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INSTITUTE OF HEALTH SCIENCES

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PH.D. THESIS

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stage from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, and I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

27/02/2025

Esma AYBAKAN

PREFACE AND ACKNOWLEDGEMENT

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

°C	Centigrade Degree
Amv RT	Avian Myeloblastosis Virus Reverse Transcriptase
APS	Ammonium Persulfate
BCA	Bicinchoninic Acid
BME	B-Mercaptoethanol
bp	Base Pair
cDNA	Complementary DNA
Cq	Quantification Cycle
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CV	Column Volume
dCTP	Deoxycytidine Triphosphate
ddH_2O	Double Distile Water
dGTP	Deoxyguanidine Triphosphate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
dsDNA	Double Strand DNA
DTT	Dithiothreitol
E. coli	*Escherichia Coli*
ECL	Enhanced Chemiluminescence
EtBr	Ethidium Bromide
FPLC	Fast Protein Liquid Chromatography
Fw	Forward Primer
g	G-Force
G2P	Gene 2 Protein
GFP	Green Flourescent Protein
gr	Gram
HCl	Hydrochloric Acid
HDA	Helicase Dependent Amplification
His	Histidine

HPLC	High Pressure Liquid Chromatography
HRP	Horseradish Peroxidase
IMAC	Immobilized Metal Affinity Chromatography
IgG	Immunoglobulin G
INAAM	Isothermal Nucleic Acid Amplification Methods
IPGT	Isopropyl ß-D-1-Thiogalactopyranoside
Kan	Kanamycin
kb	Kilobase
KCl	Potassium Chloride
L	Liter
LAMP	Loop-Mediated Isothermal Amplification
LB	Luria Bertani
LBA	Luria Bertani Agar
LIC	Ligation Independent Cloning
M	Molar
mg	Milligram
$MgCI_2$	Magnesium Chloride
mL	Mililiter
mM	Millimolar
mm	Millimeter
NAAT	Nucleic Acid Amplification Testing
NaCl	Sodium Chloride
NEAR	Enzyme Amplification Reaction
ng	Nanogram
nm	Nanometer
OD	Optic Density
ORTE	Observable Real Time Electrophoresis
p1GFP	Pet His6 GFP TEV LIC Cloning Vector
P2	Protein 2 of M13KE
pAb	Polyclonal Antibody
PAGE	Polyacrylamide Gel Electrophoresis
PC	Positive Control
PCR	Polymerase Chain Reaction
pH	Potential Hydrogen

PMA	P2 Mediated Amplification
POC	Point Of Care
psi	Pounds Per Square Inch
Q-PCR	Quantitative Polymerase Chain Reaction
RCA	Rolling Circle Amplification
RF	Replicative Form
RNA	Ribonucleic Acid
RPA	Recombinase Polymerase Amplification
rpm	Rotation Per Minute
rRNA	Ribosomal Ribonucleic Acid
RT	Room Temperature
Rv	Reverse Primer
SDS	Sodium Dodecyl Sulfate
SEC	Size Exclusion Chromatography
ssDNA	Single Strand DNA
TAE	Tris Acetic Acid
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline Tween-20
TEMED	N, N,N′,N′ -Tetramethyl ethylenediamine
TEV	Tobacco Etch Virus
TS	Test Sample
TSS	Transformation And Storage Buffer
U	Unit
UV	Ultraviolet Light
V	Volt
v:i	Vector To Insert Molar Ratio
w/v	Weight/Volume
w/w	Weight/Weight
µg	Microgram
µL	Microliter
µM	Micromolar

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Development of Isothermal Nucleic Acid Amplification Methods at Room Temperature

Nucleic acid testing for infectious diseases at the point of care is beginning to enter clinical practice in developed and developing countries. In this field, polymerase chain reaction-based systems are still relatively complex and expensive. The detection of infectious diseases with nucleic acid amplification tests (NAATs) is only carried out in central laboratories equipped with high-level devices and operated by skilled personnel in this field. Developing countries have limited financial resources and cannot implement commercially available complex NAAT systems. Point-of-care NAAT systems can provide access to the diagnostic methods needed in regions with low resources and high disease rates. The difficulties, costs and complexity of implementing tests can be reduced by using a real-time monitorable isothermal amplification method. A functional protein called Protein 2 (P2) of M13 bacteriophages takes a role in phage replication by nicking only the single strand of double-stranded DNA on a specific sequence in the f1 replication origin. The expression of the P2 occurs at 37°C by phage infected host bacterial cells. This suggests that the P2 can nick the DNA strand under similar temperatures or lower conditions at in vitro. Various NAAT methods have been developed using enzymes that can nick on a single strand of DNA. However, the high operating temperatures of these enzymes and the requirement of heating equipment increase the cost and limit the applicability of the method. Also, previous studies have not more information about the biochemical properties and enzymatic activity of P2. In this study, it is expected that developing the new isothermal nucleic acid amplification method using P2 at room temperature by designing a pathogen-specific primer pair and obtaining more data on P2.

Keywords: Bacteriophage nicking enzyme P2, Isothermal nucleic acid amplification, Nicking enzyme amplification reaction, Nucleic acid amplification tests

ÖZET

Oda Sıcaklığında İzotermal Nükleik Asit Çoğaltım Yöntemlerinin Geliştirilmesi

Klinik enfeksiyöz hastalıkların tanısında nükleik asit testlerinin (NAAT) kullanımı gün geçtikçe artmaktadır. Bu alanda polimeraz zincir reaksiyonu temelli sistemler, günümüzde hala kompleks ve yüksek maliyetlidir. Enfeksiyöz hastalıkların NAAT ile tespit edilmesi yalnızca merkezi laboratuvarlarda, üst düzey cihazlar ve alanında yetkin personeller aracılığıyla gerçekleştirilmektedir. Sınırlı finansal kaynaklara sahip gelişmekte olan ülkeler, ticari olarak mevcut olan karmaşık NAAT sistemlerini uygulayabilme olanağına sahip değildir. Hasta başı NAAT sistemleri, düşük kaynağa sahip ve hastalık oranı yüksek olan bölgelerde ihtiyaç duyulan tanı yöntemlerine erişimi sağlayabilir. Gerçek zamanlı izlenebilir bir izotermal çoğaltım yöntemi kullanılarak testlerin uygulanmasındaki zorluklar, maliyet ve karmaşıklık azaltılabilir. M13 bakteriyofajlarına ait Protein 2 (P2) olarak adlandırılan fonksiyonel bir protein, f1 replikasyon orijininde bulunan özgün bir dizi üzerinde çift zincirli DNA'nın yalnızca tek zincirini keserek faj replikasyonunda rol alır. P2'nin sentezi, M13 fajları ile enfekte olmuş konak bakteri hücreleri tarafından 37°C sıcaklıkta gerçekleşir. Bu durum, proteinin DNA zinciri kesim reaksiyonunu in vitro olarak benzer sıcaklık ya da daha altındaki koşullarda da gerçekleştirebileceğini düşündürmüştür. DNA'nın tek zinciri üzerinde kesim yapabilen enzimlerin kullanıldığı çeşitli NAAT yöntemleri geliştirilmiştir. Fakat bu yöntemlerde kullanılan enzimlerin çalışma sıcaklıklarının yüksek olması ve dolayısıyla ısıtıcı bir ekipman gerektirmesi maliyeti arttırarak yöntemin uygulanabilirliğini kısıtlamaktadır. Aynı zamanda P2'nin biyokimyasal özelliklerinin ve enzimatik aktivitesine dair çalışmaların yeterli düzeyde olmadığı görülmüştür. İlgili çalışma ile P2 hakkında daha fazla verinin elde edilmesiyle birlikte, patojene özgün primer çifti tasarlanarak, P2'nin kullanıldığı oda sıcaklığında yeni bir izotermal nükleik asit çoğaltım yönteminin geliştirilmesi beklenmektedir.

Anahtar Sözcükler: Nükleik asit çoğaltım yöntemi, İzotermal PZR, Hızlı tanı kiti, Oda sıcaklığında PZR

1 INTRODUCTION AND AIM

M13 bacteriophages are filamentous viruses that carry the single-stranded DNA and infect only male Enterobacteria through a lysogenic life cycle. Protein 2 (P2), an essential replication-associated protein, takes a role as nicking enzyme in the replication of the phage genome via rolling circle amplification model. Despite decades of research, the molecular details of the P2 protein features and its nicking mechanism have not been sufficiently studied in vitro. Previous studies have identified a specific recognition sequence in the f1 origin; however, information about the minimal recognition site of the enzyme, its enzymatic activity, and its biochemical functionality remains limited. This study aims to examine P2's nicking features after nearly 40 years and to provide insights into its potential applications in nucleic acid amplification technologies. Some human pathogenic viruses share the same replication mechanism as M13 phages. Due to this similarity, M13 phages can serve as a powerful model organism for predicting the spread and treatment of viruses in viral emergence studies. Virus-host interactions provide important insights into viral replication and molecular processing mechanisms, making them valuable for the development of diagnostic tools. Isothermal Nucleic Acid Amplification Methods (INAAM) have become a popular research field for developing simple and cost-effective diagnostic methods for point-of-care testing. Unlike traditional PCR, these methods amplify DNA at a constant temperature without requiring an expensive thermal cycler. One type of INAAM is nicking enzyme-mediated amplification reactions, which relies on the use of an endonuclease and a polymerase with strand displacement activity. However, even these methods require higher temperatures than room temperature. This study aims to utilize P2 as a nicking enzyme to develop a novel INAAM-based diagnostic approach that is low-cost, accessible and capable of efficient operation at room temperature. This research could lead to the development of novel, cost-effective diagnostic tests for detecting pathogens in low-resourced area.

2 BACKGROUND

Bacteriophages, also called phages were discovered and first named by Fèlix d'Herelle in 1917 (1,2). Bacteriophages are a type of virus that are obligate parasites of bacteria and infect only male Enterobacteria that carry the F pilus (F episome, F^+) (1–5). They are classified according to their host organisms, genetic material, morphology, and structure (1,2,5,6). The M13 phages are filamentous bacteriophages that carry single-stranded DNA (ssDNA) and have a lysogenic life cycle (3,4). While phage coat proteins are present on the phage particle, the functional proteins are synthesized in infected host cells by host enzymes using phage genomic DNA (4). These functional proteins are present only in the bacterial host cell and play roles in the production and packaging of the newly generated phage particles. M13 infection begins with the binding of a fusion protein of the phage, called Protein 3 (P3), to the F episome of the host bacteria (4,5). When the single-strand DNA (ssDNA) genome of the phage (NC_003287.2) is transferred into the cell, RNA polymerase generates a primer from the (+) strand and a complementary strand (−) is synthesized from the replication origin by bacterial DNA Polymerase III, converting ssDNA into a double-stranded DNA form called replicative form (RFI DNA) (4,5,7). Then, the amplification of the DNA genetic material of the phage particles is achieved by the rolling circle amplification mechanism shown in Figure 1.1. The *gene II* sequence of the M13 phage (NC_003287.2) encodes Protein 2 (P2), also called *gene II* Protein (Gp2), which functions as a replication associated protein G2 (UniProt-EC:6.5.1.1 and EC:3.1.21) (8–10). P2 recognizes a specific gene sequence at the f1 origin (ori) and creates a nick on one strand of the (+) double-stranded replicative DNA (RF DNA) form, which is the first step of DNA replication (3,11). Host DNA Polymerase recognizes this 3’ OH end of the nicked strand and initiates polymerization using the template DNA. When the synthesis of the complete genomic DNA strand is completed, P2 cuts the strand again at the same specific nicking site and produces new progeny (+) DNA strands (11,12). Viral proteins, including Protein 2, 5 and 10 (P2, P5 and P10) remain in the cytoplasm and play a role in viral genome replication, assembly, and packaging of the phage. Protein 1, 4 and 11 (P1, P4 and P11) create a transport complex in the inner and outer membranes, while Protein 6, 7, 8, 9 and 3 (P6, P7, P8,

P9 and P3) accumulate in the membrane before packaging (13). The released progeny ssDNA is coated with P5, and this complex is transported to the membrane for the export complex that consists of P1, P11, and P4 along with phage structure proteins, and then ejected from the bacterial cell without lysis of the host cell membrane (Figure 1.1) (11,14–18).

