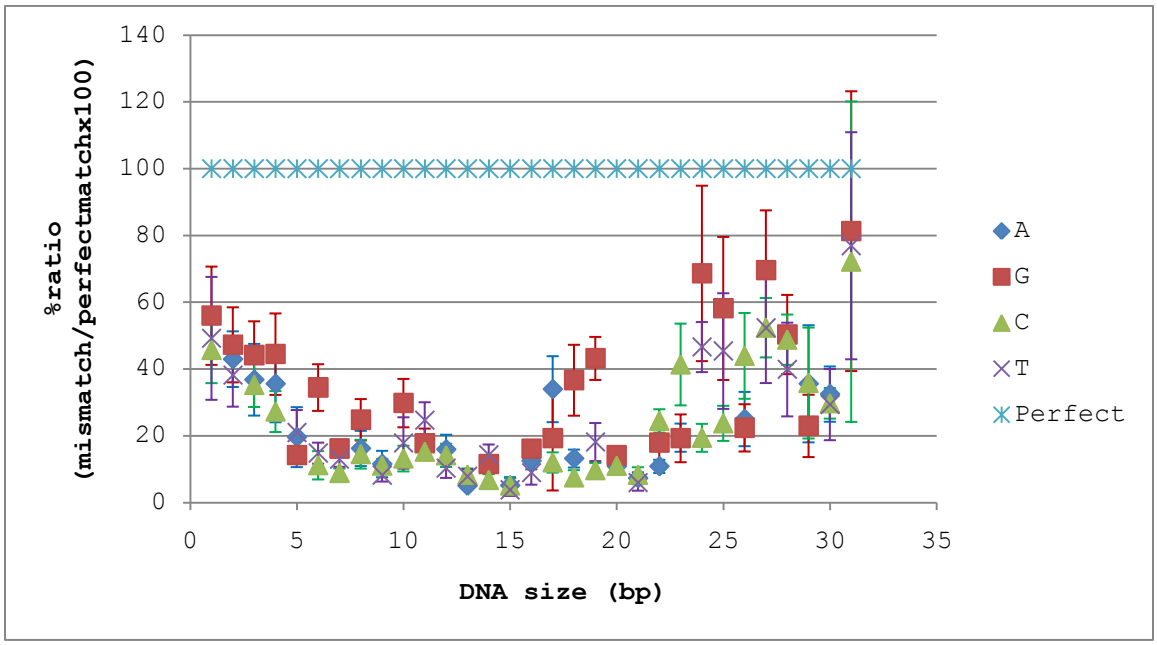


Figure 3.7 The analysis of DNA purification efficiency by the location and sequences of F195. F31, F195, R265, and R297

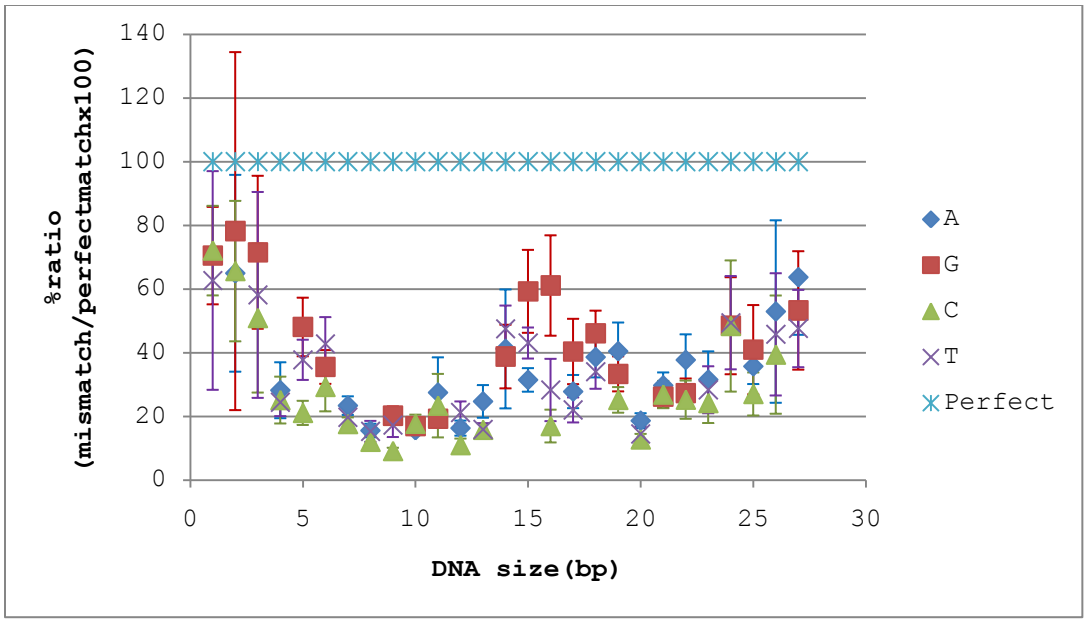


Figure 3.8 The analysis of DNA purification efficiency by the location and sequences of R265.

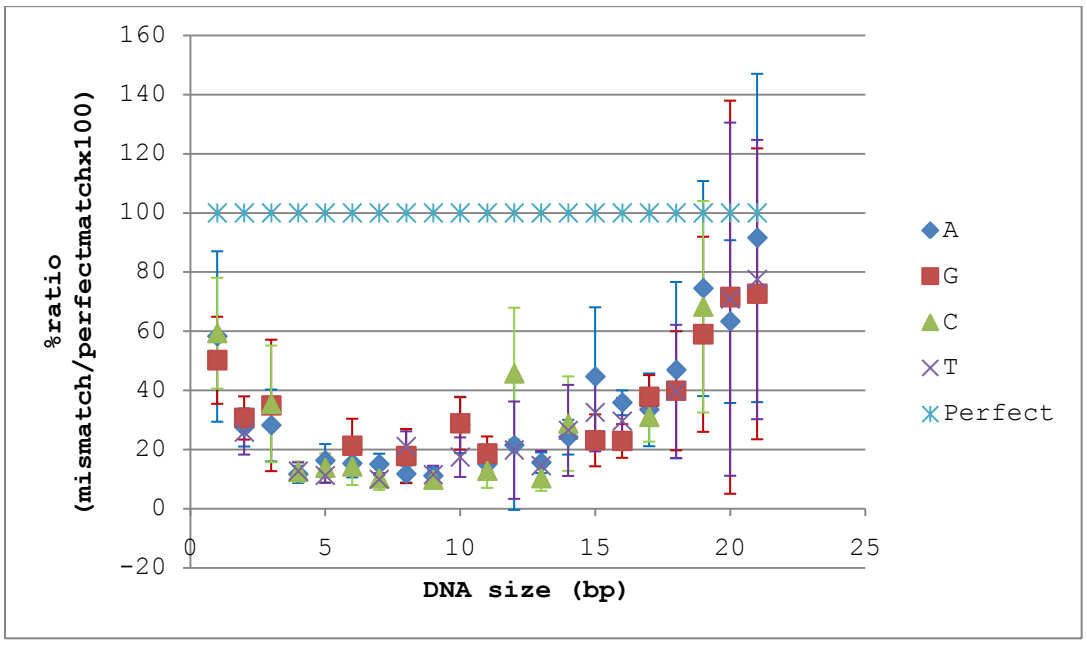


Figure 3.9 The analysis of DNA purification efficiency by the location and sequences of R297.

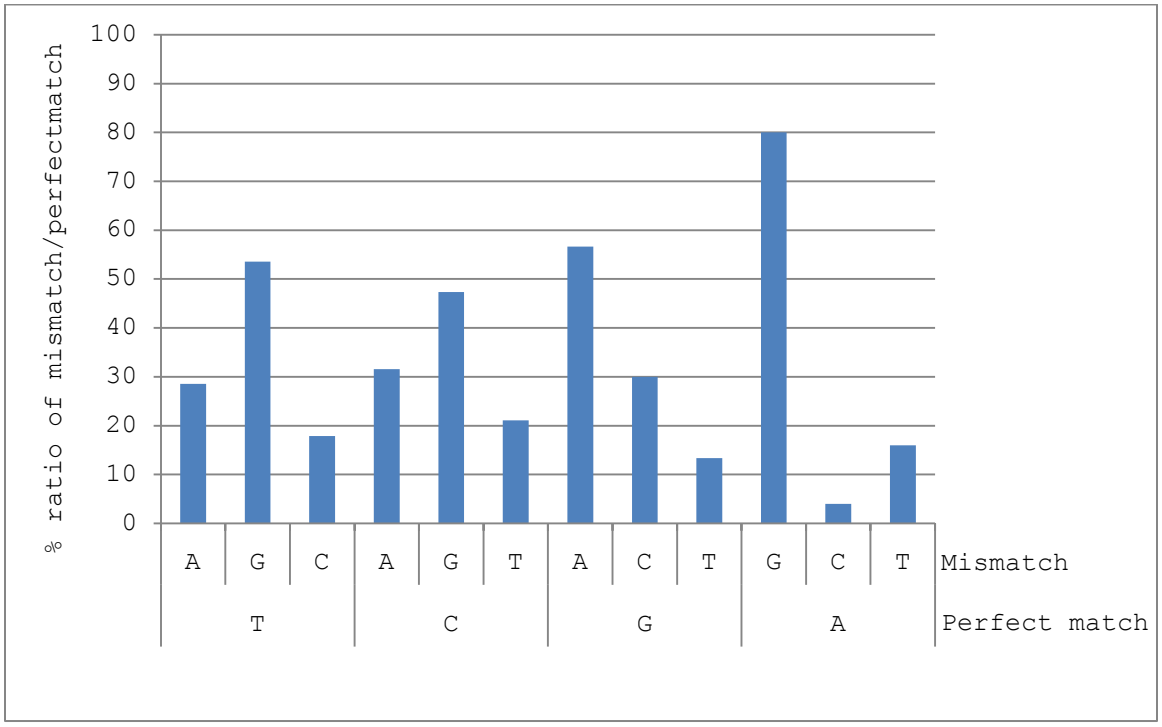


Figure 3.10 The analysis of DNA purification efficiency by the sequences of 4 capture probes.

The ratios of remaining mismatch DNA sequences were compared in the same sequence of perfect match DNA in Figures 3.6, 7, 8, 9, and 10. The high ratio of mismatch DNA to perfect match DNA meant that it was difficult to be eliminated and had the low DNA purification yield. In T-nucleotide of perfect match, the order of remaining mismatch DNA sequences was G, A, and C in Figure 3.10, so guanine monomer had the strongest nonspecific binding force among the monomers. The order of remaining mismatch DNA was A, G, T in C-nucleotide, A, C, T in G-nucleotide, and G, C, T in A-nucleotide. When the order of the purification yield by mismatch DNA sequence was compared by the different locations in capture probes, the pattern of purification was the same. In conclusion, guanine was the most difficult monomer to be purified by DNA hybridization in all mismatch DNAs, and adenine monomer was followed. The purification yield of Thymine monomer was similar with one of cytosine. The reason of purification yield difference among monomers is heteroatom. Heteroatom is any atom that is not carbon or hydrogen and makes stronger hydrogen bonding than carbon atom. Guanine has 6 heteroatoms and adenine has 5. Thymine and cytosine have 4. So, Guanine could have the strongest hydrogen bonding when mismatch DNAs are stacking in duplex form.