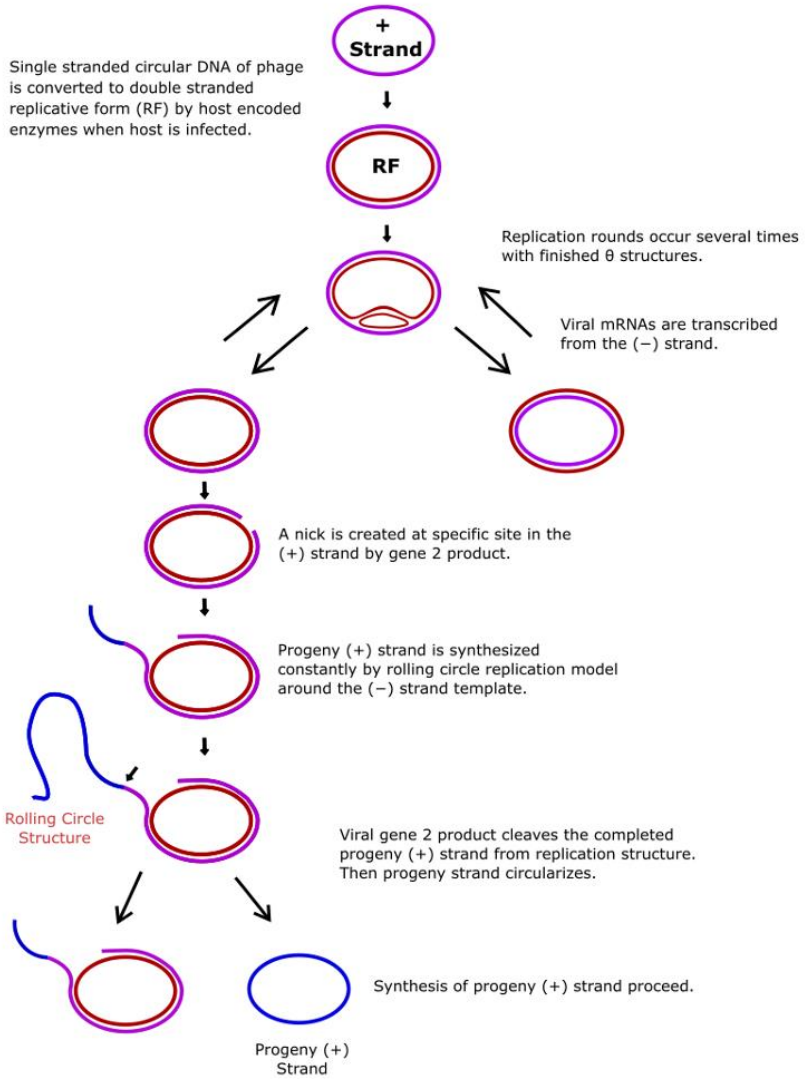


Figure 1.1: Rolling circle DNA amplification mechanism. Phage single-stranded circular DNA is converted to the double-stranded replicative form (RF) by host enzymes after infection. P2 creates a nick in one strand of the RF. Then DNA polymerase recognizes the nicked 3′ end and synthesizes a new DNA strand. P2 then cleaves the new DNA strand again at the same specific recognition site [Regenerated according to Sambrook (2001) (4) via Inkscape Project software, v. 1. 3. 2].

DNA sequences of many filamentous phages are 98% identical to each other (14,19,20). Previous studies have shown that the specific recognition sequence for P2 is localized in the f1 ori of DNA (3,11,12,21). These studies found that the nicking position is in the sequence 5'-CTTT/ATT-3', which is localized in the intergenic region of the phage genome between the 5781st and 5782nd nucleotides (22,23). One of the strands folds to form a hairpin of two secondary structures, known as A1 and A2, in the f1 ori sequence (Figure 1.2) (21,24–26). It has been suggested that P2 creates these loops by bending the DNA strands and then cutting them (Figure 1.3) (27,28). P2 not only cuts the RF DNA but also seals the newly generated ssDNA, which can be further used as a template for replication or as genetic material for new phage particles (22).

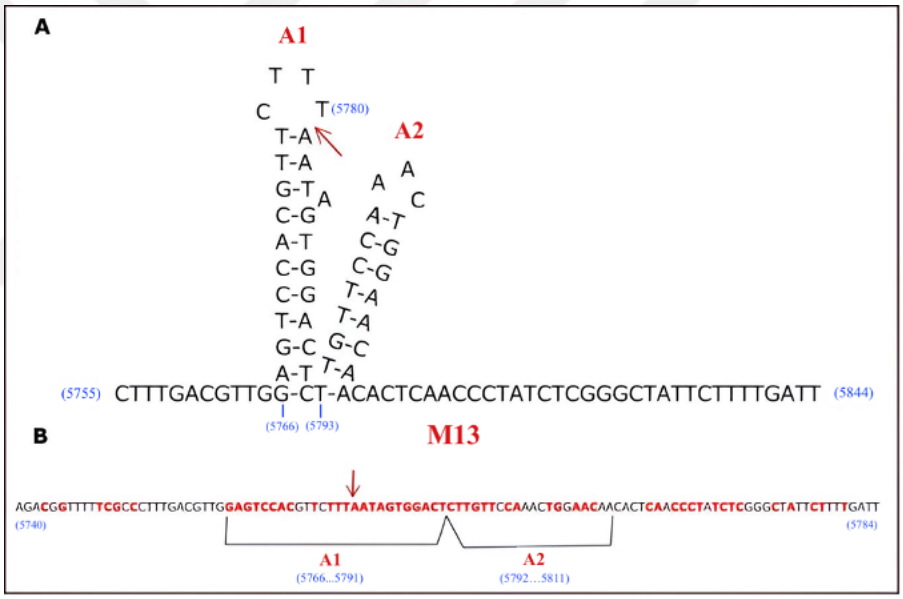


Figure 1.2: P2 recognition site in the M13 filamentous bacteriophage genome (NC_003287.2). **(A)** A1 and A2 are two loop structures of the recognition site, and the arrow indicates the nicking site. **(B)** The linearized DNA sequence of recognition site. Red-inked nucleotides represent the required sequences for recognition of P2 from previous studies (24–26).

For the nicking action, researchers have suggested that, firstly P2 creates loops by bending the DNA strands and then cutting the one of the strands (Figure 1.3) (28,29). The most recent study published about the features of the P2 enzyme is older than 40

years, which is why we do not have enough information about the P2 protein nicking reaction, as well as its characteristics in vivo and in vitro.

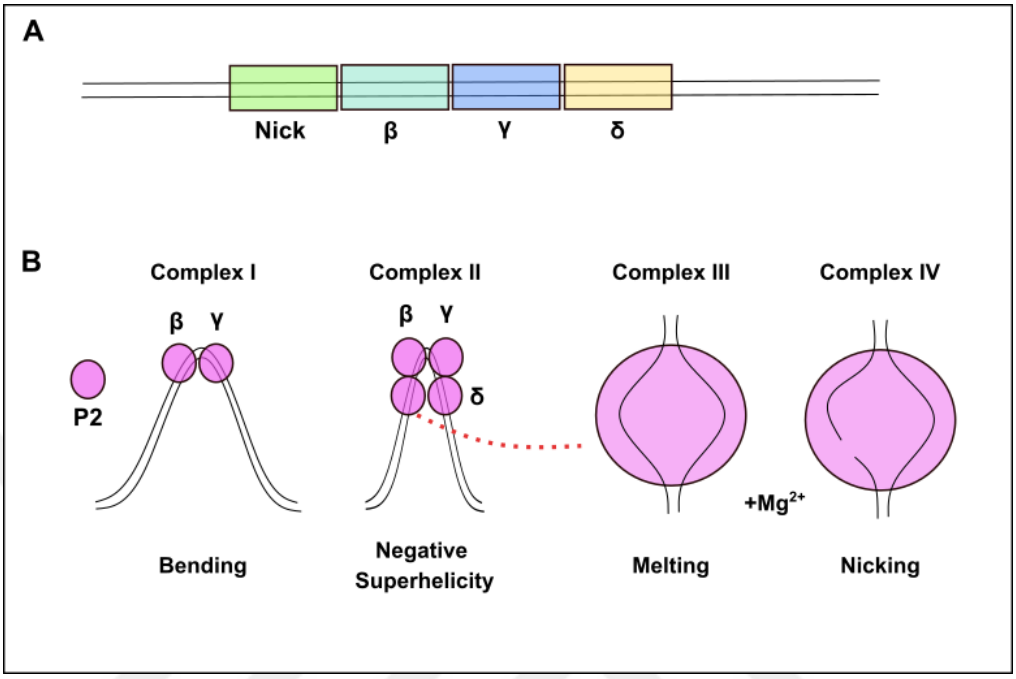


Figure 1.3: Conformational change in DNA created by P2 during the nicking process. (**A**) Representation of the DNA sequence segments (β, γ and δ) on the recognition site. (**B**) When P2 binds to the recognition site, it bends the DNA to create negative superhelicity. P2 nicks one of the DNA strands after the melting step, which involves denaturation of the strands. [Regenerated according to Horiuchi K. (1997) (28) via Inkscape Project Software, v. 1. 3. 2].

Viruses are important due to their ability to infect new host populations. They are major public health issues when their hosts are human, such as Human Immunodeficiency Virus (HIV) and severe acute respiratory syndrome (SARS) pandemics. Heuristic methods can be useful to detect emerging diseases before they spread (30). Emerging viruses are those that have entered new populations of hosts, and they are generally evaluated by epidemiological or patient-based approaches. Current patient-based approaches are not sufficient (30). Alternative methods that exploit the evolutionary ecology of viruses may be better at predicting the emergence and evolution of viruses. For example, working on metazoan viruses presents great challenges, and the development of new models is desirable. Much research has shown that bacteriophages are appropriate model organisms for virus emergence studies

because the population parameters can easily be manipulated (30). They have various advantages in terms of expanding host range parameters such as population size, growth rate, mutation rate (30).

To clarify the molecular mechanisms of cellular processes, multiple aspects of filamentous phage life have been utilized as model systems. Intensive research in this field has produced important quantitative data on M13 biology. Many of these studies on DNA replication, mRNA degradation, and mRNA processing in M13 phages were particularly useful (13). The DNA replication mechanism of M13 phages can be used as a model for ssDNA virus amplification. In this context, nicking activity features of P2 can be important for establishing the specific mechanisms for endonuclease-mediated DNA amplification systems.

Nowadays, researchers are focused on the signal amplification systems for diagnostic tests and the Nucleic Acid Amplification Techniques (NAATs) are becoming more common techniques in this field. These techniques are used for bio-detection methods with high specificity. NAATs are classified into two groups depending on their reaction temperatures. The first one is the non-isothermal Polymerase Chain Reaction (PCR) method which is performed by a thermal cycler and thermostable DNA Polymerase enzyme. The other one, called the isothermal method, uses only one constant temperature for DNA amplification in reaction. Typically, PCR is commonly used in clinical diagnosis for specific detection of bacteria or viruses from patient samples like saliva, smears, and body secretions in the medical field. However, PCR-based based methods may have some disadvantages, such as time delays when working with large sample amounts, difficulties in sending samples to other laboratories for the testing, and the need for expensive thermal cycling machines and well-trained personnel for analysis (31). Additionally, the high price of thermal stable enzymes, limited accessibility to this method of the low-cost laboratories, and limited availability to point of care (POC) tests are other disadvantages of this technique. As a result, researchers are focusing on isothermal techniques for nucleic acid amplification methods in signal amplification systems (32). Isothermal Nucleic Acid Amplification Methods (INAAM) include many different techniques such as

loop-mediated amplification (LAMP), rolling circle amplification (RCA), Helicase dependent amplification (HDA), CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) mediated, etc. (33–35). Despite these methods successfully denaturing the double-stranded DNA strands without high temperature like conventional PCR methods, there is still a need for an initial temperature or a special chemical for the reaction. Therefore, developing simple, specific, low-cost, and accessible new techniques might be very important in this field (36). On the other hand, all NAATs have different advantages and disadvantages depending on their features (Table 1.1) (35). Nicking Enzyme Amplification Reaction (NEAR), which the subtype of isothermal nucleic acid amplification method that is based on using of a nicking enzyme. The enzyme can create a nick site on one of the DNA strands as an endonuclease. DNA Polymerase enzyme, which has strand displacement activity, can synthesize the new strand from the created 3'OH prime while displacing the old strand in front of it. While DNA synthesis continues, nicking enzyme-specific target sequences will be recreated, and reaction steps will cycle. Meanwhile, the displaced strands come together with overlapping sequences, creating a new template with free primers for DNA Polymerase. The targeted DNA site can be amplified by cycling these reactions (37–39). P2 might be used as a nicking enzyme for a new NEAR-based amplification method that can be used for the determination of ssDNA or RNA-based microorganisms by creating the amplification product with "P2-tagged" primers that carry a P2 recognition site. Then signal amplification can increase by tandem amplification as shown in Figure 1.4.

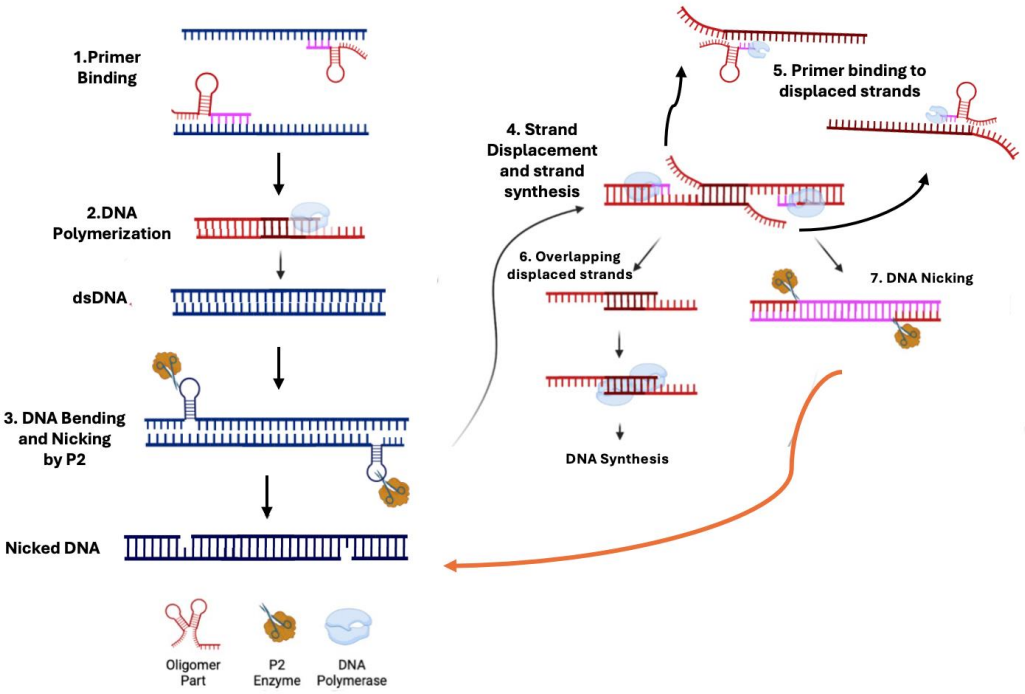


Figure 1.4: P2 mediated DNA amplification reaction. PMA bases on using the P2 nicking enzyme that able to create a nick site on the DNA strand as an endonuclease. A specific primer pairs, which carry a P2 recognition site, bind to the template DNA and the DNA Polymerase enzyme synthesizes the new strand by using the 3'end of primer (step 1 and 2). P2 nicks at newly created recognition sites and DNA polymerase displaces the old strand while synthesizing the new one (step 3 and 4). Displaced strands can overlap with their matched sequence parts and the DNA polymerase synthesizes new complementary strands (steps 6). Also, primer pairs can bind to these displaced strands at same time (step 5). Displaced strands overlapping, primer binding, polymerization and nicking steps repeat. The targeted DNA site can be amplified by cycling these reactions (Created with BioRender.com).

Table 1.1: Comparison of General NAATs [Modified according to Kang T., et.al. (35)]

NAAT	Temperature (°C)	Efficiency (min)	Advantages	Disadvantages
PCR	50-90	45-60	Highly Specific	Requires trained professionals, thermal cycler, unsuitable for POC
RCA	Around 37	60-120	Versatility in application, isothermal	Challenges for big production, purification, and storage
LAMP	60-65	60	specific and isothermal	Complexity of primer design, possible false-positive results
RPA	37-42	20-40	Simple, selective, and flexible	Requires purification and protein design afterward.
CRISPR-related	Around 37	60	Optimal sensitivity, and versatility in combining with other techniques	Still in the experimental stage

Many isothermal or non-isothermal NAATs use a higher temperature than the room temperature (20-25°C) as given in Table 1.1. Despite not requiring complex thermal cycling machine, it still needs a thermal heating machine for the reaction. Developing new techniques easily adaptable to low temperatures will be crucial for decreasing the cost and accessibility of tests. This study will lead to the development of new technologies that work at room temperature. Also, we suggest that P2 can be used as a nicking enzyme for isothermal nucleic acid amplification methods at room temperature. This is because the P2 is produced by M13 phage-infected bacterial cells naturally. This production occurs at the optimal bacterial growth temperature, which is 37°C. These findings suggest that the protein could catalyze the nicking reaction at a similar or lower temperature in vitro. P2 could be used as an endonuclease enzyme with specific primers for Nicking Enzyme Amplification Reaction (NEAR) under the room or bacterial incubation temperature. Then, the amplified DNA products from

specific targets can be detected as a signal for the diagnosis of many pathogens. The developed nucleic acid endonuclease-mediated amplification reaction method could be combined with a lateral flow assay to design a rapid diagnostic test with low cost, high specificity, sensitivity, cost-effectiveness, and accessibility. This study plans to develop an isothermal nicking enzyme-triggered nucleic acid amplification system for diagnostic purposes that works at a lower temperature than the current diagnostic tests by using the M13 P2. This is the first study to explore the idea of using the P2 protein in nucleic acid amplification-based signal amplification technologies. For this purpose, the cloning, production, purification, and activity tests of the P2 protein have been performed in this thesis. In the last several years, there have not been any studies about the P2 enzyme, and all data that were previously published are outdated (12,40–48). In this study, also the nicking features of P2 have been determined in detail. While the exact limitations of the minimal P2 recognition sequence in f1 ori were not given in previous studies and other natural replication proteins were included in the reaction, we used different parts of this recognition sequence as a substrate for the P2-GFP enzyme to investigate the region essential for nicking activity without any cooperative effect of the other proteins.

All the output data of this study will contribute more information about P2 in scientific fields. Developing novel diagnostic tests which are able to work at room temperature may provide access to much-needed diagnostic methods in low-resource, high-disease burden areas. Developing the appropriate devices from this technology by further research should be affordable, robust, and easy to use by minimally trained personnel. Also, it should be stable, include ready-to-use reagents, and be simple, maintenance-free instrumentation. It should give clear, actionable results while being suitably sensitive and specific.

3 MATERIALS AND METHODS

3.1. Materials and Equipments

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

Table 3.1: Chemicals

Chemical	Brand	Catalog ID
%30 Polyacrylamide Solution	Neofroxx / DE	1106ML500
5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside / Xgal	Goldbio / USA	X4281C
Acetic Acid (glacial)	Sigma-Aldrich / USA	1.00063
Agarose	Geneon / DE	604-001
Ammonium Per Sulphate / APS	Neofroxx / DE	1610GR100
Ampicillin	GeneMark / TP	GAS02
B-PER Solution	Thermo Fisher Scientific / USA	78243
Bacteriological Agar	BioShop / CA	AGR001.R
BCA Protein Assay Kit	Thermo Fisher Scientific / USA	A55864
Beta-mercaptoethanol	Merck / USA	M6250-10ML
Boric Acid	Merck / USA	B6768-500G
Bovine Serum Albumin (BSA)	Sigma-Aldrich / USA	A3311-50G
Bromophenol Blue	Sigma-Aldrich / USA	114391-5G
Clarity Western ECL Substrate	Bio-Rad / USA	1705061
Coomassie Brillant Blue R250	BioFroxx / DE	1912GR100
dCTP	Genemark / Taiwan	GD002-C
dGTP	Genemark / Taiwan	GD002-G
Dimethyl sulfoxide / DMSO	Neofroxx / DE	1610GR100
Dithiothreitol (DTT)	Bio-Rad / USA	1610611
DNA Ladder 1 kb	GeneMark / TP	GMM02
DNA Ladder 100 bp	GeneMark / TP	GMM100
DNA Loading Dye	GeneMark / TP	DL02-1
DNA Polymerase I Large Fragment	NEB / USA	M0210S
DNA Polymerase Master Mix	Genemarkbio / TP	RP02-II
DNAse I Enzyme	Roche / USA	10104159001
dNTPs	GeneMark / TP	GM007
Dyna-Bead	Invitrogen / USA	10470515
EtBr	Sigma-Aldrich / USA	E1510-10ML
Ethanol	Amasya Seker Fabrikasi	-

Table 3.1: Chemicals (Continue)

Chemical	**Brand**	**Catalog ID**
Ethylenediaminetetraacetic acid / EDTA	Bioshop / CA	EDT002
Formamide	Thermo Scientific / USA	17899
Gel Elution Kit	Zymo-Research / USA	D4007/D4008
Glycerol	Neofroxx / Germany	1073LT001
Glycine	Isolab	927.033
Goat pAb IgG-HRP Antibody	Abcam / UK	ab205718
HCl	Merck / USA	100317
HisTrap FF 5 mL Column	Cytiva / USA	17525501
HisTrap HP 1 mL Column	Cytiva / USA	17531901
HPLC Grade EtOH	Sigma-Aldrich / USA	09-0851
Imidazole	Sigma-Aldrich / USA	I5513
Isopropyl β- d-1-thiogalactopyranoside / IPTG	Genemarkbio / Taiwan	GMSJ0012-0005
Kanamycine	GeneMark / TP	GAS10
KCl	Sigma-Aldrich / USA	529552
Klenow Buffer	NEB / USA	B7002SVIAL
Laemli Protein Loading Dye	GeneMark / TP	GM47-b-50
LIC-Qualified T4 DNA Polymerase	Novagen / USA	70099-3
Luria Bertani Liquid Medium (Miller)	Caisson / USA	LBP01-500GM
Lysozyme	GeneMark / TP	GB28
M13KE DNA	NEB / USA	E8101S
Maxi Prep Plasmid Isolation Kit	Zymo-Research / USA	D4202
Methanol	Merck / USA	34860
$MgCl_2$	Merck	M8266
MyTaq™ HS DNA Polymerase	Bioline / London	BIO-21111
N,N,N′,N′-Tetramethyl ethylenediamine / TEMED	Bio-Rad / California	1610800EDU
NaCl	Merck / USA	106406
NaOH	Merck / USA	UN1832
Ni-TA Beads	Gold Bio / USA	H-350-5
Orange G	TiBO / TR	NS03
PCR Clean-Up Kit	Genemarkbio / TP	DP04
PCR Master Mix	GeneMark / TP	RP02-II
pET His6 GFP TEV LIC	Addgene / USA	29663-DNA.cg
Phi 29 DNA Polymerase Buffer	NEB / USA	B0269SVIAL
Phi29 DNA Polymerase	NEB / USA	M0269L
PMSF	Thermo Scientific / USA	215740010
Protease Inhibitor Coctail	Roche / USA	11836153001
Q-PCR Master Mix	GeneMark / TP	QPSY01
Rabbit pAb to 6× His Antibody	Abcam / UK	ab9108

Table 3.1: Chemicals (Continue)

Chemical	Brand	Catalog ID
Rainbow Protein Weight Marker	GeneMark / TP	GMP0671
rCut Smart Buffer	NEB / USA	B6004S
recAlbumine	NEB / USA	B9200SVIAL
Sephadex G-100	Cytiva / USA	17006001
Sephadex G-50	Cytiva / USA	17004302
Skim Milk	BioRad / USA	1706404
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich / USA	L3771
Sodium Phosphate	Sigma-Aldrich / USA	S3139
Sorbitol	Sigma-Aldrich / USA	S1876
SspI Restriction Enzyme	NEB / USA	R0132
Sybr Gold	TiBO / TR	NS02
Sybr Green	TiBO / TR	NS01
T4 Polynucleotide Kinase	NEB / USA	M0201S
TA Cloning Kit	Bio Basic / SG	BS435
TEMED	BioRad / USA	1610800
TEV-Protease Plus Enzyme	Promega / USA	V6101
Thiourea	Sigma-Aldrich / USA	T8656
Triton X-100	Sigma-Aldrich / USA	X100-500ML
Trizma Base	Sigma-Aldrich / USA	T1503-1KG
Tween 20	Sigma-Aldrich / USA	P9416
UPLC BEH SEC Column	Waters / USA	186005226
Urea	Sigma-Aldrich / USA	U5378
Xcylene Cyanol FF	Sigma-Aldrich / USA	X4126
XhoI Restriction Enzyme	NEB / USA	R0146S
Zymoclean Gel DNA Recovery Kit	Zymo-Research / USA	D4007

Table 3.2: Instruments

Instrument	Brand
–80°C Refrigerator	Arctico
+4°, –20°C Refrigerators	Kirsch
Agarose Gel Electrophoresis Equipment	Bio-Rad
Autoclave	Nüve
Biosafety Level 2 Hood	Thermo Scientifici
Centrifuge	Thermo Scientific
CFX96 Real-Time PCR Thermal Cycler	Bio-Rad
Chemidoc Gel Imaging System	Bio-Rad
Freeze Dryer	Labconco
Heat Block	Dlab
Ice Machine	ITV
Incubator	Thermo Scientific
Incubator with Shaking	Thermo Scientific

Table 3.2: Instruments (Continue)

Instrument	Brand
Magnetic Mixer	Thermo Scientific
Membrane Transfer System	Bio-Rad
Micropipette Set	Eppendorf
Microwave oven	Arcelik
Minispine	Biosan
Nanodrop	Thermo Scientific
ORTE (Observable Real-Time Electrophoresis)	TiBO
Pasteur Oven	Nuve
pH Meter	Thermo Scientific
Polyacrylamide Gel Electrophoresis Equipment	Bio-Rad
Power Supply	Invitrogen
Shaker	Wisd Lab. Instruments
Sonicator	Bandelin
Spectrophotometer	Thermo Scientific
Thermal Cycler	Bio-Rad
Top bench Centrifuge	Thermo Scientific
Vortex	VWR
Water Bath	Weightlab Instruments
Water Double Distillation System	Mili-Q / Merck

3.1 Plasmid Amplification

The pET His6 GFP TEV LIC cloning vector (1GFP) was purchased from Addgene, USA. The plasmid was transformed into *Escherichia coli* (*E. coli*) cells and amplified to obtain a lot of plasmids for cloning experiments.