3.3.3 The estimation of DNA purification yield in assembly PCR

In PCR, oligonucleotides are assembled to long DNA by their complementary DNA sequences. DNA purification was performed before PCR to increase the purity of assembled product. After DNA purification, the purity of PCR with oligonucleotide templates in assembly PCR could be less than the one using traditional long template DNA. The reason is that template DNA is an oligonucleotide, so each oligonucleotide has 5’ and 3’end, and has more chance to have mismatch DNA at the end of target DNAs than long template during purification.

In the previous experiment, we found DNA purification system to be efficient to eliminate mismatch DNA on microfluidic chip, but its yield was different depending on the location and the sequence of mismatch DNA.

In this experiment, the yield of DNA purification in assembly PCR was investigated. Two synthetic oligonucleotide sets with or without purification were prepared as template DNAs in Figure 3.11. After SPPCR, one set had a purification step on a microfluidic chip and the other one did not. Template sets were assembled by PCR and amplified by second PCR. Expected size of product was 278

bp. PCR products were purified by gel extraction kit and ligased with pcrSMART vector. Ligased products were transformed into *E.coli* competent cells with electroporation and cultured on nutrient agar plate at 37°C for overnight. After 16 hours, 40 grown colonies on agar plate were picked up and cultured in 2 mL of liquid media at 37°C for overnight. Finally, liquid media samples were mini-prepped and sent to DNA Sequencing Core at the University of Michigan to compare the number of mismatch sequences.

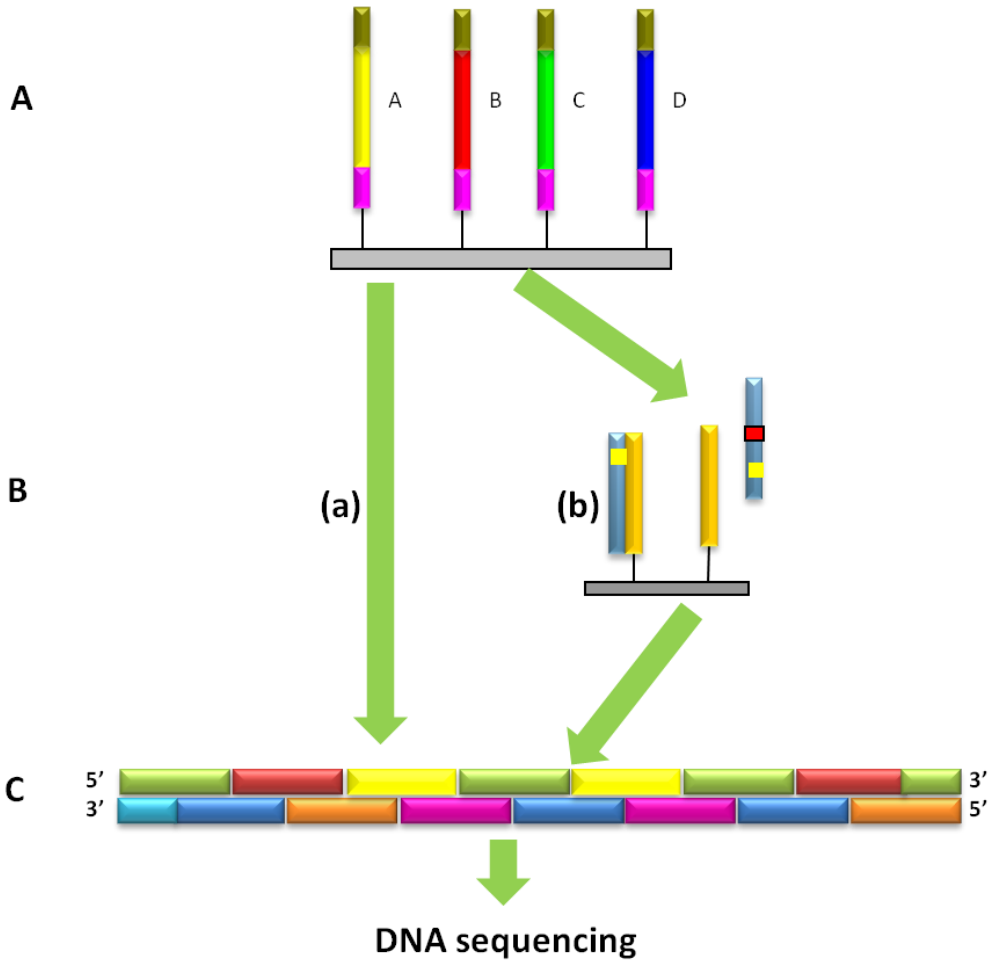


Figure 3.11 The analysis of DNA purification efficiency in assembly PCR. **A.** Two template sets were synthesized on a chip. **B.** They were treated without (a) or with (b) purification. **C.** Template sets were assembled by PCR and their sequences were compared.

Table 3.2 The investigation of DNA error between with purification and without purification.

	Average no. of errors in 278bp	Substitution	Deletion	Insertion
Purification	1.5	86.36%	9.09%	4.55%
No purification	2.2	58.33%	41.67%	

The result of purification in assembly PCR was described in Table 3.2. In 278 bp of enhanced green fluorescence protein gene, purification step reduced the number of error from 2.2 to 1.5 bp, 32% of mismatch DNAs. Especially, mismatch DNA form of deletion and insertion was eliminated efficiently. Thus, DNA purification step was required to increase the purity of target gene in assembly PCR.

3.4 Conclusions

Mismatch DNA could be produced during DNA synthesis process by the substitution, insertion, and deletion of DNA monomer leading to target DNAs with sequence errors. Thus, the elimination of mismatch DNAs is an important process to increase the purity of assembled gene.

For this purpose, DNA purification was tested by the location and different monomers of mismatch DNA in capture probes. When mismatch DNAs were located in the middle of capture probes, over 80% of them was removed. When the mismatch DNAs were in 3 bases at both ends of capture probes, 10% of them were cleared in the synthetic DNAs. So, the mismatch DNA location was found to be a key factor of the purification yield.

In the effect of DNA sequence in purification step, G monomer had the lowest purification yield followed by A, T, and C. The order of DNA purification yield by DNA monomer does not depend on the location on the capture probes.

DNA purification yield was also tested in assembly PCR, which uses synthetic oligonucleotides as a template DNA. The sequence of assembled gene with or without purification was analyzed and 32% of mismatch DNA was eliminated by DNA

purification step. Mismatch DNA of deletion and insertion in the synthetic oligonucleotides was purified more efficiently than substitution. In conclusion, DNA purification system on a microfluidic chip was an efficient process to eliminate mismatch DNA and to increase the purity of products.

3.5. Bibliography

1. Huber C. G., Oefner P. J., Bonn G. K. (1993). "High-Resolution Liquid Chromatography of Oligonucleotides on Nonporous Alkylated Styrene-Divinylbenzene Copolymers." Analytical Biochemistry 212(2): 351-358

2. R Frank, D Müller, and C Wolff (1981). "Identification and suppression of secondary structures formed from deoxy-oligonucleotides during electrophoresis in denaturing polyacrylamide-gels." Nucleic Acids Res. 9(19): 4967-4979

3. McBride et al. (1988). "A New, Reliable Cartridge for Rapid Purification of Synthetic DNA." BioTechniques, 6, 362-367

4. Karl H. Hecker, Stacy M. Green, Kaoru Kobayashi (2000). "Analysis and purification of nucleic acids by ion-pair reversed-phase high-performance liquid chromatography." Journal of Biochemical and Biophysical Methods 46(1-2): 83-93

5. Zon. (1990). "Purification of Synthetic Oligodeoxyribonucleotides." Ch. 14 in High-Performance Liquid Chromatography in Biotechnology: 301-397

6. Webster, K.R. et al. (1991). "A Rapid Method for Purification of Synthetic Oligoribonucleotides." BioTechniques 11: (05) 658-661

7. D.J. Lockhart, H. Dong, M.C. Byrne, M.T. Follettie, M.V. Gallo, M.S. Chee, M. Mittmann, C. Wang, M. Kobayashi, H. Horton et al. (1996). " Expression monitoring by hybridization to high-density oligonucleotide arrays." Nat Biotechnol 14 (1996): 1675-1680

8. L. Wodicka, H. Dong, M. Mittmann, M.H. Ho and D.J. Lockhart (1997). " Genome-wide expression monitoring in Saccharomyces cerevisiae." Nat Biotechnol 15: 1359-

1367

9. J.G. Hacia, L.C. Brody, M.S. Chee, S.P. Fodor and F.S. Collins (1996). ”Detection of heterozygous mutations in BRCA1 using high density oligonucleotide arrays and two-colour fluorescence analysis.” Nat Genet 14: 441–447

10. J.G. Hacia (1999). “Resequencing and mutational analysis using oligonucleotide microarrays.” Nat Genet 21: 42–47

11. J. Vinje and M.P. Koopmans (2000). “Simultaneous detection and genotyping of ‘Norwalk-like viruses’ by oligonucleotide array in a reverse line blot hybridization format.” J Clin Microbiol 38: 2595–2601

12. Wilkins M.H.F., A.R. Stokes A.R. & Wilson, H.R. (1953). "Molecular Structure of Deoxypentose Nucleic Acids" Nature 171: 738–740

13. Franklin R. and R.G. Gosling (1953). “Molecular Configuration in Sodium Thymonucleate.” Nature 171:740-7

14. Breslauer KJ, Frank R, Blöcker H, Marky LA (1986). "Predicting DNA duplex stability from the base sequence". PNAS 83 (11): 3746-37

15. Vainrub A, Pettitt BM (2002). “Coulomb blockage of hybridization in two-dimensional DNA arrays.” Phys Rev E Stat Nonlin Soft Matter Phys 66: 041905

16. Gadgil C, Yeckel A, Derby JJ, Hu WS (2004). “A diffusion-reaction model for DNA microarray assays.” J Biotechnol 114: 31-45.