3.1.1 The preparation of competent cells by chemically

Competent cells were prepared according to the Chung et. al. protocol (49). *E. coli* DH5α and BL21 bacterial cells were inoculated separately in Luria Bertani (LB) medium at 180 rpm, 37°C for overnight. Growth cultures were diluted with 1:100 volume of the fresh media and incubated at 180 rpm, 37°C. In the meantime, TSS Buffer and microcentrifuge tubes were put on the ice. Once cell concentration reached OD 0.3 at 600 nm, the culture was put on the ice for 10 minutes. The culture was centrifuged at 3,000 rpm, 4°C for 10 minutes. The supernatant was removed, and the

cell pellet was resuspended with 10% volume of TSS Buffer and gently pipetted. The cell suspension was aliquoted to microcentrifuge tubes at 50 µL per tube, and tubes were frozen at cold ethanol (−80°C). Tubes were stored at −80 °C until used.

3.1.2 Transformation

Sambrook et. al. (2001) transformation protocol was modified in this study (4). Briefly, competent *E. coli* DH5α cell suspension was thawed on ice. The 100 ng of pET His6 GFP TEV LIC cloning vector plasmid DNA (p1GFP) was added into cells and mixed by tapping. The tubes were incubated at 42°C water bath for 90 seconds. Then tubes were put on ice for 5 minutes. 800 µL of LB medium pre-warmed at 37°C was added into tubes and inoculated at 180 rpm, 37°C for 1 hour. Then, 100 µL of bacterial culture was plated on LB Agar (LBA) containing 50 µg/mL Kanamycin (Kan) plates which were prewarmed at 37°C. Then the plates were incubated at 37°C for overnight.

3.1.3 Plasmid isolation and purification

One of the transformant colonies was cultured in LB Medium including 50 µg/mL Kan at 180 rpm, 37°C for overnight. The culture was centrifuged at 10,000× g, 4°C for 5 minutes. The supernatant was discarded. The plasmid was isolated by using the Zymo Maxi Prep Plasmid Isolation Kit (Zymo-Research, USA). The concentration and purity of plasmids were determined by spectrophotometrically via Nanodrop (Thermo Fisher Scientific, USA) (50). Plasmids were analyzed by agarose gel electrophoresis.

3.2 Engineering of Recombinant DNA

For the engineering of the recombinant DNA, amino acid sequence of P2 were converted to nucleotide sequences and codon optimization was performed for *E. coli* species by using IDT DNA Codon Optimization Tool (51). Translation reading frames of cloned DNA sequences were verified with an online protein translation tool (Expasy) (60).

3.3 Cloning of P2 Protein Gene

3.3.1 Preparation of vector

The p1GFP was digested with SspI restriction enzyme (NEB, USA) at 37°C for 30 minutes (Table 3.3). Then the enzyme was inactivated at 65°C for 20 minutes. The digested DNA bands were cut and purified from the 0.8% agarose gel using the gel elution kit (Zymo-Research, USA). The DNA concentration of purified DNA was determined by Nanodrop and analyzed by 0.8% agarose gel electrophoresis.

Table 3.3: SspI Digestion Reaction

Reaction Ingredients	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
Buffer	10×	1×	2
SspI Restriction Enzyme	10 U/µL	6 U	0.6
DNA	100 ng/µL	600 ng	6
Nuclease free-ddH_2O			11.4
Total			20

3.3.2 Amplification of inserts

3.3.2.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed to amplify *gene II* insert DNA from M13KE replicative DNA, which was purchased (NEB, UK). For this purpose, forward and reverse primers were designed with LIC tags as given in Table 3.4 by using Snapgene Viewer (version 4.2.11). Designed primers were verified by using the Primer Designing Tool (NIH) and Oligo Analyzer Tool (IDT) (52,53). Gradient PCR was performed with between 63-72°C for annealing temperature (Table 3.5 and 3.6).

Table 3.4: Insert Amplification Primers

Primer Name	Nucleotide Sequence	Size (bp)	GC%	Tm (°C)
LIC Forward primer	*TACTTCCAATCCAATGCA**-ATGATTGACATGCTAGTTTTACGA	42	37.5	62.9
LIC Reverse primer	*TTATCCACTTCCAATGTTATTA**-TATGCGATTTTAAGAACTGGCTC	45	33.3	61.7

**LIC tag*

Table 3.5: PCR Ingredients for Amplification of Inserts

Components	Stock Concentration	Final Concentration	Volume (µL)
Mytaq Reaction Buffer	5×	1×	10
Bioline MyTaq Hot Start Polymerase Enzyme	5 U/µL	2.5 U	0.5
Template DNA	8 ng/µL	16 ng	2
Forward Primer	10 µM	0.4 µM	2
Reverse Primer	10 µM	0.4 µM	2
Nuclease free-ddH_2O			33.5
Total Volume			50

Table 3.6: PCR Conditions for Amplification of Inserts

Initial Denaturation	95 °C	1 minute
*Denaturing	95 °C	10 seconds
*Annealing	64 °C	15 seconds
*Extension	72 °C	12 seconds
Final Extension	72 °C	5 minutes
Final Temperature	4 °C	∞

*30 cycles

The PCR products were analyzed by 1% agarose gel electrophoresis then amplicons were purified from the gel by using gel elution kit (Zymo-Research, USA). DNA concentration was determined by nanodrop.

3.3.3 T4 DNA polymerase treatment

The digested vector DNA and insert DNA were treated with dGTP and dCTP respectively as given in Table 3.5. All reactions were included LIC-Qualified T4 DNA Polymerase enzyme (Novagen, USA) (Table 3.7 and 3.8) (54).

Table 3.7: T4 DNA Polymerase Reaction Components

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
DNA	166.5 ng/µL	250 ng	1.5
Buffer	10×	1×	2
Enzyme	5 U/µL	5 U	1
dGTP for Vector/ *dCTP for Insert*	100 mM/ *100 mM*	2.5 mM/ *2.5 mM*	0.5/ *0.5*
DTT	100 mM		1
ddH_2O			13.5
Total Volume			20

Table 3.8: T4 DNA Polymerase Reaction Conditions

Temperature (°C)	**Time**
23	40 minutes
75	20 minutes
4	∞

3.3.4 Annealing reaction

Vector/insert molar ratio was calculated by using the ligation calculator of NEB Biocalculator Tool, as given Table 3.9 (55). Annealing reaction of vector and inserts which were treated with LIC-Qualified T4 DNA Polymerase enzyme, was performed as given in Table 3.10.

Table 3.9: Vector: Insert Molar Ratios

	Vector: Insert Molar Ratios				
	1:1	**1:2**	**1:3**	**1:5**	**1:7**
Required Mass of Insert*	10.13 mg	20.26 ng	30.39 ng	50.64 ng	70.9 ng
Required Mass of Vector**	50 ng	50 ng	50 ng	50 ng	50 ng
Vector Volume	4 µL	4 µL	4 µL	4 µL	4 µL
Insert Volume	1 µL	2 µL	3 µL	5 µL	7 µL

*1230 bp
**6072 bp

Table 3.10: Annealing Reaction Conditions

Temperature (°C)	Time
95	1 minute
72	30 seconds
*72 to 25	**30 seconds
25	5 minutes

*Δ-4 °C gradient
**Each temperature for 30 seconds

3.3.5 Transformation

Sambrook et. al. (2001) transformation protocol was modified in this study (56). Briefly, the competent *E. coli* BL21 cell suspension was thawed on ice. The 4 µL of the annealing reaction sample was mixed with competent cells. The negative control of transformation was including an equal volume of sterile ddH_2O instead of any DNA sample, and the positive control included 200 ng of *p1GFP*. The tubes were incubated at 42°C in a water-bath for 90 seconds. Then tubes were put on ice for 5 minutes. 500 µL of LB medium which was pre-warmed at 37°C was added into the transformant cells and cultured at 180 rpm, 37°C for 60 minutes. 200 µL of the culture was seed

onto an LBA with Kan (50 μg/mL) plates which were pre-warmed at 37°C and incubated at 37°C for overnight.

3.3.6 Colony screening

The transformant cells were selected according to their Kan resistance on Kan selective plates and colonies were analyzed with colony PCR.

3.3.6.1 Colony polymerase chain reaction

Polymerase chain reaction was performed for colony screening. For this purpose, PCR was performed as given in Table 3.11 and 3.12 with forward and reverse primers that given in Table 3.4. Three colonies of each plate were picked up with a sterile loop. The sample was first plated into the fresh LB, Kan (50 μg/mL) media and then subjected to the PCR tube. The colony of positive control of transformation which including backbone DNA was used as a negative control of recombination. The prepared fresh cultures were incubated at 180 rpm, 37°C for 3 hours. PCR products were analyzed by gel electrophoresis. The cultures of the recombinant positive colonies were stored with 20% glycerol at −80°C.

Table 3.11: Colony PCR Components

Components	**Stock Concentration**	**Final Concentration**	**Volume (μL)**
Mytaq Reaction Buffer	5×	1×	5
Bioline MyTaq Hot Start Polymerase Enzyme	5 U/μL	1.5 U	0.3
Template DNA			Touched to Colony
Forward Primer	10 μM	0.4 μM	1
Reverse Primer	10 μM	0.4 μM	1
Nuclease free-ddH_2O			17.7
Total Volume			25

Table 3.12: PCR Conditions for Colony Selection

Initial Denaturation	95 °C	3 minutes
*Denaturing	95 °C	10 seconds
*Annealing	64 °C	10 seconds
*Extension	72 °C	12 seconds
Final Extension	72 °C	5 minutes
Final Temperature	4 °C	∞
*30 cycles		

3.3.6.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed with 8 µL of colony PCR products that were mixed with 3 µL volume of 3:1 loading dye (1× Orange G:1/100 Sybr Gold). 5 µL of 1 kb DNA Ladder (GeneMark, TP) was mixed with 1 µL of 1/100 Sybr Gold dye. Samples were analyzed by 1% agarose gel electrophoresis and gel was imaged by using the ChemiDoc Gel Imaging System (Bio-Rad, USA).

3.3.6.3 Plasmid isolation and DNA purification

One of the positive colonies was cultured in LB Medium including 100 µg/mL Kan at 180 rpm, 37°C for overnight. The culture was centrifuged at 10,000 × g, 4°C for 5 minutes. The supernatant was discarded, and plasmid was isolated by using the Zymo Maxi Prep Plasmid Isolation Kit. Concentration of plasmids was determined spectrophotometrically by using Nanodrop (Thermo Fisher Scientific, USA) (50). Plasmids were analyzed by 0.8% agarose gel electrophoresis and plasmid was sent to Eurofins Genomics Company for Sanger DNA sequencing (appx. 1-4). Sequences were verified by DNA sequence alignment (appx. 5).

3.4 Protein Expression

3.4.1 Optimization of expression conditions

The recombinant *E. coli* BL21 cells were incubated in 350 mL volume of LB media including 50 µg/mL Kan at 200 rpm, 37°C for overnight. The growth cultures

were diluted with 1:100 volume of the fresh LB media with Kan and incubated at the same conditions until the cell concentration reached OD ~0.6 at 600 nm. The culture was cooled down onto the ice and it was divided into different sterile flasks as 50 mL then protein expression was induced by adding 0.1, 0.5, and 1 mM of isopropyl β- d-1-thiogalactopyranoside (IPTG) separately. While one group of the culture was incubated at 20°C for slow protein induction, other was at 37°C for fast protein induction with 180 rpm shaking for 18 hours. 1 mL of culture was collected from each expression culture at end of the 1, 2, 3, 4, 5, and 18 hours. Collected samples were centrifuged at 5,000 × g, 4°C for 10 minutes. Cell pellets and supernatants were collected and fluorescent lightening of P2-GFP was imaged by using an observable real-time electrophoresis machine (ORTE) (TiBO, TR). The non-induced culture was used as a lightening control.

3.4.2 Detection of P2

The collected cell pellets from the different groups at different time courses were separated into soluble and insoluble protein fractions by using the B-PER solution (Thermo Fisher Scientific, USA). For this purpose, ~40 mg cell pellet was resuspended in 160 µL volume of the B-PER solution and incubated at room temperature (RT) for 15 minutes. Lysate was centrifuged at 15,000 × g for 5 minutes and the supernatant was separated from the pellet. The pellet was resuspended in 120 µL volume of B-PER solution (Figure 3.1). Then all samples were analyzed by denaturing, non-denaturing Polyacrylamide Gel Electrophoresis (PAGE), and Western Blotting.