17. Hagan MF, Chakraborty AK (2004). “Hybridization dynamics of surface immobilized DNA.” J Chem Phys 120: 4958-4968

18. Sorokin NV, Chechetkin VR, Chechetkin MA, Vasiliskov VA, Turygin AY, Mirzabekov AD (2003). “Kinetics of hybridization on the oligonucleotide microchips with gel pads.” J Biomol Struct Dyn 21: 279-288

19. Held GA, Grinstein G, Tu Y (2003). “Modeling of DNA microarray data by using physical properties of hybridization.” Proc Natl Acad Sci 100: 7575-7580

20. Peterson, A. W., R. J. Heaton, et al. (2001). "The effect of surface probe density on DNA hybridization." Nucleic Acids Res. 29(24): 5163-8

21. Peterson AW, Wolf LK, Georgiadis RM (2002). “Hybridization of mismatched or partially matched DNA at surfaces.” J Am Chem Soc 124: 14601-14607

CHAPTER 4

GENE SYNTHESIS FROM OLIGONUCLEOTIDES BY ON-CHIP ASSEMBLY PCR

4.1 Introduction

Gene synthesis is to synthesize the artificially designed gene using DNA monomer. It was first demonstrated by Har Gobind Khorana in 1970 [1]. These days, lots of companies produce commercial synthetic gene with below $1 per base because the gene synthesis could be applied in many fields. First, De Novo gene synthesis can produce totally new gene with interesting function such as protease cleavage sites, even though there is no template DNA. Second is the combinatorial biology which is the generation of target genes for molecular diagnostics and pathogen detection [2]. Third is the gene therapy to develop vectors with high stability to transfer and express the gene [3-5]. Gene synthesis can also modify protein codon in order to make over expression of specific protein [6].

Traditional gene synthesis from oligonucleotides is produced by the chemical synthesis method using phosphoramidites [7]. This method was well defined and produced over 1 µg in one column. However, it requires 20 columns to produce 20 different oligonucleotide sequences. When the size of target gene is over 700 bp, over 45 different oligonucleotides were required. It means gene synthesis would need 45 columns. Thus, chemical synthesis method may have a high cost, time, and steps.

To figure out this problem in our laboratory, oligonucleotides were synthesized through light-directed synthesis on microfluidic chips using photogenerated acid [8]. This method allows simultaneous multi-sequence oligonucleotide synthesis on one chip. In theory, over 10,000 different oligonucleotides could be synthesized at one chip, but, in this study, 20 different oligonucleotides were synthesized on a chip due to the yield of solid phase PCR (SPPCR) and assembly PCR.

Gene assembly from oligonucleotides was performed by PCR [9-14]. Oligonucleotides of target gene were synthesized on microfluidic chips and covered by SPPCR. After restriction enzyme treatment, PCR products were in double stranded (ds) form. In assembly PCR, they were used

as template DNA, which had 20 different oligonucleotides. When template DNA is double stranded DNA in assembly PCR, there is a competition between complementary DNA of template and other template DNA with overlapping sequences. The length of overlapping sequence in template DNA is 50% less than one of full template sequence, so complementary DNA has more matched sequence and higher binding force. This means complementary DNA has much higher opportunity of template hybridization than template with overlapping sequence in PCR. If the number of ds template oligonucleotides is small like 4 or 6, there is still a chance which templates can be hybridized and elongated properly with overcoming competition, even though it is a few [15]. However, as the number of different ds oligonucleotides is increased, the chance of desired template assembly is reduced. Thus, ds templates were converted to single stranded (ss) DNA by the treatment of restriction enzymes and lambda exonuclease [16]. Assembly PCR was carried out with single stranded templates, and EGFP gene was produced.

To demonstrate the assembly of multifunctional genes, EGFP gene and ZeoR gene were synthesized and assembled. Their expression was analyzed by gene cloning method.

4.2 Materials and Methods

4.2.1 Target gene Design and synthesis

Enhanced green fluorescence protein (EGFP) was tested as a target gene which has 717 bp in length [17-18]. Target gene was divided 3 fragments with 250 bp and 20 different oligonucleotides. EGFP was divided by Gene2Oligo Software [19] and DNA layout for synthesis was composed by Microsoft Excel Software (Redmond, WA, USA). Synthetic oilgonucleotides had a 41.51^{o}C of average hybridization temperature and 15.5 nucleotide of average hybridization unit.

EGFP oligonucleotides were synthesized on 7 channel serpentine chip following designed DNA layout and deprotected by ethylenediamine-ethanol solution [20]. They were recovered by SPPCR from the chip surface. Synthetic PCR products were treated by the restriction enzymes BsaI and MlyI (New England Biolab, Ipswich, MA, USA) to eliminate universal primer site.

4.2.2. Convert single stranded DNA

Double stranded DNA was converted to single stranded DNA form to prevent competitive reaction between

overlapping target DNAs and complementary DNAs of each DNA in Figure 4.1. 1 µg of SPPCR products were treated by 10 units of MlyI (New England Biolab, Ipswich, MA, USA) at 37°C for 1 hour. MlyI restriction was checked on 2% agarose gel. Synthetic oligonucleotides were treated with 5 units of Antarctic Phosphatase(New England Biolab, Ipswich, MA, USA) at 37°C for 1 hour to remove phosphate group at 5' end of them. They were treated by 10 units of BsaI (New England Biolab, Ipswich, MA, USA) at 50°C for 1 hour and complementary strands of them were removed by 5 units of lambda exonuclease at 37°C for 30 mins. Single stranded DNAs were verified by 15% native polyacrylamide gel analysis. 10 µL of DNA samples were mixed with 2.5 µL of loading gel and put the polyacrylamide gel and run at 45 V for 15 hours. They were stained by SYBRgold (Invitrogen Corporation, CA, USA) at 37°C for 30 mins in dark room and analyzed on UV transiluminator with 350 nm of wavelength.

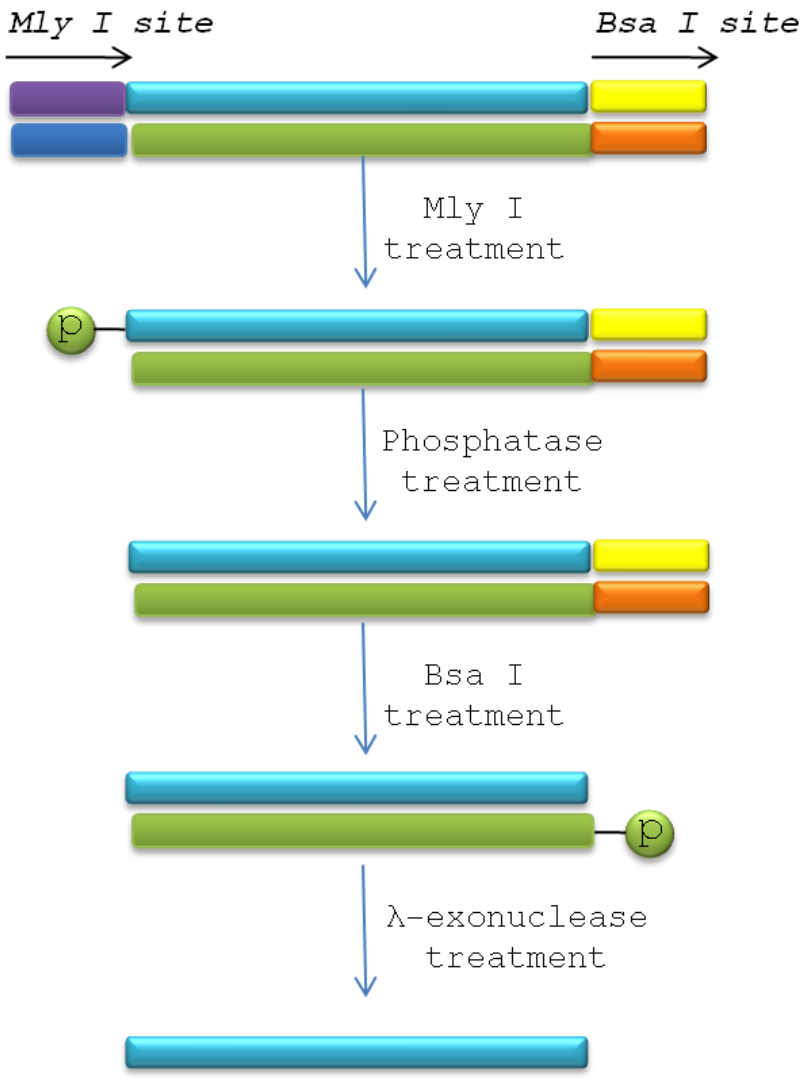


Figure 4.1 The scheme of single stranded DNA conversion by restriction enzymes and lambda exonuclease.

4.2.3. Passivation of chip surface

On-chip PCR requires passivation step to prevent surface adsorption of PCR reagents. Bovine Serum Albumin (BSA) (SIGMA-ALDRICH, St. Louis, MO, USA), formamide (SIGMA-ALDRICH, St. Louis, MO, USA), and PEG8000 (SIGMA-ALDRICH, St. Louis, MO, USA) were used as passivation materials. Chip chambers were fully filled with passivation material solutions (10% w/v in water) and another chip chamber was filled with nuclease free water as a control. They were incubated at room temperature for 30 min, and helium gas was used to blow and dry the liquid out of the chambers for 30 sec.

4.2.4. Assembly PCR of synthetic oligonucleotides

Single stranded oligonucleotides were assembled on a microfluidic chip. After passivation process with PEG 8000, PCR mixture was prepared with 0.5 U of Vent DNA polymerase, 10 mM dNTPs, buffer, 10 ng of single stranded templates, and water in 50 µL. 15 µL of PCR mixture was inserted into chip chamber with synthetic oligonucleotides and mineral oil was dropped on the holes of chip backside and slide glass was put to prevent the evaporation during PCR in

Figure 4.2. Assembly PCR was performed in a machine under 95°C, 2 min for 1 cycle; 95°C, 30 sec/35°C, 1 min,/ 35°C(+3°C/cycle), 1 min for 11 cycles; 95°C, 30 sec/45°C, 30 sec, / 68°C, 30 sec for 30 cycles, 68°C, 5 min for 1 cycle.

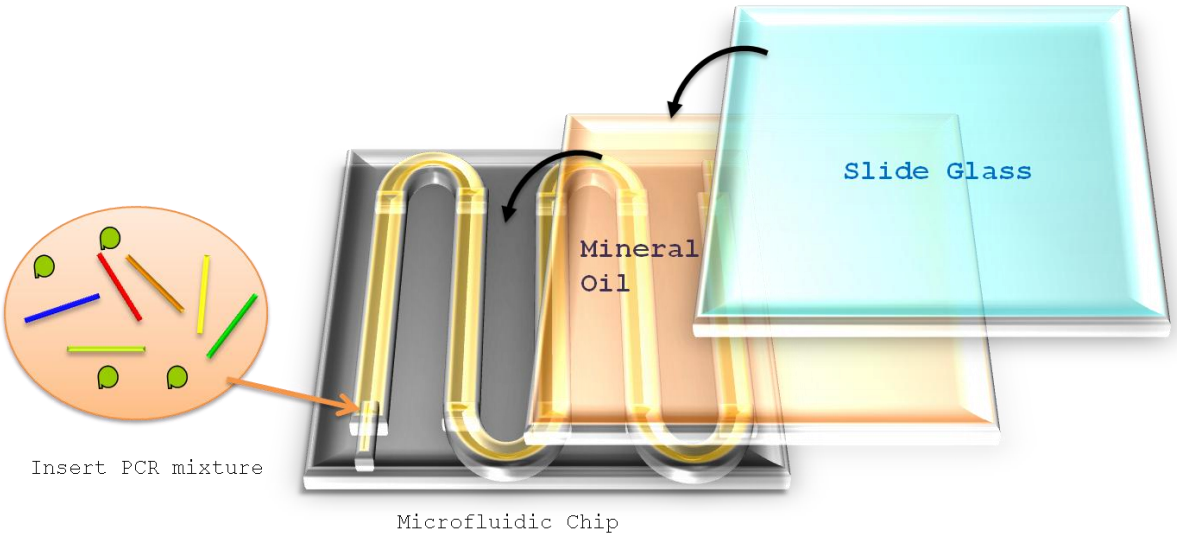


Figure 4.2 The procedure of on-chip PCR preparation.

Assembly PCR products were amplified again with 0.5 U of Vent DNA polymerase, 50 pmol of forward and reverse primers, 10 mM dNTPs, buffer, 10 ng of single stranded templates, and water in 50 µL. They were analyzed on 1% agarose gel. In this step, EGFP fragments were synthesized.

EGFP fragments were assembled by fusion PCR to make full EGFP gene. Fusion PCR had 10 ng of EGFP 1, 2, and 3 as

a template, 0.5 U of Vent DNA polymerase, 50 pmol of forward and reverser primers, 10 mM dNTPs, buffer, 10 ng of single stranded templates, and water in 50 µL. They were analyzed on 1% agarose gel.

4.2.5 Gene expression

Assembled full EGFP gene was amplified with primer set with BglII and EcoRI binding sites, and purified on agarose gel by a gel purification kit (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI). They were treated with BglII and EcoRI (New England Biolabs, Inc., MA) to expose hybridization sites with pET29b, and purified by alcohol precipitation to change buffer. For gene expression, pET29b vector (QIAGEN Inc., Valencia, CA) was treated with BglII and EcoRI, and ligated to purified synthetic EGFP gene (1:3 ratio, mole/mole) using 20 units of T4 DNA ligase (New England Biolabs, Inc., MA) at 16^{o}C for overnight. Ligated product was heated at 65^{o}C for 20 min to inactivate T4 DNA ligase activity and transformed into *E.coli* EXPRESS BL21 (DE3) Electrocompetent Cells (Lucigen, Madison, WI). 25 µL of competent cells was transformed into chilled 1.6 mL eppendorf tube and 1 µL of ligated product were added. They were mixed with micropipette tip stirring and then,

transferred to chilled cuvette. Transformation was performed in a cuvette with a 0.1 cm gap by the electroporation machine. The settings for electroporation were 10 μF, 600 Ohms, and 1800 Volts. Time constants were 3.5 to 4.5 msec. After transformation, competent cells were diluted in 975 μL of express media from Lucigen (Madison, WI, USA) and cultured at 250 rpm and 37°C for 1 hour. 100 μL of cultured media and concentrated one were spread on nutrient agar plates containing Kanamycin as an antibiotic and incubated at 37°C for overnight.

Grown colonies were picked up and spread out on nutrient agar plate with Isopropyl β-D-1-thiogalactopyranoside (IPTG, Invitrogen Corporation, CA) to induce protein expression. After checking the color of colonies, they were inoculated in 2 mL of liquid nutrient media and cultured at 250 rpm and 37°C for 16 hours. Liquid media was concentrated by centrifuge machine at 5000 rpm for 3 minutes and mini-prepped by QIAprep Spin Miniprep Kit(QIAGEN Inc., Valencia,CA). Mini-prepped samples were sent to the DNA Sequencing Core at the University of Michigan and their sequences were analyzed.

4.2.6 The assembly of EGFP and Zeocin Resistance gene

Two functional genes were assembled using EGFP and Zeocin resistant gene (ZeoR) [21-22]. ZeoR gene sequence was 345 bp in length. ZeoR was divided by Gene2Oligo and synthesized on microfluidic chip [23]. They were recovered from the chip by SPPCR and assembled by PCR after the conversion of single stranded DNA by MlyI, Antarctic phosphatase, BsaI, and lambda exonuclease treatment. Assembly PCR was performed with 0.5 U of Vent DNA polymerase, 50 pmol of forward and reverser primers, 10 mM dNTPs, buffer, 10 ng of single stranded templates, and water in 50 μL under 95°C, 2 min for 1 cycle; 95°C, 30 sec/35°C, 1 min,/ 35°C(+3°C/cycle), 1 min for 11 cycles; 95°C, 30 sec/45°C, 30 sec, / 68°C, 30 sec for 30 cycles, 68°C, 5 min for 1 cycle.

In order to conjugate EGFP and ZeoR, bridge DNA was designed to have the sequence, 'CTCGAGTCGCGC' and XhoI digestion site. EGFP gene had a bridge DNA at 3'end and ZeoR gene had it at 5'end by PCR using primer set with bridge DNA sequence. Both genes were treated with XhoI at 37°C for 1 hour and small cut fragments were removed by QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) to prevent self-ligation between two genes and cut

fragments. They were ligated by T4 DNA ligase (New England Biolab, Ipswich, MA, USA) at room temperature for 1 hour.

Ligated product with 1104 bp in length was identified by XhoI treatment on 1% agarose gel. This product was also inserted into pET 29b vector and transformation was done. All protocols followed EGFP transformation in *E.coli* EXPRESS BL21 (DE3) Electrocompetent Cells (Lucigen, Madison, WI, USA).

4.3 Results and discussion

4.3.1 Passivation effect in on-chip PCR

On-chip PCR requires the passivation process for the best amplification yield. Different passivation materials were tested during on-chip PCR, such as polyethyleneglycol (PEG) (SIGMA-ALDRICH, St. Louis, MO, USA), Bovine serum albumin (BSA) (SIGMA-ALDRICH, St. Louis, MO, USA), and formamide (SIGMA-ALDRICH, St. Louis, MO, USA). They were mixed with PCR solution and PCR was carried out on a chip. Among these materials, PEG had the best amplification yield when product band intensities were compared [24]. To find the optimal concentration of PEG, it was mixed with PCR solution to have from 0.1 to 5% final concentration and on-chip PCR was performed. PCR without PEG was also done as a negative control reaction. PCR products were analyzed on 1% agarose gel and scanned by image J software (Wayne Rasband, National Institutes of Health, MD, USA) [25].

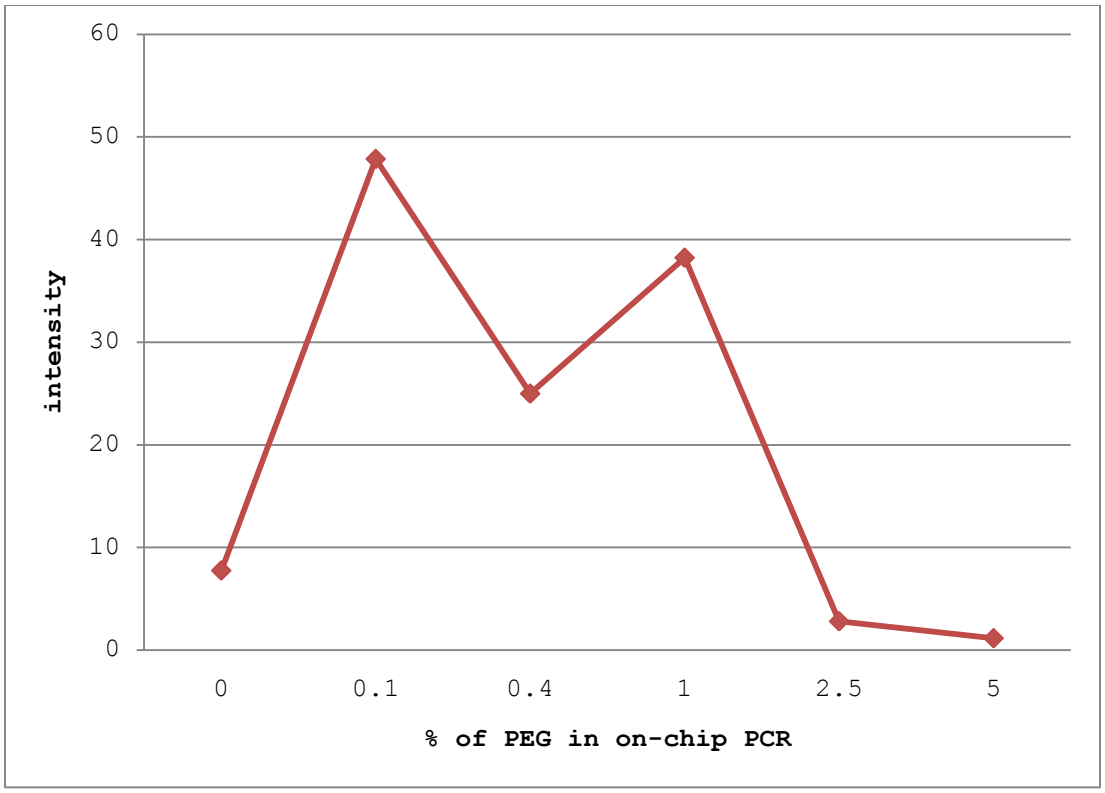


Figure 4.3 The result of PEG passivation during on-chip PCR. PCR chip has 0.1, 0.4, 1, 2.5, and 5% final concentration of PEG.

In Figure 4.3, product band intensity was the highest in 0.1% of PEG. Thus, optimal concentration of PEG was decided to be 0.1%.

4.3.2 The limit of template concentration of on-chip PCR

The limitation of template concentration was measured during on-chip PCR. PCR solution was prepared in 50 μL and template was diluted to have from 0.1 to 10,000 fmol/50 μL. When PCR solution was inserted into a chip channel, 15 μL of 50 μL PCR solution was used. Mineral oil and slide glass was used for sealing of on-chip PCR. After on-chip PCR in thermo PCR machine, products were recovered from the chip by micropipette and amplified again. PCR products were analyzed on 1% agarose gel and scanned by image J software (Wayne Rasband, National Institutes of Health, MD, USA).