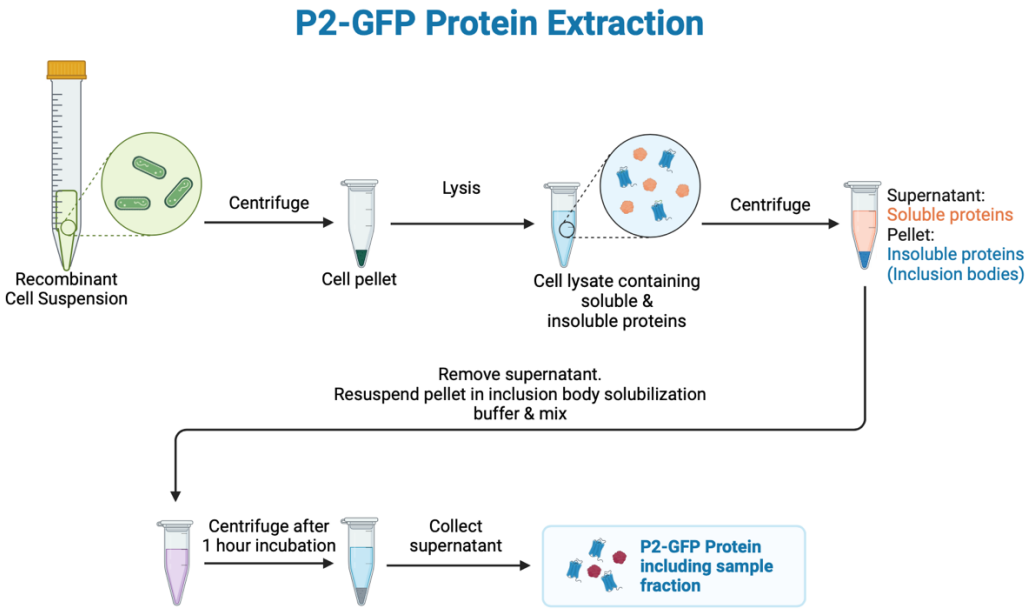


Figure 3.1: P2-GFP protein extraction. It was performed first by adding B-PER solution onto cell pellets to remove the soluble protein fraction from inclusion bodies. The inclusion body pellet was resolved in the freshly prepared inclusion body dissolving solution for 1 hour at room temperature. The mixture was centrifuged and the supernatant that included the dissolved P2-GFPs was collected.

3.4.2.1 Denaturing polyacrylamide gel electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (10%) (Table 3.13) was performed with 5% stacking gel (Table 3.14) for analyzing the protein samples (Table 4). 16 µL volume of each sample was denatured at 95°C for 5 minutes with laemli loading buffer (GeneMark, TP). Rainbow protein marker (GeneMark, TP) and samples were loaded into the gel in symmetrical order. Samples were run with SDS-PAGE running buffer that 1× Tris-Glycine (25 mM Tris-HCl, 192.4 mM Glycine, 0.1% SDS) at 85 V for 30 minutes and 150 V for 2 hours. After electrophoresis, gels were horizontally split into two parts as symmetrical. Half of the gel was stained by Coomassie R250 staining solution (0.1 % Coomassie Blue R250 (w/w), 30 % methanol, 5 % acetic acid) and the unstained gel part was used for Western Blotting analysis. Coomassie stained gel was destained by destaining solution (40% methanol, 10% acetic acid, 50% distilled water).

Table 3.13: SDS-PAGE Separating Gel Components

10% (w/v) Acrylamide Gel	**2 Gels/ 1mm (10 mL)**
30% (w/v) Acrylamide-Bisacrylamide Solution (29:1)	3.4 mL
1.5 M Tris-HCl pH:8.8 Buffer	2.6 mL
10% SDS	100 µL
ddH_2O	3.8 mL
10% APS*	100 µL
TEMED*	10 µL
* Added last and APS used freshly	

Table 3.14: SDS-PAGE Stacking Gel Components

5% (w/v) Acrylamide Gel	**2 Gels/ 1mm (5 mL)**
30% (w/v) Acrylamide-Bisacrylamide Solution (29:1)	0.67 mL
1 M Tris-HCl pH:6.8 Buffer	0.65 mL
10% SDS	50 µL
ddH_2O	3.58 mL
10% APS*	50 µL
TEMED*	5 µL

3.4.2.2 Western blotting

The proteins were transferred onto a nitrocellulose membrane from SDS-Polyacrylamide gels by using the small gel mode of a semi-dry gel transfer system (Bio-Rad, USA) at 25 V for 30 minutes. The membrane was incubated with 5% skim milk powder in Tris-buffered saline (TBS) supplemented with 0.05% Tween 20 (1× TBS-T) for 1 hour at RT for blocking of non-specific binding sites. The membrane was then washed three times with washing buffer (1× TBS-T). After washing, the membrane was incubated with a 1:1000 dilution of primer antibody, ab9108 Rabbit pAb to 6× His (stock 2 mg/mL) (Abcam, UK), prepared in dilution buffer (5% skim milk in 1× TBS-T) at RT for 3 hours with shaking. The membrane was washed again three times with the same washing buffer and then incubated with a 1:20,000 dilution

of HRP-conjugated secondary antibody, ab205718 Goat pAb IgG-HRP (stock 2 mg/mL) (Abcam, UK), prepared in dilution buffer at RT for 1 hour. The membrane was washed with washing buffer three times and developed using Clarity Western ECL substrate (Bio-Rad, USA) for 5 minutes. Subsequently, luminescent images of the stained blots were captured using the ChemiDoc Gel Image System (Bio-Rad, USA). The transferred SDS-PAGE gel was stained by Coomassie Brilliant Blue R250 to compare the transfer efficiency.

3.4.2.3 Non-denaturing polyacrylamide gel electrophoresis

Native Polyacrylamide Gel (Native-PAGE) (12%) was performed as given in Table 3.15. 13 µL of each sample was mixed with 2× Native-PAGE loading dye (62.5 mM Tris-HCl pH:6.8, 25% glycerol, 1% bromophenol blue) and run with running buffer (25 mM Tris-HCl pH:8.3, 192 mM Glycine) at 150 V for 2.5 hours (57). Gel was imaged by using the Chemidoc Gel Imaging System (Bio-Rad, USA). Each band of soluble fraction samples at different conditions for 5-hour and 18-hour incubation were cut from gel and the gel pieces were crushed in Tris buffer (50 mM Tris-HCl pH: 7.5, 150 mM NaCl, 1× Protease inhibitor cocktail (Roche, CH)). Then samples were run on SDS-PAGE.

Table 3.15: Native-PAGE Components

%12 (w/v) Native Polyacrylamide Gel	**Volume**
%30 (w/v) Acrylamide-Bisacrylamide Solution (29:1)	4 mL
0.375 M Tris-HCl (pH:8.8)	5.89 mL
TEMED*	10 µL
%10 APS*	100 µL
* Added last and APS used fresh	

3.4.3 Protein expression

The recombinant *E. coli* BL21 cells were incubated in LB with Kan (50 µg/mL) media at 200 rpm, 37°C for overnight. The growth culture was diluted with 1:100

volume of the fresh LB with Kan media and incubated at the same conditions until the cell concentration reached OD ~0.6 at 600 nm. The culture was cooled down on ice and protein expression was induced by adding 0.5 mM of IPTG. Culture was incubated at 180 rpm, 37°C for 5 hours. The culture was centrifuged at 5,000 × g for 15 minutes and cell pellets were stored at −80°C.

3.5 Protein Purification

The 459 mg of the cell pellet was dissolved in 1.84 mL B-PER (Thermo Fisher Scientific, USA) solution with 10 µL of 20 mg/mL Lysozyme and 133.28 µL of 25 mg/mL DNAse I, and then it was incubated at RT for 15 minutes according to the B-PER protocol. Lysate was centrifuged at 15,000 × g for 5 minutes and fractions were collected. The pellet was resuspended in 1 mL of B-PER solution, and the protein concentrations of all samples were measured by using a BCA Kit (Thermo Fisher Scientific, USA).

3.5.1 Protein purification by dyna-beads

The desired lysate volume for the Dyna-Bead (Invitrogen, USA) protein purification protocol was calculated by using protein concentration results measured by the BCA Kit, according to the protein binding capacity of beads (1-5 µg per µL). The pellet of lysate was dissolved in inclusion body dissolving solution that was altered to using Triton X-100 instead of SDS (2M Thiourea, 6M Urea, 1% Triton X-100) because beads were not suitable for SDS. Additionally, 0.05 mg/mL of Lysozyme, 4 µL of 25 mg/mL DNAse I, and 1 mM PMSF were added into the solution and then incubated at RT for 30 minutes. The same protocol for the sample preparation step was tried with 0.1% Tween-10 and sonicated at 40% power, 30 pulses 10 seconds for 5 cycles with 1-minute intervals on ice. According to the kit protocol, 200 µL volume of magnetic bead solution (Merch, USA) was added into sterile eppendorf tubes and beads were collected by using a magnetic attraction stand. The solution was removed, and the released beads were equilibrated for 1 minute in equilibration buffer 1 (100 mM Sodium Phosphate, 10 mM Tris-HCl pH:8.0, 8M Urea) or equilibration

buffer 2 (2M Thiourea, 6M Urea, 1% Triton X-100). Then the solution was removed by the same technique. Beads were mixed with 1 mL volume of lysate and incubated at RT for 30 minutes with shaking. At the end of the incubation, beads were collected, and the unbound sample was removed. Beads were washed nine times with wash solution (50 mM Sodium Phosphate, 300 mM NaCl, 10 mM Imidazol, pH:8.0). After the washing steps, bounded proteins were eluted from beads by using elution buffer (50 mM Sodium Phosphate, 300 mM NaCl, 300 mM Imidazol, pH:8.0) and this elution step was performed twice. Eluted fractions were stored at −20°C until use. Samples were analyzed by SDS-PAGE.

3.5.2 Protein purification by immobilized metal affinity chromatography

For His-Tag protein purification, immobilized metal affinity chromatography (IMAC) was performed by preparing a 3 mL volume of Nickel coated agarose beads that Ni-Beads (GoldBio, USA) into a 15 mL volume-sized empty chromatography column (Bio-Rad, USA). The column was washed twice with 5-column volumes (CV) of Tris buffer (20 mM Tris-HCl, 100 mM NaCl, pH:7.4), and after washing steps, the column was equilibrated with 5 CV of the same buffer. Dissolved proteins in the inclusion body solution (2M Thiourea, 6M Urea, 2% SDS) were buffer exchanged with the Tris buffer by using Amicon Ultra-15 50K Centrifugal Filter Device (Millipore, Merck, USA). 1 mL of prepared protein sample was loaded into the column with 5 CV of Tris buffer and incubated at 4°C for overnight with slight shaking. The next day, all the buffer was poured from the column, and flow-through samples were collected. Column washed with 10 CV of wash buffer 1 (20 mM Tris-HCl, 100 mM NaCl, 15 mM Imidazole, pH:7.4) and 5 CV of wash buffer 2 (20 mM Tris-HCl, 300 mM NaCl, 22.5 mM Imidazole, pH:7.4). All fractions were collected. Finally, bounded proteins were eluted with elution buffer (20 mM Tris-HCl, 500 mM NaCl, 300 mM/500 mM Imidazole, pH:7.4). The protein elution fraction's buffer was exchanged to dilution buffer (20 mM Tris-HCl, 100 mM NaCl, 10% glycerol, 1mM DTT, 50 µg/mL BSA, pH:7.4) buffer to remove residual imidazole by using Amicon Ultra-15 50K Centrifugal Filter Device (Millipore). The sample was verified using SDS-PAGE and Western Blotting (Figure 3.2). All stock samples were stored at −20°C until use.

3.5.3 Protein purification by passive gel elution method

The passive gel elution protocol was used as a second purification method. For this reason, all purified protein fractions were run on SDS PAGE, and bands were cut after running. Gel pieces were crushed in Tris-HCl buffer (50 mM Tris-HCl pH: 7.5, 150 mM NaCl, 1× Protease inhibitor cocktail) by mortar and pestle. The sample was incubated at 30°C for overnight. The sample was centrifuged at 10,000 × g for 10 mins and supernatant was collected. The supernatant sample was verified by SDS-PAGE and Western Blotting. Then purified protein sample was dialyzed with 2L of 20 mM Tris-HCl pH: 6.8, 0.1 mM DTT buffer at 4°C for overnight. Then, the buffer was exchanged with 2L of 20 mM Tris-HCl pH: 6.8 and it was dialyzed at 4°C for 5 hours.

3.5.4 Sample preparation for SEC and FPLC

For sample preparation, 1.52 gr of the cell pellet containing P2-GFP was thawed on ice. 3.2 mL of the B-PER solution was added into the pellet and incubated at RT for 15 minutes according to the B-PER protocol. Lysate was centrifuged at 15,000 × g for 5 minutes and the pellet included inclusion bodies was resuspended in 3 mL of B-PER solution again for washing. This washing step was repeated 5 times. The final cell pellet was dissolved in 8 mL volume of inclusion body dissolving solution (2M Thiourea, 6M Urea, 2% SDS) and incubated at RT for 1 hour. Then it was centrifuged at 16,000 × g for 10 minutes and supernatant was collected for further purification steps.