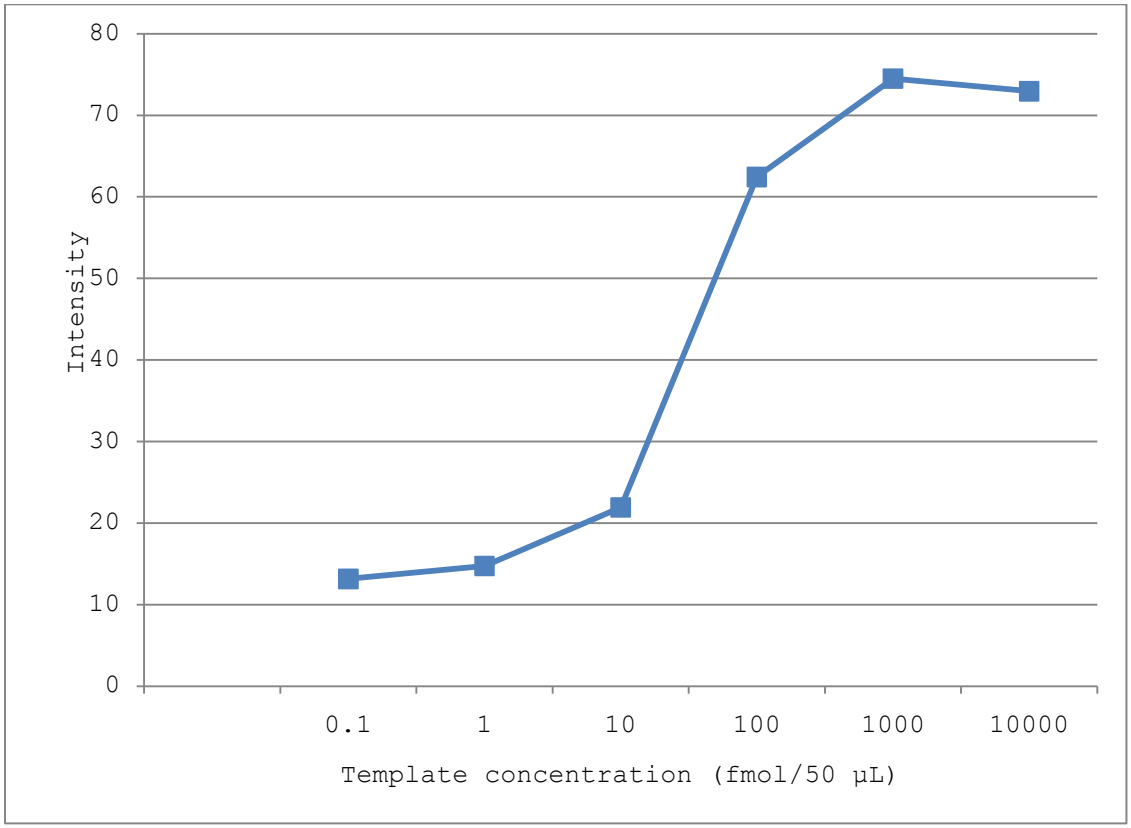


Figure 4.4 On-chip PCR with different concentration of template.

In Figure 4.4, the template DNA could be amplified when the concentration was 0.1 fmol in 50 μL. 0.01 fmol of template could not be amplified properly (data not shown). Thus, the limit of template concentration in on-chip PCR was decided to be 0.1 fmol in 50 μL.

4.3.3 The conversion of single stranded DNA

PCR products had a double stranded DNA form, which was consisted of template DNA and complementary DNA. In assembly PCR, designed template oligonucleotides had overlapping sequences with another template oligonucleotide and hybridized with their overlapping sequences. Overlapping length of each oligonucleotide was 50% less than the full length. When template DNA is in single stranded DNA form, template DNAs would be assembled by PCR following their overlapping sequences of other oligonucleotides. But, when template DNAs are in double stranded DNA form, there must be a competition between complementary DNAs of template DNAs and other template DNAs with overlapping sequences. Complementary DNAs had a full matched sequence of template DNA and another template with overlapping DNAs just had a half of matched sequence. This means complementary DNA had a higher binding force and more

binding opportunities than another template DNA during PCR cycles. Thus, assembly PCR with double stranded template DNAs could not work properly because of the binding between template DNAs and complementary DNAs.

In this experiment, double stranded DNA was converted to single stranded DNA by enzyme treatments. Two different restriction enzyme sites of MlyI and BsaI were initialized at the end of PCR products. First, PCR products were treated with MlyI and Antarctic phosphatase to remove the phosphate group of product DNAs. Then, they were treated with BsaI and had the phosphate group at 5'end of complementary DNAs. Lambda exonuclease treatment could remove complementary DNAs with phosphate groups and template DNAs were in a single stranded DNA form.

The measurement of lambda exonuclease activity

Lambda exonuclease activity was tested with commercial double stranded DNAs which were 36 bp in length and with or without phosphate group at 5'end. 1 µg of double stranded DNAs were treated with 1 unit of lambda exonuclease at 37°C in 7 tubes in 30 min and the DNA degradation by lambda exonuclease in each tube was stopped on ice for every 5 min. The Lambda exonuclease activity was analyzed on 2% agarose gel. Band intensities were scanned by Image J software.

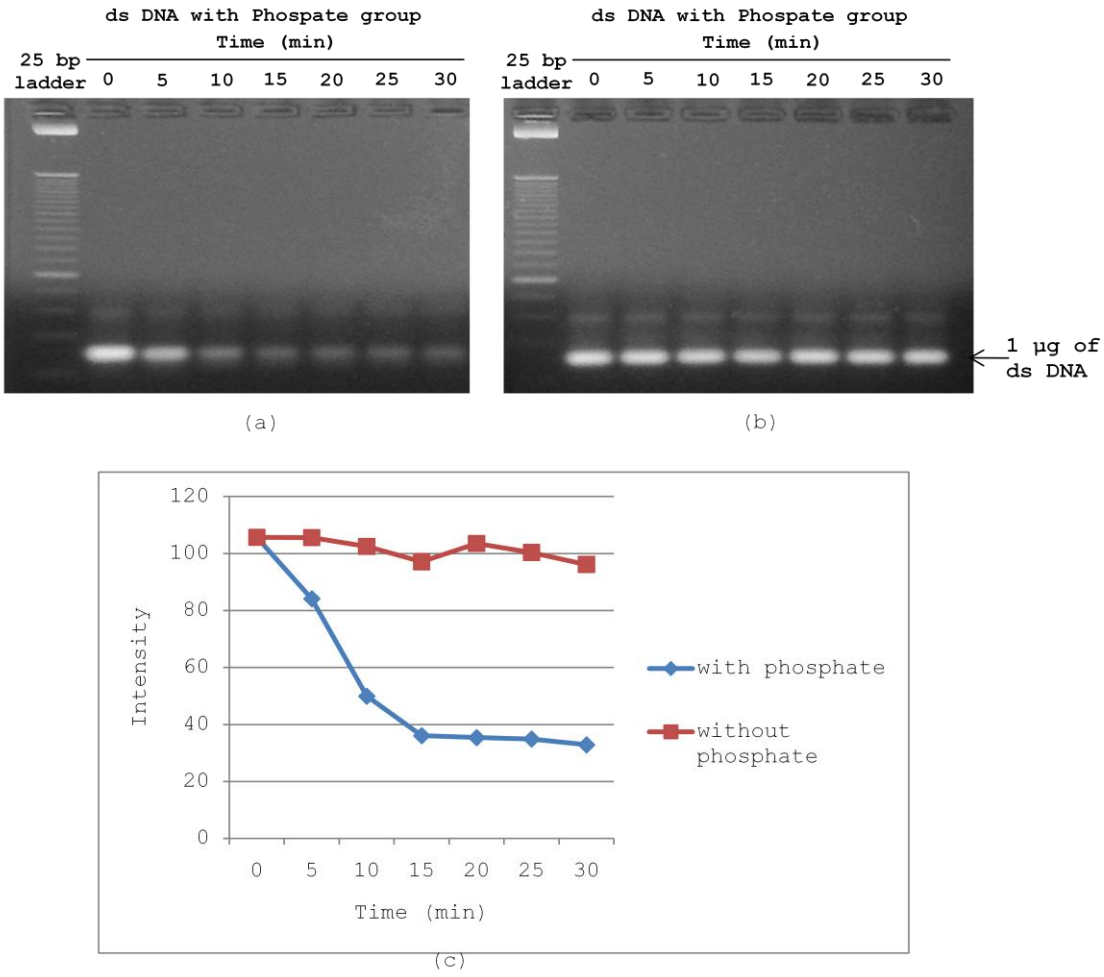


Figure 4.5 The determination of lambda exonuclease activity. 1 µg of double stranded DNA with (a) or without (b) phosphate group was treated with lambda exonuclease at 37°C for 30 mins. They were analyzed on 2% agarose gel. The change of band intensities in (a) and (b) were plotted and compared (c).

In Figure 4.5, double stranded (ds) commercial DNAs with phosphate group were degraded rapidly by 15 min, and slowly after 15 min. The intensities of ds DNAs without phosphate group had no critical change in 30 min. Thus, 1 unit of lambda exonuclease could remove 1 µg of DNA in 15 min efficiently.

The conversion of single stranded DNA

SPPCR products in double stranded form were converted to single stranded DNA by the treatment of enzymes. They were treated with MlyI and Antarctic phosphatase. After heat inactivation of Antarctic phosphatase activity, they were treated with BsaI. Finally, SPPCR product with phosphate group at the 5'end of complementary DNAs were mixed with lambda exonuclease in 15 min and analyzed on 15% native polyacrylamide gel with commercial DNAs as a marker.

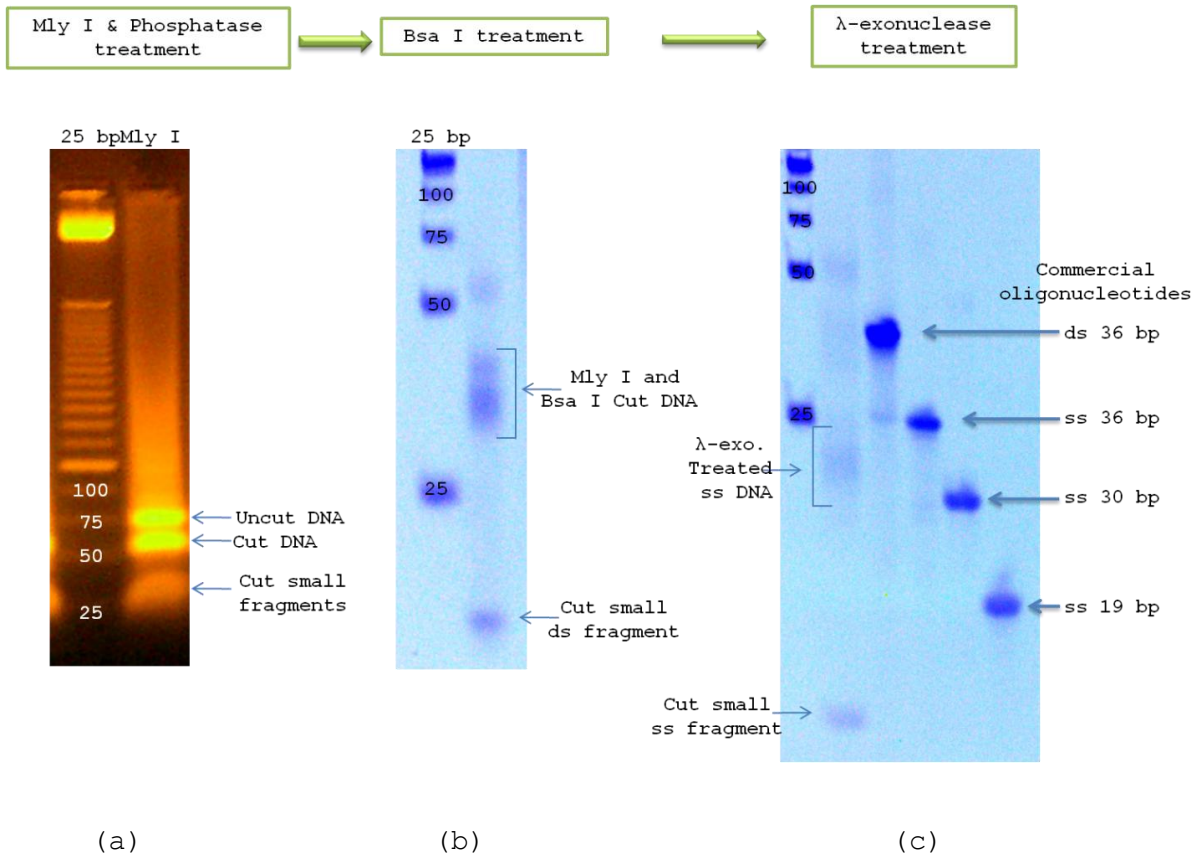


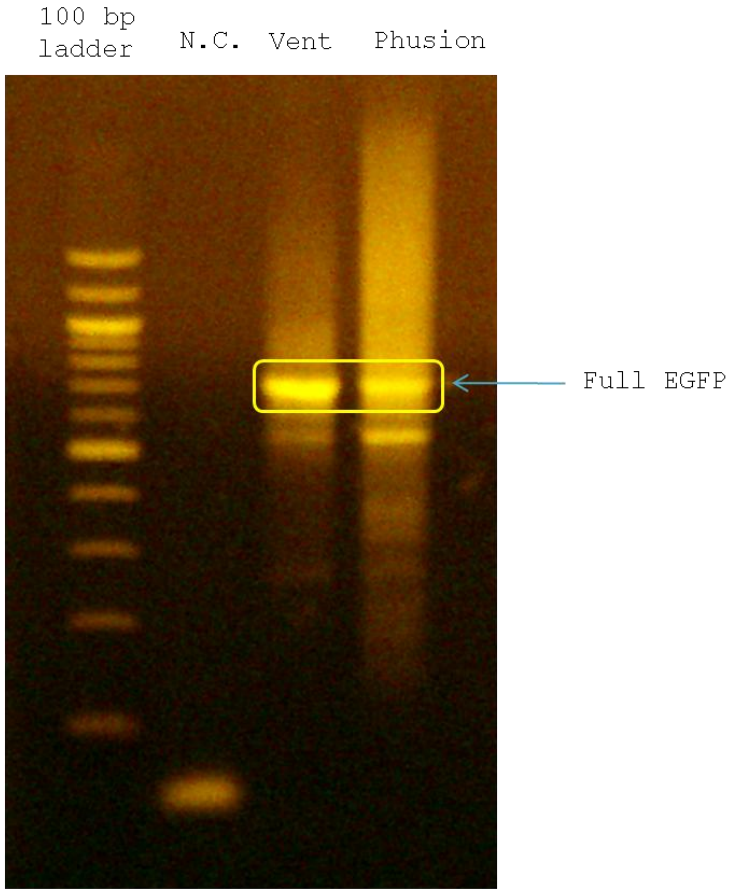
Figure 4.6 The result of ss DNA conversion by restriction enzymes and lambda exonuclease. 1 µg of double stranded SPPCR products were treated with MlyI and analyzed on 2% agarose gel (a). They were digested by BsaI and loaded on 15% native polyacrylamide gel. To make ss DNA, PCR products were treated with lambda exonuclease at 37°Cfor 15 mins. They were loaded on 15% native polyacrylamide gel with size marker DNAs (c).

In Figure 4.6 (a), MlyI removed 5’ end part of SPPCR products properly. Even though there were uncut DNAs due to less reaction time or amount of MlyI, half of products moved down below 55 bp. After Antarctic phosphatase and BsaI treatment, most of ds SPPCR products were located between 25 and 50 bp, and meant that BsaI digested their 3’end efficiently in Figure 4.6 (b). Lambda exonuclease removed complementary DNAs, and SPPCR products were in single stranded form in Figure 4.6 (c). Thus, ds synthetic DNAs were converted to single stranded form and assembly PCR was performed with them successfully.

4.3.4 The assembly of synthetic EGFP

The oligonucleotide set of EGFP gene was synthesized on a chip. They consisted of 3 gene fragments of 250 bp in length, and a set of 20 oligonucleotides. After oligonucleotide synthesis, they were recovered by SPCPR from the chip surface. Then, they were converted to single stranded DNA form by the treatment of restriction enzymes and lambda exonuclease, and assembled by PCR. Each assembled EGFP fragment was verified and purified on 1% agarose gel, and combined by fusion PCR. Template DNA in fusion PCR was EGFP fragments and primer set was first

forward oligonucleotide and last reverse one in full EGFP gene. Fusion PCR used two kinds of DNA polymerase enzyme, such as vent DNA polymerase (New England Biolab, Ipswich, MA, USA) and phusion high fidelity polymerase (New England Biolab, Ipswich, MA, USA), because they have high amplification yield and proofreading function compared with *Taq* DNA polymerase (New England Biolab, Ipswich, MA, USA). Expected size of EGFP gene was 717 bp in length. After fusion PCR, they were ligased to pcrSMART vector and transformed into electrocompetent cells. After miniprep, they were sent to DNA Sequencing Core at the University of Michigan to analyze their purity.



(a)

Avg.(in 717 bp)	Substitution	Deletion	Insertion
3.06	2.06	0.65	0.35
% Error	67.28	21.15	11.57

(b)

Figure 4.7 The result of EGFP gene assembly by PCR from synthetic oligonucleotides. Assembled EGFP gene was loaded on 2% agarose gel (a). The average number of error in synthetic gene was analyzed (b).

In Figure 4.7 (a), full EGFP gene was assembled over 700 bp marker in both vent and phusion high fidelity DNA polymerases. EGFP gene was produced successfully from synthetic oligonucleotide on a chip. Next, their sequences were analyzed and compared with 18 samples. In Figure 4.7 (b), synthetic EGFP gene had 3.06 bp of average mismatch sequence in 717 bp and 67% of mismatch DNA were caused by substitution and deletion, followed by insertion.

4.3.5. EGFP gene expression

Assembled EGFP gene was expressed for enhanced green fluorescent protein in pET29b vector. Even though there was one mismatch DNA, EGFP could not make green fluorescence. To verify the expression yield of synthetic EGFP, it was inserted into pET29b vector and transformed into electrocompetent BL21 (DE3) cells. They were cultured on nutrient agar plate with IPTG at 37^{o}C for 2 days. Colonies were streaked again on agar plate with IPTG for easy verification. The color was scanned and a picture was taken on UV transilluminator at 350 nm.

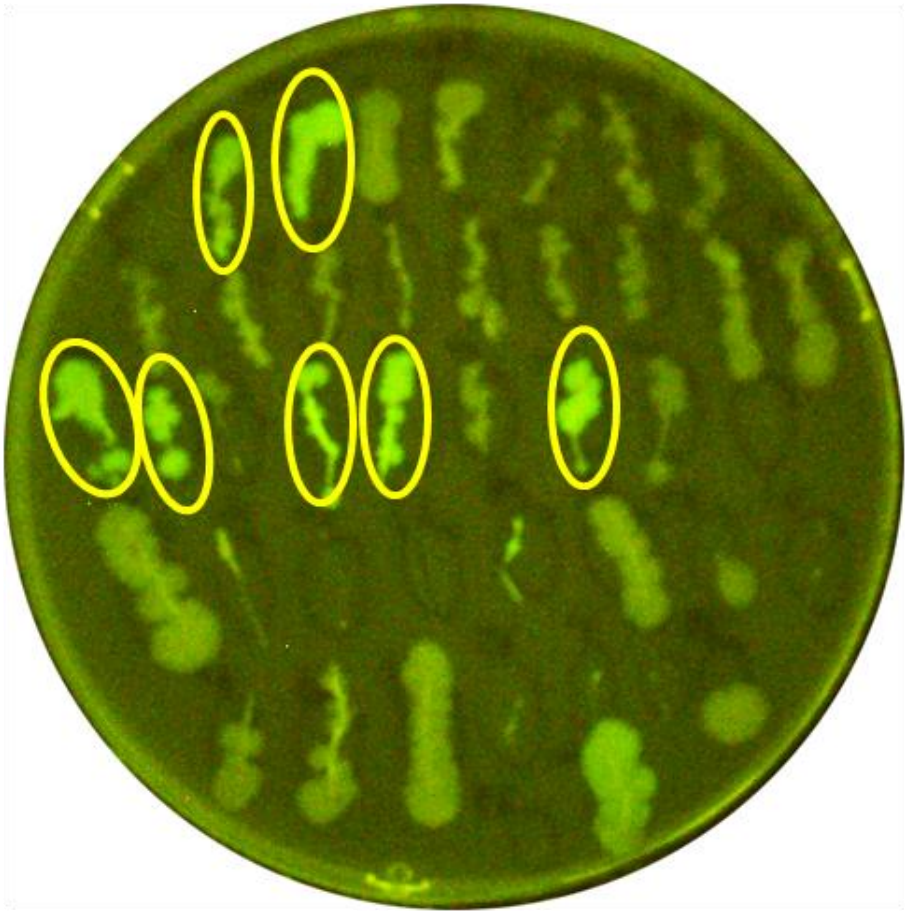


Figure 4.8 One streaked bar of cell represented for one colonies. 28 colonies was streaked and 7 colonies in yellow circles produced green fluorescence.

In Figure 4.8, 7 of 28 colonies had a green fluorescence, so 25% of synthetic EGFP gene produced green signal.

4.3.6. The assembly of two functional genes: EGFP and Zeocin resistant gene (ZeoR)

In this experiment, gene synthesis demonstrated that multi-functional genes could be synthesized and their functions can be combined. Two functional genes were synthesized and combined. Target genes were EGFP with 717 bp and Zeocin resistant gene (ZeoR) with 345 bp. Zeocin resistance gene had a resistance for Zeocin antibiotic, so bacteria with ZeoR can survive on nutrient agar plate with Zeocin. They were synthesized on a microfluidic chip by designed DNA layout, collected and assembled by PCR. Full EGFP and ZeoR genes were connected separately with 12 bp in length of bridge DNA with XhoI binding sequence. After XhoI treatment of them, full EGFP gene and ZeoR gene were combined by ligation.