3.5.5 Protein purification by size exclusion chromatography

Different gel matrixes were prepared by using 1 mg of Sephadex G-100 (Sigma Aldrich, USA) powder in 20 mL volume of Tris-HCl buffer (10 mM Tris-HCl, pH:7.4) and 3 gr of Sephadex G-50 (Sigma Aldrich, USA) in 35 mL Tris-HCl buffer. Sephadex G-100 and G-50 were swelled at 90°C for 5 hours and 1 hour respectively. The column matrix was loaded into empty columns separately. For creating the stable column bed, they were kept at 4°C for overnight. Then each column was washed with Tris-HCl buffer for 2 CV. 4 mL of the prepared sample was loaded into a column and flowed

with 15 mL volume of Tris-HCl buffer with ~100 µL/min flow rate. Samples were collected at different time points for 75 minutes and GFP lightening was measured at OD_{504} nm (1 abs=1mg/mL). The samples were analyzed by SDS-PAGE, Western Blotting, and BCA Protein Concentration Kit. 15 mL of purified sample was lyophilized to 1.5 mg and resuspended in 10 mM Tris-HCl (pH 8.5), 20% glycerol, and 1 mM DTT buffer. Protein concentration was determined by using the BCA Kit, and samples were stored at −20°C for further analysis.

3.5.6 Protein purification by HPLC

Sample that in inclusion body dissolving solution and buffer exchanged one that in 10 mM Tris-HCl pH:7.4 were purified by using UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 4.6 mm column (Water, USA) and Agilent 1260 Infinity HPLC-FLD system. Tris-HCl buffer (10 mM Tris-HCl, pH:7.4) constantly flow-through with 0.3 mL/min flow rate and maximum pressure adjusted to 4000 psi. 20 µL volume of sample was injected into the column and 35 minutes run time was performed. Peaks were determined at excitation 509 nm or 516 nm and emission 504 nm wavelengths as recommended by Zhong et.al. (58). Peaks were collected manually and stored at −20°C. Samples were lyophilized and all samples were analyzed by SDS-PAGE. Different Tris-HCl buffers (10 mM Tris-HCl pH:6.8, 7.0, 7.4) were tried with the same protocol for the determination of different pH effects.

3.5.7 Protein purification by FPLC

Different parameters were tried for the determination of the optimum purification method by ÄKTA Pure System (Cytiva, USA). 1 mL sized HisTrap HP and 5 mL sized HisTrap FF columns (Cytiva, USA) were used separately with the gradient elution method. The dissolved GFP-P2s protein mixture was defrosted on the ice and buffer exchange was performed with ~15 mL Tris buffer (10 mM Tris-HCl, pH:7.4) via Amicon Ultra-15 50K Centrifugal Filter Device (Millipore, USA) to remove denaturants. Purification of P2-GFP was performed with fast protein liquid chromatography (FPLC) by ÄKTA Pure Protein Purification System (Cytiva, USA)

and Histidine binding Nickel Affinity Column HisTrap HP (1 mL) column (Cytiva, USA). The column was equilibrated with 5-CV of Tris buffer with 1 mL/min flow rate. The sample was loaded into the column with 0.250 mL/min flow rate from a filled 1 mL sample loop. The column was washed with 10 CV of wash buffer (10 mM Tris-HCl, 500 mM NaCl, 20 mM Imidazole, pH:7.4) with 1 mL/min flow rate. Elution was performed utilizing 20 CV of elution buffer (10 mM Tris-HCl, 500 mM NaCl, 500 mM Imidazole, pH:7.4) with 0.25 mL/min flow rate, and a 280 nm UV detector was used to determine the isolated peaks were collected. Analysis was accomplished using UNICORN 7.0 software. The protein elution fraction was buffer exchanged to Tris-HCl buffer to remove residual imidazole, and the sample was concentrated by using an Amicon Ultra-15 50K Centrifugal Filter Device (Millipore, USA). BCA assay was performed to determine the total protein concentration of the purified sample. The sample was verified using SDS-PAGE, and Western Blotting (Figure 3.2). The purified P2-GFP samples were stored in 20 mM Tris-HCl (pH: 7.4), 1 mM DTT, and 20% glycerol stock solution. P2-GFP enzyme stocks were stored at −20°C.

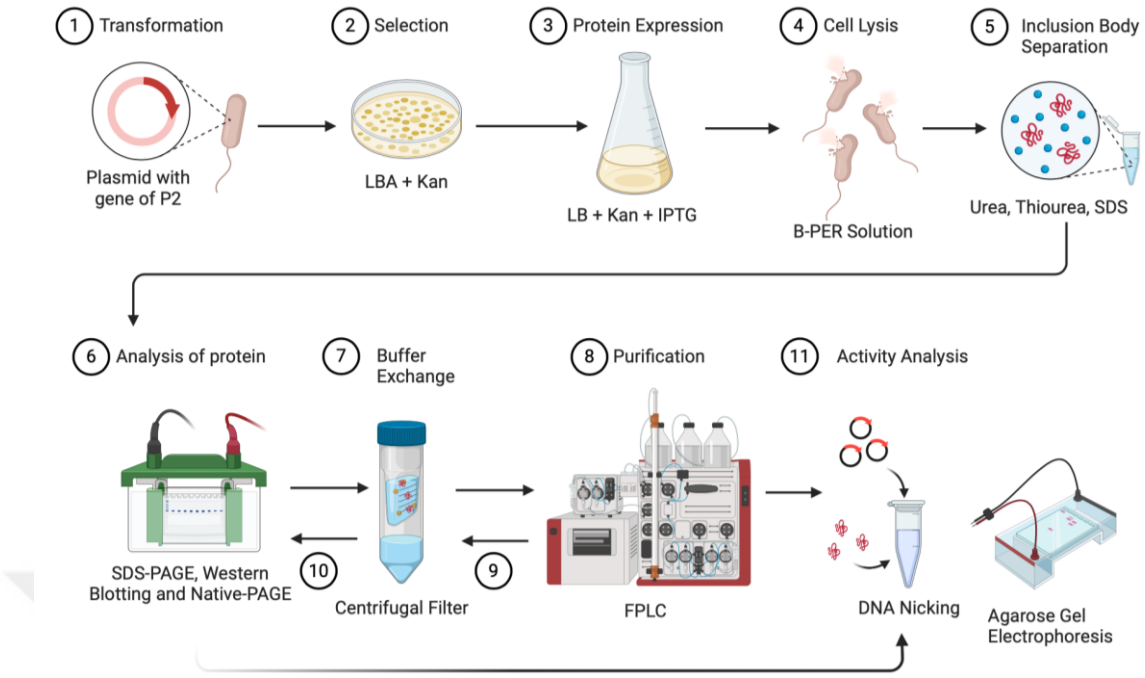


Figure 3.2: P2-GFP production process schema. After the transformation of the recombinant plasmid, P2-GFP was expressed, and cell lysate was analyzed by using SDS-PAGE, Native-PAGE, and Western Blotting methods. P2-GFP was purified from the collected sample mixture by FPLC and samples were analyzed again by the same methods.

Fluorescence of samples were imaged using 200 µL of the purified sample placed into 96-well black-bottom plates (Corning, USA) under blue epi-illumination with a standard filter in the ChemiDoc gel imaging system (Bio-Rad).

3.6 TEV Digestion

GFP and His-Tag protein part of P2-GFP was cleaved with TEV-Protease Plus enzyme (Promega) at room temperature for 6 hours as given in Table 3.16. The 2 mL volume of Nickel-coated agarose bead solution (Ni-Beads (GoldBio, USA)) was centrifuged at 3,000 rpm for 10 mins and supernatant was removed. The beads were mixed with 3 CV of Tris buffer (20 mM Tris-HCl, 100 mM NaCl, pH:7.4), and equilibrated at 4°C for 20 minutes with slightly shaking. The sample was centrifuged, and supernatant was removed at end of the equilibration time. This washing step was performed one more time. The TEV-digested sample was loaded into column bed and incubated at 4°C with slightly shaking for overnight to binding. The next day, flow-

through samples were collected as Histidine-GFP fusion protein part removed P2. Bounded proteins which including digested Histidine-GFP proteins were eluted with elution buffer (20 mM Tris-HCl, 500 mM NaCl, 400 mM Imidazole, pH:7.4). All stock samples were stored at −20°C until use.

Table 3.16: TEV Protease Digestion Reaction

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
ProTev Buffer (Promega)	20×	1×	5
DTT	100 mM	1 mM	1
Fusion Protein		20 µg	
ProTev Plus Enzyme	10U/µL	20U	2
Nuclease free-ddH_2O			Up to 100
Total Volume			100

3.7 Residual DNA Control of Purified P2

The purified P2-GFP sample was analyzed for presence of residual DNA with universal 16S rRNA primers which 27F1 forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 534R reverse (5'-ATTACCGCGGCTGCTGG-3') primers by Real-Time PCR (Q-PCR) as given in Table 3.17 and 3.18. P2-GFP sample was used as a test sample and DH5α *E. coli* genomic DNA was used as a positive control of PCR. PCR products were analyzed by Cq values, melting curve and 1% agarose gel electrophoresis by EtBr Dye.

Table 3.17: Q-PCR Ingredients for Amplification of 16S rRNA Site

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
Q-PCR Master Mix (GeneMark)	2×	1×	10
Template/ P2-GFP protein sample			1
Forward Primer	10 uM	0.2 uM	0.5
Reverse Primer	10 uM	0.2 uM	0.5
Nuclease free-ddH_2O			8
Total Volume			20

Table 3.18: Q-PCR conditions for 16S rRNA PCR

Initial Denaturation	95 °C	2 minutes
*Denaturing	95 °C	20 seconds
*Annealing	52 °C	30 seconds
*Extension^	72 °C	1 minute
Melting Curve	52-95 °C	
*40 cycles		
^ Sybr green reading		

3.8 Activity Tests

3.8.1 DNA nicking

3.8.1.1 Linear DNA nicking

3.8.1.1.1 Preparation of substrates

For cDNA conversion was performed by using the inactivated Sars-CoV-2 virus particles were obtained from LabCell (Acibadem University). The inactivated virus solution was incubated at 55°C for 5 minutes to melt virus package. cDNA conversion reaction was performed as given in Table 3.19 at 42°C for 1 hour, 85°C for 5 minutes by a thermal cycler (Bio-Rad, USA). cDNA sample was stored at −20°C.

Table 3.19: cDNA Conversion Reaction

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
Amv Buffer (NEB)	10×	1×	2
Amv RT Enzyme (NEB)			1
Sars-CoV19 virus			5
dNTPs	10 mM	0.5 mM	1
Thermo Random Primers	10x	1x	2
Nuclease free-ddH_2O			10
Total Volume			20

Different substrates which are given in Table 3.20 were produced by using different primer pairs depend on DNA template (Table 3.21, and 3.22). Primers were designed as shown in Figure 3.3. Substrates were amplified by PCR as given in Table 3.23, 3.24 and 3.25.

Table 3.20: List of Substrates

Substrate Name	**Description**	**Primer Pairs (Set)**	**Product Size (bp)**
Substrate I	dsDNA: f1 ori amplification	P2 Ct1-Fw / P2 Ct Rv **(Set 1)**	191
Substrate II	dsDNA: Sars_CoV-2 N1 site amplification with "whole" part of the recognition site included primers	N1P2-Fw / Std 1 P2Rv **(Set 2)**	264
Substrate III	dsDNA: Sars_CoV-2 N1 site amplification with "A1 and A2 loop" part of the recognition site included primers	N1P2 A12-Fw / Std 1 P2 A12 Rv **(Set 3)**	208
Substrate IV	dsDNA: Sars_CoV-2 N1 site amplification with "A1 loop" part of the recognition site included primers	N1P2 A1-Fw / Std 1 P2 A1 Rv **(Set 4)**	158

Table 3.20: List of Substrates (Continue)

Substrate Name	Description	Primer Pairs (Set)	Product Size (bp)
Substrate V	dsDNA: Sars_CoV-2 N1 site amplification with "truncated A1 loop" part of the recognition site included primers	A1L Fw / A1L Rv **(Set 5)**	114
Substrate VI	dsDNA: Sars_CoV-2 N1 site amplification with "smallest truncated A1 loop" part of the recognition site included primers	A2L Fw / A2L Rv **(Set 6)**	104
Substrate VII	dsDNA: dsOligomer (by Klenow)	N1-P2H-Fw / P2 CtB Fw **(Set 7)**	110 bp
Substrate VIII	ssDNA: Oligomer	N1-P2H-Fw (110 bp)	110

Table 3.21: List of Primers specific to p1GFP for Substrate I

Primer Name	Nucleotide Sequence (5'-3')	Size (bp)	GC%	Tm (°C)
P2-CtF1 Fw	AGGGTTCCGATTTAGTGCTT	20	45	54
P2 Ct Rev	AATCAAAAGAATAGCCCGAGATAG	24	37.5	52.7