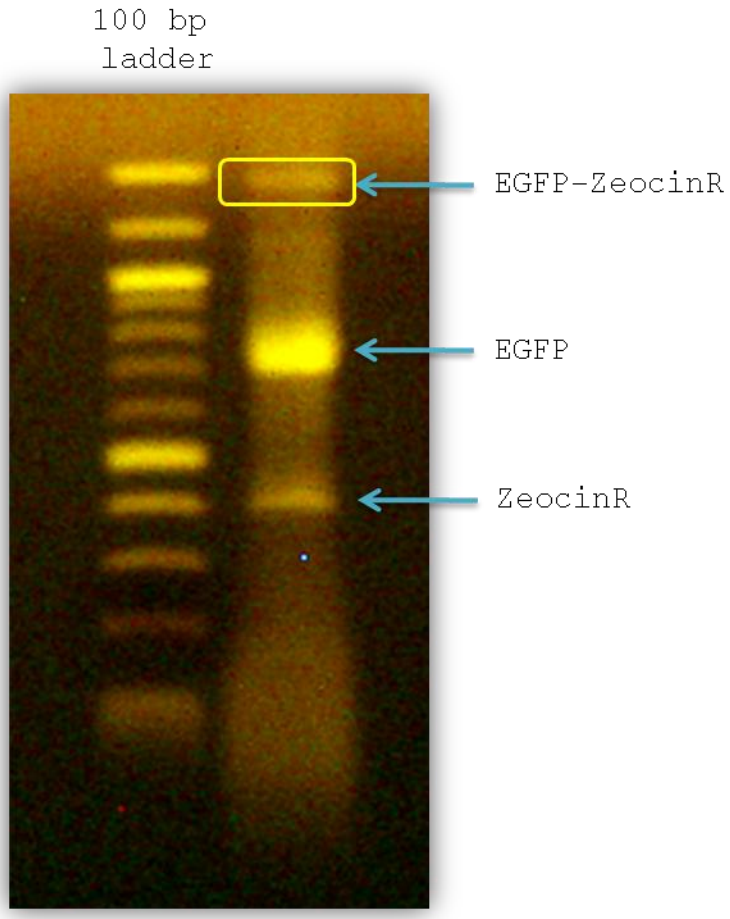


Figure 4.9. The assembly of EGFP-ZeoR complex on 2% agarose gel.

In Figure 4.9, EGFP-ZeoR product was assembled and located between 1100 bp and 1200 bp. Assembled EGFP-ZeoR product had bridge DNA and restriction enzyme sites at both ends, so the overall size looked closed to 1200 bp.

4.4 Conclusions

In this chapter, EGFP gene was synthesized and assembled on a microfluidic chip. The passivation process with PEG material enhanced the yield of on-chip PCR [24]. To remove the competition between complementary DNAs and templates, complementary DNAs were eliminated by restriction enzymes and lambda exonuclease. EGFP gene was assembled from template of single stranded oligonucleotides by assembly PCR and 25% of it was expressed.

Multifunctional gene, EGFP-ZeoR gene was designed and synthesized on a microfluidic chip. It was assembled by assembly PCR on a chip properly. This showed the ability to design and synthesize modified target genes and totally new genes. Finally, we found our system can produce desired target gene from oligonucleotides with high specificity and potential with high throughput gene assembly system.

4.5 Bibliography

1. Khorana HG, Agarwal KL, Büchi H, Caruthers MH, Gupta NK, Kleppe K, Kumar A, Otsuka E, RajBhandary UL, Van de Sande JH, Sgaramella V, Terao T, Weber H, Yamada T (1972). "Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast." J Mol Biol. 72(2):209-17

2. Pelletier J, Sidhu S (August 2001). "Mapping protein-protein interactions with combinatorial biology methods". Curr. Opin. Biotechnol. 12 (4): 340–7

3. Morgan, R. A., M. E. Dudley, et al. (2006). "Cancer regression in patients after transfer of genetically engineered lymphocytes." Science 314(5796): 126-9.

4. Ott, M. G., M. Schmidt, et al. (2006). "Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1." Nat Med **12**(4): 401-9

5. Brown, B. D., M. A. Venneri, et al. (2006). "Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer." Nat Med 12(5): 585-91

6. Riccardo, S., G. Tortoriello, et al. (2007). "The coding/non-coding overlapping architecture of the gene encoding the Drosophila pseudouridine synthase." BMC Mol Biol 8: 15

7. Caruthers MH, Barone AD, Beaucage SL, Dodds DR, Fisher EF, McBride LJ, Matteucci M, Stabinsky Z, Tang JY, Chemical synthesis of deoxyoligonucleotides by the phosphoramidite method. Methods Enzymol, 1987. 154: 287-313

8. Gao X, Gulari E, Zhou X, In situ synthesis of oligonucleotide microarrays. Biopolymers, 2004. 73: 579-596

9. Dillon, P. J. and C. A. Rosen (1990). "A rapid method for the construction of synthetic genes using the polymerase chain reaction." Biotechniques 9(3): 298-300

10. Stemmer, W. P., A. Crameri, et al. (1995). "Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides." Gene 164(1): 49-53

11. Rydzanicz, R., X. S. Zhao, et al. (2005). "Assembly PCR oligo maker: a tool for designing oligodeoxynucleotides for constructing long DNA molecules for RNA production." Nucleic Acids Res 33(Web Server issue): W521-5

12. Xiong, A. S., Q. H. Yao, et al. (2006). "PCR-based accurate synthesis of long DNA sequences." Nat Protoc 1(2): 791-7

13. Reisinger, S. J., K. G. Patel, et al. (2006). "Total synthesis of multi-kilobase DNA sequences from oligonucleotides." Nat Protoc 1(6): 2596-603

14. Wu, G., J. B. Wolf, et al. (2006). "Simplified gene synthesis: a one-step approach to PCR-based gene construction." J Biotechnol 124(3): 496-50

15. TerMaat, J. R., E. Pienaar, et al. (2009). "Gene synthesis by integrated polymerase chain assembly and PCR amplification using a high-speed thermocycler." J Microbiol Methods 79(3): 295-300

16. Little, J. W. (1981). "Lambda exonuclease." Gene Amplif Anal 2: 135-45

17. Tsien, R. Y. (1998). "The green fluorescent protein." Annu Rev Biochem 67: 509-4

18. Thastrup O, Tullin S, Kongsbak Poulsen L, Bjørn S (1995). "Fluorescent Proteins". US patent

19. Rouillard, J. M., W. Lee, et al. (2004). "Gene2Oligo: oligonucleotide design for in vitro gene synthesis." Nucleic Acids Res 32(Web Server issue): W176-80

20. Hogrefe, R. I., M. M. Vaghefi, et al. (1993). "Deprotection of methylphosphonate oligonucleotides using a novel one-pot procedure." Nucleic Acids Res 21(9): 2031-8

21. Calmels, T., M. Parriche, et al. (1991). "High efficiency transformation of Tolypocladium geodes conidiospores to phleomycin resistance." Curr Genet 20(4): 309-14

22. Drocourt, D., T. Calmels, et al. (1990). "Cassettes of the Streptoalloteichus hindustanus ble gene for transformation of lower and higher eukaryotes to phleomycin resistance." Nucleic Acids Res 18(13): 4009

23. Rouillard, J. M., W. Lee, et al. (2004). "Gene2Oligo: oligonucleotide design for in vitro gene synthesis." Nucleic Acids Res 32(Web Server issue): W176-80

24. Yong-Mei Xia, Zhi-Shan Hua, Onnop Srivannavit, Ayse Bilge Ozel and Erdogan Gulari (2007). “Minimizing the surface effect of PDMS–glass microchip on polymerase chain reaction by dynamic polymer passivation.” J Chem Technol Biotechnol 82:33–38

25. http://rsbweb.nih.gov/ij

CHAPTER 5

LIGATION AND TRANSFORMATION ON A MICROFLUIDIC CHIP

5.1 Introduction

Transformation is a genetic alteration process by which a foreign DNA is inserted into competent cells [1-2]. It is used for the protein expression of foreign DNA. In our project, this process is required to check whether synthetic DNA is translated to protein in a cell properly.

In molecular biology, transformation is performed in an Eppendorf tube on ice bowl and water bath [2-3]. To have a potential of high throughput system in our experiment, it was tried on a microfluidic chip [5-7]. After EGFP gene synthesis, it was ligased with a plasmid on a chip and ligation mixture was transferred into the chip channel. Transformation was performed on a heat block.

In this chapter, the feasibility and potential of on-chip transformation was tested and analyzed.

5.2 Materials and Methods

5.2.1 Preparation of EGFP gene and pET 29b vector

Assembled full EGFP gene was amplified by primer set with BglII and EcoRI binding sites and purified on agarose gel by a gel purification kit (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI). They were treated by BglII and EcoRI (New England Biolabs, Inc., MA), and purified by alcohol precipitation to change buffer. For gene expression, pET29b vector (QIAGEN Inc., Valencia, CA) was treated with BglII and EcoRI.

5.2.2 Ligation on a microfluidic chip

50 µL of ligation solution was prepared with pET29b and EGFP gene (1:3, mole/mole), 20 units of T4 DNA ligase (New England Biolabs, Inc., MA), buffer, and water. 15 µL of ligation solution was inserted into a microfluidic chip and the other 25 µL was transferred into 1.6 mL eppendorf tube as a control. Ligation was performed at room temperature for 1 hour and the mixture was heated at 65°C for 20 min to inactivate T4 DNA ligase [4].

5.2.3 Transformation of ligated product on a microfluidic chip

EXPRESS BL21 (DE3) Chemically Competent Cell (Lucigen, Madison, WI) was thawed on ice for 15 min and 40 µL of it was transferred to chilled 1.6 mL eppendorf tube. 1 µL of ligation solution was added in competent cell and stirred by a micropipette tip. 15 µL of ligation solution was inserted into a microfluidic chip and incubated on ice for 30 min. 40 µL of competent cell and 1 µL of ligation mixture was incubated in a tube as a control.

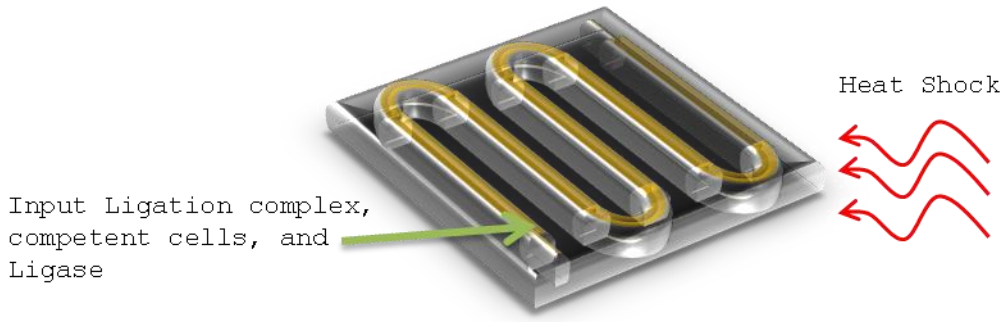


Figure 5.1 On-chip DNA ligation and transformation of synthetic EGFP gene.

After 30 min, they were put on heat block and did heat shock at 42°C for 45 sec in Figure 5.1. They were transferred to ice immediately and diluted in 960 µL of expression media from Lucigen (Madison, WI, USA). After the culture was incubated at 37°C for 1 hour, 100 µL of cultured media was spread out on nutrient agar plate with kanamycin. The remaining media was concentrated at 5000 rpm for 3 min and suspended again in 100 µL of expression media. It was also spread on nutrient agar plate with kanamycin.

5.3 Results and discussion

5.3.1. Transformation yield on a microfluidic chip

Ligation samples were prepared on a tube and chip. Transformation was performed on a chip and in a tube as a control in Figure 5.2. All transformed solutions were spread on agar plate with kanamycin.