Table 3.22: List of HPLC grade Primers specific to Sars Cov-2

Primer Name	Nucleotide Sequence (5'-3')	Size (bp)	GC %	Tm (°C)
*N1P2 Fw	CTTTGACGTTGGAGTCCACGTTCTTT**AAT AGTGGACTCTTGTTCCAAACTGGAACA ACACTCAACCCTATCTCGGTCTATTCTT TTGA-TT**GACCCCAAAATCAGCGAAAT	110	41.8	71
N1P2-A1 Fw	CGTTGGAGTCCACGTTCTTT**AATAGTGGA CTCTACAC**GACCCCAAAATCAGCGAAAT	57	45.6	68.6
*N1P2-A12 Fw	CTTTGACGTTGGAGTCCACGTTCT-TT**AATAGTGGACTCTTGTTCCAA-ACTGGAACAACACTC**GACCCCAA-AATCAGCGAAAT	82	43.9	70.6
Std1 P2 Rv	CTTTGACGTTGGAGTCCACGTTC-TTT**AATAGTGGACTCTTGTTCC-AAACTGGAACAACACTCAACC-CTATCTCGGTCTATTCTTTTGA-TT**TGCGTTCTCCATTCTGGTTA	110	41.8	70.8
Std 1 Rv	TGCGTTCTCCATTCTGGTTA	20	45	53.9
Std1 P2-A1 Rv	CGTTGGAGTCCACGTTCTTT**AA-TAGTGGACTCTACACT**GCGTTC-TCCATTCTGGTTA	57	45.6	68.1
Std 1 P2-A12 Rv	CTTTGACGTTGGAGTCCACGTTC-TTT**AATAGTGGACTCTTGTTCC-AAACTGGAACAACACTC**TGCGT-TCTCCATTCTGGTTA	82	43.9	70.3

Underlined and **bold** letters show P2 digested parts at recognition site

*N1: 2019- nCoV_N1 Forward Primer derived

Table 3.22: List of HPLC grade Primers specific to Sars Cov-2 (Continue)

Primer Name	Nucleotide Sequence (5'-3')	Size (bp)	GC%	Tm (°C)
A1L Fw	ACGTTCTTT**AATAGTGACCCCA-AAATCAGCGAAAT**	35	37.1	69.8
A1L Rv	ACGTTCTTT**AATAGTTGCGTTC-TCCATTCTGGTTA**	35	37.1	69.2
A2L Fw	GTTCTTT**AATGACCCCAAAATC-AGCGAAAT**	30	36.7	66.6
A2L Rv	GTTCTTT**AATTGCGTTCTCCAT-TCTGGTTA**	30	36.7	65.9

Underlined and **bold** letters show P2 digested parts at recognition site

*N1: 2019- nCoV_N1 Forward Primer derived

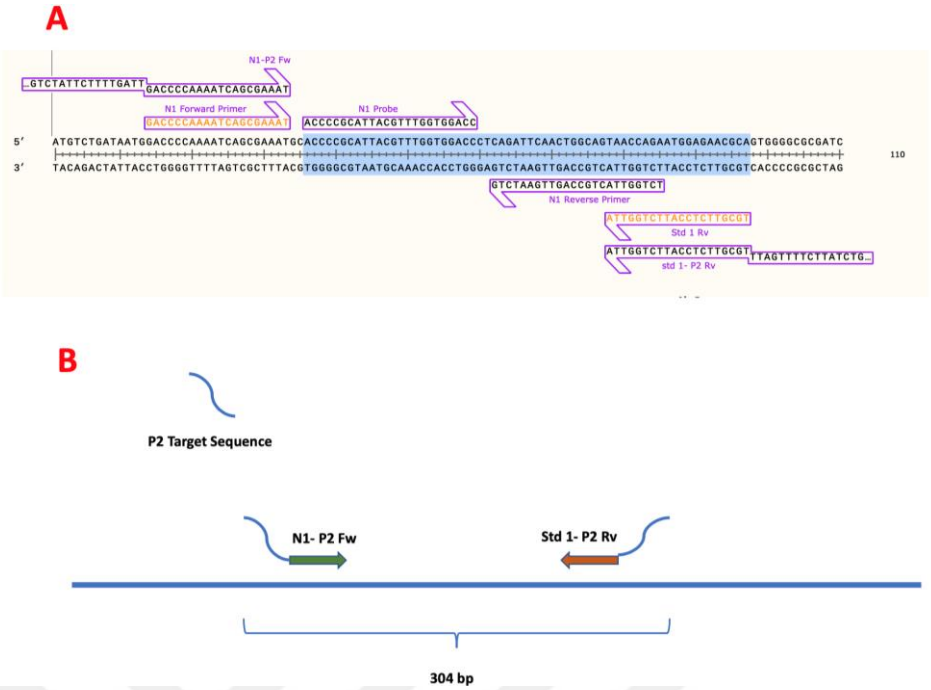


Figure 3.3: Representation of designed primer bindings and PCR products. **A.** Designed primers are shown on Sars Cov-2 cDNA sequence. **B.** Designed primers with P2 recognition site and expected product size from Sars Cov-19 cDNA template are shown. Gradient PCR was performed for substrate 2,3 and 4 primer pairs separately.

Table 3.23: PCR Ingredients for Amplification of Substrate I

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
Genemark Master Mix	5×	1×	10
Template DNA	50 ng/µL	100 ng	2
Forward Primer	10 µM	0.4 µM	2
Reverse Primer	10 µM	0.4 µM	2
Nuclease free-ddH_2O			34
Total Volume			50

Table 3.24: PCR Ingredients for amplification of cDNA Templated Substrates

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
Genemark Master Mix	5×	1×	10
Template DNA (cDNA)			1
Forward Primer	10 µM	0.4 µM	1
Reverse Primer	10 µM	0.4µM	1
Nuclease free-ddH_2O			17
Total Volume			25

Table 3.25: Gradient PCR Conditions for Substrates

Initial Denaturation	95 °C	3 minutes
*Denaturing	95 °C	30 seconds
*Annealing	54-64 °C	20 seconds
*Extension	72 °C	30 seconds
Final Extension	72 °C	5 minutes
Final Temperature	4 °C	∞
*35 cycles		

PCR products were analyzed by 1.5% agarose gel electrophoresis with EtBr to obtain optimal annealing temperature. Amplicons were amplified by PCR under optimal PCR condition and purified from the 1.5% agarose gel by using the gel elution kit (Zymo-Research, USA). DNA concentration of purified amplicon was determined via nanodrop and analyzed by 2% agarose gel electrophoresis with EtBr.

Substrate VII was obtained as given in Table 3.26 with DNA Polymerase I Large Fragment (Klenow Fragment) (NEB, USA) by duplication of the oligomer strand with using a reverse primer which specific to this oligomer. The reaction was incubated at 25 °C for 1 hour and the enzyme was inactivated at 75 °C for 20 minutes then the sample was kept at 4°C. Each of the forward and reverse primers was used separately as a reaction control. Products were analyzed on urea gel with 1× Sybr Gold staining.

Table 3.26: Ingredients of Substrate VII Conversion Reaction

Components	Stock Concentration	Final Concentration	Volume (µL)
NEB Klenow Buffer 2.0	10×	1×	5
Klenow Fragment	5 U/µL	5 U	1
dNTPs mix	10 mM	0.2 mM	1
Forward Primer	10 µM	0.6 µM	3
Reverse Primer	10 µM	0.6 µM	3
Nuclease free-ddH_2O			37
Total Volume			50

3.8.1.1.2 Nicking reaction

Nicking reactions for linear DNA samples that substrates I, II, III, IV and V were performed by using each SEC (Sephadex G-100), HPLC, Passive Gel Elution and FPLC purified P2-GFP protein samples. The reaction was performed according to the protocol which given in Table 3.27 for SEC purified; Table 3.28 for HPLC or passive gel elution method purified. Samples were incubated at 37 °C for 3 hours and inactivated at 75 °C for 20 minutes. Reaction samples were analyzed on urea gel with 1× Sybr Gold staining. Non-purified protein sample in the inclusion body dissolving solution was used as a control sample.

Table 3.27: Ingredients of Nicking Reaction of Linear DNA Substrates with SEC Purified P2-GFP

Components	Stock Concentration	Final Concentration	Volume (µL)
NEB Klenow Buffer 2.0	10×	0.5×	2.5
NEB rCut Smart Buffer	10×	0.5×	2.5
Substrate DNA		500 ng	x
P2-GFP	0.55 µg/µL	72 / 40 / 11 / 6 µg	y
Nuclease free-ddH_2O			50–(x+y)
Total Volume			50

Table 3.28: Ingredients of Nicking Reaction of Linear DNA Substrates with HPLC or Passive Gel Elution Method Purified P2-GFP

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
NEB Klenow Buffer 2.0	10×	0.5×	2.5
NEB rCut Smart Buffer	10×	0.5×	2.5
Substrate DNA	75 ng/µL	525 ng	7
P2-GFP	1.5 µg/mL	37.5 ng	25
Nuclease free-ddH_2O			13
Total Volume			50

3.8.1.1.3 Product analysis by urea gel

Denaturing conformational DNA polyacrylamide gel (%15) was prepared with urea as given in Table 3.29. Gel was pre-run in 1× TBE buffer at 50°C for 1 hour. Samples were prepared with 2× Loading Dye 1 (90% formamide, 0.5% EDTA, 0.1% xylene cyanol FF, 0.1% bromophenol blue) or 2× Loading Dye 2 (90% DMSO, 0.5% EDTA, 0.1% xylene cyanol FF, 0.1% bromophenol blue) and incubated at 95°C for 5 minutes then loaded into wells. Gel was run at 120V, 50°C for 3 hours. Gels were fixated in fixation solution (5% EtOH, 5% metOH, 1× TBE) for 20 minutes with shaking for 2 times then dyed with 1× Sybr Gold Solution in 1× TBE or 0.5 µg/mL EtBr solution for 1 hour and visualized by ChemiDoc Gel Imaging System (Bio-Rad, USA). Non-dyed gels were stained with a silver staining kit according to the kit protocol (Bio-Rad, USA).

Table 3.29: Urea-PAGE Gel Components

15% (w/v) Acrylamide Gel	**2 Gels/ 1mm (10 mL)**
30% (w/v) Acrylamide-Bisacrylamide Solution (29:1)*	5 mL
Urea*	4.8 gr
10× TBE	1 mL
30% APS**	33 µL
TEMED**	5 µL
*Heated together	
** Added last and APS used freshly	

3.8.1.2 Circular DNA nicking

The first enzymatic activity test of P2-GFP was performed according to Meyer and Geider's protocol (59). The cleavage reaction of P2-GFP was performed in the presence of 20 mM Tris-HCl pH:8.0, 5 mM $MgCI_2$, 5 mM β-mercaptoethanol (BME), 20% sorbitol, 80 mM KCl and 4 µg BSA with p1GFP plasmid as a substrate for 20 minutes at 30°C. The reaction was stopped with 60 mM EDTA (59). Digestion reactions were performed at different conditions to determine the optimal reaction conditions as given in Table 3.30. In another reaction performed with 0.5× rCut Smart Buffer (NEB, USA) the which were 25 mM Potassium Acetate, 10 mM Tris-acetate, 5 mM Magnesium Acetate, 50 µg/ml Recombinant Albumin (pH 7.9); and 0.5× Klenow Fragment's Buffer (NEB 2.0) which were 5 mM Tris-HCl, 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT (pH 7.9) buffer mix. 300 ng p1GFP with 16 ng P2-GFP were incubated at 30°C for 30 minutes. TEV Digested P2, which is GFP removed, and M13KE RF DNA was used as a natural substrate for P2 nicking reaction control under same conditions.

Table 3.30: Optimization Conditions of P2-GFP Nicking Reaction

Reagents	**Conditions**
$MgCI_2$ (mM)	10, 8, 6, 5, 2 ,1, 0
KCl (mM)	160, 80, 40, 20, 10, 5, 0
BME (mM)	19.83, 14.87, 9.92, 5, 0
P2-GFP (ng)	128, 64, 48, 32, 16, 8, 4, 2, 0
Temperature (°C)	37, 36.4, 35.5, 34.5, 32.8, 31.3, 30.4, 30, 22-25*
Time (min)	15, 30, 45, 60
*Room temperature	

After the first activity tests, the next nicking assays were typically performed with 80 fmol of f1 origin-containing p1GFP plasmid DNA (300 ng) and 220 fmol of P2-GFP enzyme in 20 µL (11 nM) reaction. DNA was prepared with KCl buffer before the reaction. Different concentrations of the reaction buffer reagents such as β-Mercaptoethanol, $MgCI_2$, enzyme, KCl and different incubation time courses at different temperatures were performed to determine the optimal reaction conditions in 1× Reaction Buffer (20 mM Tris pH: 8.5, 9.10% Sorbitol, 5 µg BSA). The P2 insert DNA was used as a non-targeted substrate DNA because it lacks a nicking site and to control the star activity of the enzyme. The control reaction was performed with a stock solution reagent instead of P2-GFP enzyme to follow the enzyme product conversion. The reactions were stopped with 60 mM EDTA and 120 ng of the total DNA sample from the reaction was analyzed on 1% agarose gel electrophoresis with EtBr dye. Gel was imaged by using the ChemiDoc Gel Imaging system (Bio-Rad, USA). Gel images were converted to quantitative values by using Image J Software then graphics were created from these values by Prism software.