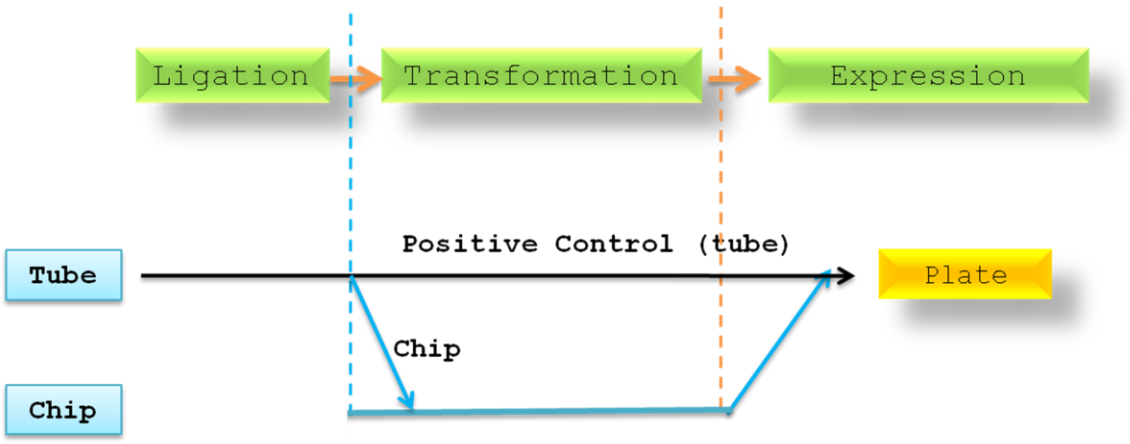


Figure 5.2 The scheme of on-chip transformation experiment.

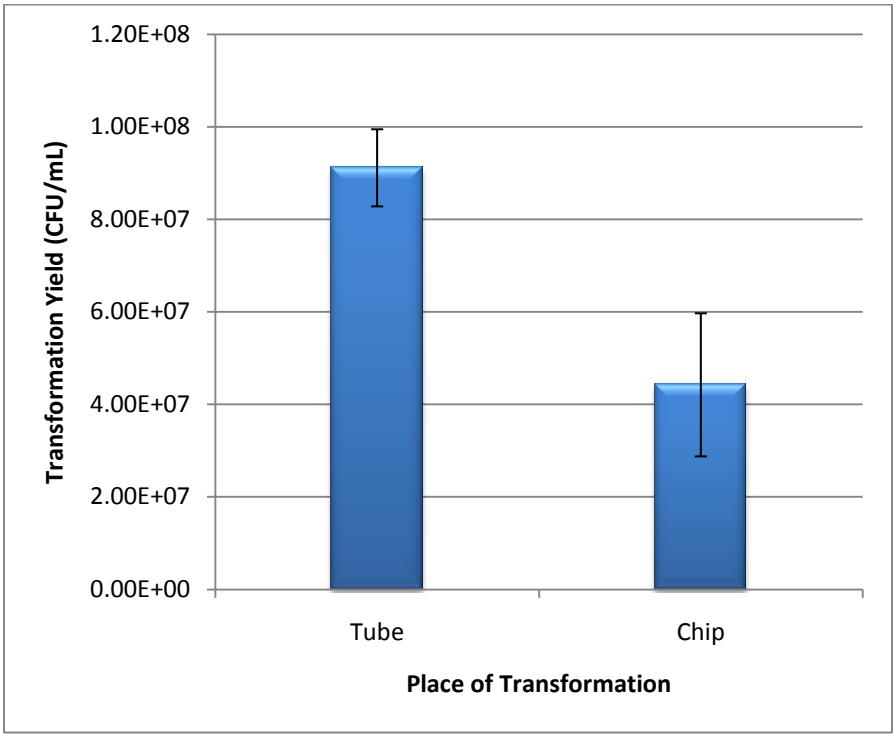


Figure 5.3 The comparison of transformation yield in a tube and a chip.

In Figure 5.3, transformation in a tube showed 9.15×10^7 CFU/mL of yield and the one in a chip had 4.42×10^7 CFU/mL. The percentage of standard deviations in tube and chip were 9.1% and 35%, respectively. The percent ratio of in chip to in tube showed 47.8%. The transformation yield in a chip reduced around 50% compared with the one in a tube, but it still had reasonable yield for protein expression. Thus, on-chip transformation system could be applied using high throughput system for protein expression.

5.4 Conclusions

In this chapter, on-chip transformation system was demonstrated with competent cells and EGFP-plasmid DNA. When the transformation yield was compared to the one in a tube, on-chip transformation had 50% yields. We found that competent cells could still have their activities on a chip when transformed with foreign DNA by using heat shock.

For a complete on-chip protein expression system, cell culture on a chip and high selective protein expression system should also be considered and developed.

5.5 Bibliography

1. Chen, I. and D. Dubnau (2004). "DNA uptake during bacterial transformation." Nat Rev Microbiol 2(3): 241-9

2. Hanahan, D. (1983). "Studies on transformation of Escherichia coli with plasmids." J Mol Biol 166(4): 557-80

3. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, (2nd Ed.): 1.76-1.84

4. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, (2nd Ed.): 1.53-1.73

5. Nagamine, K., S. Onodera, et al. (2005). "On-chip transformation of bacteria." Anal Chem 77(13): 4278-81

6. Ingham, C. J., A. Sprenkels, et al. (2007). "The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms." Proc Natl Acad Sci U S A 104(46): 18217-22

7. Jong Wook Hong, Yan Chen, W French Anderson and Stephen R Quake1 (2006). “Molecular biology on a microfluidic chip.” J. Phys.: Condens. Matter 18: S691–S701

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In this project, the main objective is the study of on-chip gene assembly system [1-6]. Target DNA is designed, synthesized, purified, assembled, and expressed on a microfluidic chip.

For this purpose, Chapter 2 revealed the design and synthesis of oligonucleotides of target gene. Enhanced green fluorescence protein (EGFP) was set up as a target gene. It was divided into oligonucleotides by Gene2Oligo software [7] and DNA layout for synthesis was prepared. Oligonucleotides of EGFP were synthesized on 3 microfluidic chips with photogenerated acid and each chip had 20 different oligonucleotides. Synthetic oligonucleotides were collected by solid phase PCR (SPPCR) from a chip surface. SPPCR was optimized by controlling the density of synthetic DNA, and they were obtained from a chip surface properly.

SPPCR potentially reduced recovery time and work involved in synthetic DNA generation.

Chapter 3 demonstrated efficient DNA purification system by hybridization. Synthetic DNAs tried to be purified by capture probes on a microfluidic chip. First, the purification yield was analyzed by the location of mismatch DNA in capture probes. We found the mismatch DNA in the middle of capture probe more distinctive as this probe was eliminated more efficiently than the ones at the ends. Second, the purification yield was measured by the sequence of mismatch DNA. Guanine base had the strongest non-specific binding force and adenine, thymine, and cytosine followed. It was analyzed in assembly PCR by the comparison between with and without purification and it showed an increase of purity of the assembled gene.

Chapter 4 showed the on-chip assembly PCR with synthetic DNAs. For the optimal amplification, passivation effect was tested by materials and their concentrations. Passivation prevented for physical adsorption of PCR reagents efficiently. EGFP fragments were assembled by PCR and finally full EGFP gene was combined from them. Multifunctional genes were also assembled. Target genes were EGFP and Zeocin resistant gene (ZeoR). They were

synthesized on a microfluidic chip and combined. This experiment showed the opportunity of multifunctional genes, which we desired.

Chapter 5 provided the potential of on-chip protein expression by the ligation and transformation on a chip [8-9]. Synthetic gene was ligased with plasmid on a chip and transferred to another chip chamber. Ligased product was transformed into competent cell on a chip and they were expressed on a nutrient agar plate.

Finally, we successfully demonstrated on-chip gene assembly from DNA design to protein expression. Even though all processes were performed separately, this project showed the potential of integrated on-chip gene assembly system.

6.2. Recommendations for future work

In this study, we demonstrated on-chip gene assembly from oligonucleotides with specificity by solid phase PCR, purification, assembly PCR, and transformation. We also suggested the potential of on-chip protein expression. This means DNA could be synthesized, purified, and transformed on an integrated microfluidic chip. It is

possible to do the automated and high throughput gene synthesis system.

For the completion of high throughput gene synthesis, there are three limitations in my project. First is cell culture in a microfluidic channel after on-chip transformation [9]. Transformed cells require air during cell culture, so aeration in a chip channel should be developed without the evaporation of cell mixture.

Second is the selection of transformed product. After on-chip transformation, plasmid without insert DNA could be grown mainly instead of insert DNA-plasmid complex. To figure out this issue, new custom plasmid should be designed which has the partial of antibiotics at the end of ligation site. In this case, synthetic gene is also designed to have the partial of antibiotics, and insert DNA-plasmid mixture has the full antibiotic sequence. Only insert DNA-plasmid mixture can be grown in an antibiotic media and selective cell culture must be performed on a chip.

Last one is the integration of all processes with micro valve and pump. Among the gene synthesis processes, all materials should be transferred and removed on time. This step makes the completion of a high throughput gene

and protein synthesis system, and can reduce the cost and time below 10% of current technology.

6.3 Bibliography

1. Zhou, X., S. Cai, et al. (2004). "Microfluidic PicoArray synthesis of oligodeoxynucleotides and simultaneous assembling of multiple DNA sequences." Nucleic Acids Res 32(18): 5409-17

2. Tian, J., H. Gong, et al. (2004). "Accurate multiplex gene synthesis from programmable DNA microchips." Nature 432(7020): 1050-4

3. Xiong, A. S., Q. H. Yao, et al. (2006). "PCR-based accurate synthesis of long DNA sequences." Nat Protoc 1(2): 791-7

4. Reisinger, S. J., K. G. Patel, et al. (2006). "Total synthesis of multi-kilobase DNA sequences from oligonucleotides." Nat Protoc 1(6): 2596-603

5. Wu, G., J. B. Wolf, et al. (2006). "Simplified gene synthesis: a one-step approach to PCR-based gene construction." J Biotechnol 124(3): 496-50

6. TerMaat, J. R., E. Pienaar, et al. (2009). "Gene synthesis by integrated polymerase chain assembly and PCR amplification using a high-speed thermocycler." J Microbiol Methods 79(3): 295-300

7. Rouillard, J. M., W. Lee, et al. (2004). "Gene2Oligo: oligonucleotide design for in vitro gene synthesis." Nucleic Acids Res 32(Web Server issue): W176-80Little, J. W. (1981). "Lambda exonuclease." Gene Amplif Anal 2: 135-45

8. Nagamine, K., S. Onodera, et al. (2005). "On-chip transformation of bacteria." Anal Chem 77(13): 4278-81

9. Ingham, C. J., A. Sprenkels, et al. (2007). "The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms." Proc Natl Acad Sci U S A 104(46): 18217-22