3.9. Stability Control

The stability of the P2 enzyme after 6 months at −20°C was tested by the optimized protocol of circular DNA nicking reaction as given in Table 3.31. Reactions were incubated at 30°C for 30 minutes and 60 minutes. Also, the P2-GFP nicking

reaction was performed in 1× phi29 DNA Polymerase Buffer (NEB, USA) (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM DTT) for the test of buffer compatibility with the same conditions. Then reactions were stopped with 60 mM EDTA, and 86 ng of the total DNA sample from the reaction was analyzed on 1% agarose gel electrophoresis with EtBr dye. The gel was imaged by using the ChemiDoc Gel Imaging system (Bio-Rad, USA). Gel images were converted to quantitative values by using Image J Software, and then graphics were created from these values by Prism software.

Table 3.31: Stability Control of P2

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
P2 Nicking Buffer*	3×	1×	7
$MgCI_2$	100 mM	10 mM	2.1
BME	286 mM	9.9 mM	0.73
BSA	5 mg/mL	5 µg	1
KCl Buffer**	5×	0.05×	1
Substrate DNA	75 ng/µL	300 ng	4
P2-GFP	16 ng/µL	16/32 ng	1/2
Nuclease free-ddH_2O			4.17/3.17
Total Volume			21

*1×: 20 mM Tris-HCl pH:8.4, 9% sorbitol

**1×: 20 mM Tris-HCl pH:8.4, 80 mM KCl

3.10 Determination of Minimal Recognition Sequence for P2 Nicking

3.10.1 Cloning of different segments of P2 nicking site

Different insert DNAs were prepared from the Sars-CoV2 virus genome by using designed primers that carry different parts of P2 recognition sites (Table 3.32). cDNA conversion was performed by using inactivated Sars-CoV2 virus particles, Amv Reverse Transcriptase enzyme (NEB, USA) with random primers (Thermo Fisher

Scientific, USA) at 42°C for 1 hour of incubation. Each insert was amplified from cDNA by PCR using suitable primer pairs as given in Table 3.33 by PCR (Table 3.24). PCR products were analyzed by agarose gel electrophoresis and amplicons were purified from the gel by using the gel elution kit (Zymo-Research, USA). DNA concentration was determined by nanodrop.

Table 3.32: List of Inserts

Insert Name	**Description**	**Primer Pairs (Set)**	**Product Size (bp)**
Insert W	dsDNA: Sars_CoV2 N1 site amplification with "whole" part recognition site included primers	N1P2 Fw / Std 1 P2 Rv	264
Insert A12	dsDNA: Sars_CoV2 N1 site amplification with "A1 and A2 loop" part recognition site included primers	N1P2 A12 Fw / Std 1 P2 A12 Rv	208
Insert A1	dsDNA: Sars_CoV2 N1 site amplification with "A1 loop" part recognition site included primers	N1P2 A1 Fw / Std 1 P2 A1 Rv	158
Insert A1L	dsDNA: Sars_CoV2 N1 site amplification with "Truncated A1 loop" part recognition site included primers	A1L Fw / A1L Rv	114
Insert A2L	dsDNA: Sars_CoV2 N1 site amplification with "Truncated smallest part of A1 loop" part recognition site included primers	A2L Fw / A2L Rv	104

Table 3.33: PCR Conditions for Inserts

Initial Denaturation	95 °C	3 minutes
*Denaturing	95 °C	30 seconds
*Annealing	**56-61°C	20 seconds
*Extension	72 °C	30 seconds
Final Extension	72 °C	5 minutes
Final Temperature	4 °C	∞

*35 cycles
**56 °C for A12 and A1 inserts; 57°C for A1L insert; 60°C for A2L insert; 61°C for W insert.

Inserts were ligated into the pUCm-T vector by using the TA Cloning Kit (Bio Basic, SG). The prepared recombinant DNA constructs were transformed into competent *E. coli* DH5α by the heat-shock method (26). The transformed cells with recombinant constructs were selected on LBA media containing 100 µg/mL Ampicillin (Amp), 200 µg/mL X-Gal, and 1 mM IPTG plates by blue-white screening. The white colonies were screened for insert genes by PCR using A1 insert amplification primers. The presence of insert genes was verified by agarose gel electrophoresis. The plasmids were isolated from the transformant culture by using a plasmid isolation kit (GeneMark, TP) and inserts were verified by PCR. The concentration and purity of plasmids were measured by nanodrop (Thermo Fisher Scientific, USA).

3.10.2 Nicking reaction of different segments of P2 recognition sequence

For the nicking of linear DNA samples, wild type recognition site of P2 which is the f1 origin part (191 bp) was amplified from the *p1GFP* plasmid. The f1 origin primers which are 5'AGGGTTCCGATTTAGTGCTT3' and 5'AATCAAAAGAATAGCCCGAGATAG3' were used by PCR as given in Table 3.23 and 3.25 with 56°C annealing temperature. PCR products were purified using a PCR clean-up kit (GeneMark, TP) and concentration was measured by nanodrop. The same concentration of plasmids as supercoiled form, f1 origin, and insert DNAs as a linear form of recognition site including parts were digested with P2 enzyme as given in Table 3.34 at RT for 2 hours, and the reaction was stopped with 50 mM EDTA. Also, plasmids were digested with *Xho I* restriction enzyme (NEB, USA) for confirmation of linear DNA migration. Digestion samples were analyzed for plasmids and PCR samples by agarose gel electrophoresis and 12% DNA-PAGE with 2× Loading Dye (90% DMSO, 0.5% EDTA, 0.1% xylene cyanol FF, 0.1% bromophenol blue) respectively (28).

Table 3.34: Nicking Reaction

Reagent	Stock Concentration	Final Concentration	Volume (µL)
Reaction Buffer	3×	1×	7
BME (1/100)	143 mM	9.9 mM	1.45
P2 Enzyme	16 ng/µL	16 ng	1
Substrate DNA in 1× KCl Buffer*	-	300 ng	-
ddH_2O	-	-	Up to 21 µL
***Preparation of Substrate DNA**			
DNA		200-300 ng	
KCl Buffer	5×	1×	

3.10.3 Data analysis

Band intensities from agarose gel images were converted to quantitative values by Image J software. Each band's intensity was compared between samples and controls. The percentage of product DNA conversion was calculated depending on the band's intensities; also differences between samples were shown by relative fold change of control. Data was converted to graphics by using Prism Software (version 10.1).

3.11 Binding Assay

The f1 origin DNA that Substrate I (Table 3.20) was amplified from p1GFP DNA with primers which given in Table 3.21 by PCR as given in Table 3.23 and 3.25. The PCR products were purified by GeneMark (TP) PCR Clean-up Kit and DNA concentrations were measured by nanodrop (Thermo Fisher Scientific, USA). DNA and P2-GFP enzymes were put together as given Table 3.35 to analyze binding feature of enzyme. BSA was used as a control protein instead of P2-GFP enzyme. Samples were incubated at 30°C for 3 hours and all sample volume was analyzed by Native Gel Electrophoresis. Gel was imaged with fluorescent lightening by ChemiDoc Gel Imaging System (BioRad, USA). Then gel was stained with EtBr solution to determine DNA bands. After all gel was stained with Coomassie Brillant Blue R-250 and imaged.

Table 3.35: Binding Reaction Samples

Sample	DNA (ng)	Protein (ng)
DNA Control	450	-
DNA + P2-GFP	450	80
P2-GFP Control	-	80
BSA Control	-	80
DNA + BSA	450	80
P2-GFP + BSA	450	80
P2-GFP 1:5 dilution	-	80

3.12 Enzyme Kinetics

Enzyme kinetic nicking reactions were performed by using 600, 300, 200, and 100 ng substrate DNA concentrations. Samples were collected and the reaction stopped with 50 mM EDTA at 15-, 30-, 60-, and 120-minute time points. Samples were analyzed by agarose gel electrophoresis. The gel image was converted to quantitative values as given in the data analysis section. Enzyme kinetics were determined by using the Michaelis Menten Equation via Prism software (version 10.1).

3.13 P2 Mediated DNA Amplification at Room Temperature

phi29 DNA Polymerase (NEB) has an inherent 3´→5' proofreading exonuclease activity, which is most prominent in its strand displacement activity. PAM was performed with qPCR as described in Table 3.36 at 30°C, and Sybr Green readings were obtained every 40 seconds over a period of 2-3.5 hours using a qPCR thermal cycler (Bio-Rad, USA). For reaction control, test tubes without P2-GFP enzyme and substrate DNA template were used. P2-GFP was denaturized at 95°C for 5 minutes to eliminate of any amplification in test samples resulting from residual DNA used by P2-GFP in both test and control groups. The final 1 mM DMSO concentration was used in the reaction to facilitate the denaturation of DNA samples, and the reaction ingredient volumes were adjusted by adding DMSO to replace the volume of nuclease-free ddH_2O. Samples were analyzed by 1% agarose gel electrophoresis after Q-PCR.

Table 3.36: Q-PCR with phi29 DNA Polymerase

Components	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
Phi29 DNA Polymerase Buffer (NEB)	10×	1×	2
Phi29 DNA Polymerase (NEB)	10 U/µL	5 U	0.5
rAlbumin (NEB)	2 mg/mL	100 µg/mL	1
dNTPs mix	20 mM	0.2 mM	0.2
$MgCl_2$	100 mM	5 mM	1
N1P2 A1 Fw Primer	10 µM	0.25 µM	0.5
Std1P2 A1 Rv Primer	10 µM	0.25 µM	0.5
pA1 DNA	1.57 ng/µL	7.85 ng	5
KCl Buffer*	5×	0.05×	1
Sybr Green	10×	1×	2
P2-GFP Enzyme	16 ng/µL	32 ng	2
Nuclease-free ddH_2O			4.3
Total Volume			20

*1×: 20 mM Tris-HCl pH:8.4, 80 mM KCl

4 RESULTS

4.1 Plasmid Amplification

The concentration of the purified pET His6 GFP TEV LIC cloning vector (p1GFP) plasmid was found 100 ng/µL (A280/A260=1.9, A_{260}/A_{230}= 2.40). Electrophoresis gel image of 300 ng of plasmid DNA (p1GFP) is given in Figure 4.1.

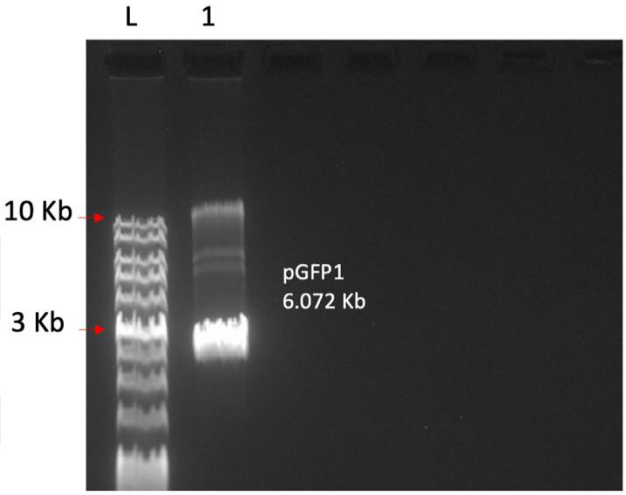


Figure 4.1: p1GFP plasmid DNA electrophoresis. L, 1 kb DNA Ladder; Lane 1, p1GFP plasmid DNA. 0.8% agarose gel with EtBr.

4.2 Engineering of Recombinant DNA

The M13KE *gene II* consists of 410 amino acids, as shown in Table 4.1, and the gene location in the plasmid cassette is depicted in Figure 4.2. The molecular weight of the P2 protein, which includes a 6×Histidine tag and GFP, is 46.2 kDa (Figure 4.3).

Table 4.1: Aminoacid Sequence of Insert

M13KE *gene II* Aminoacid Sequence	MIDMLVLRLPFIDSLVCSRLSGNDLIAFVDLSKIATLSGINLSARTVEYHIDGDLTVSGLS HPFESLPTHYSGIAFKIYEGSKNFYPCVEIKASPAKVLQGHNVFGTTDLALCSEALLLNF ANSLPCLYDLLDVNATTISRIDATFSARAPNENIAKQVIDHLRNVSNGQTKSTRSQNWE STVIWNETSRHRTLVAYLKHVELQHYIQQLSSKPSAKMTSYQKEQLKVLSNPDLLEFA SGLVRFEARIKTRYLKSFGLPLNLFDAIRFASDYNSQGKDLIFDLWSFSFSELFKAFEG DSMNIYDDSAVLDAIQSKHFTITPSGKTSFAKASRYFGFYRRLVNEGYDSVALTMPRN SFWRYVSALVECGIPKSQLMNLSTCNNVVPLVRFINVDFSSQRPDWYNEPVLKIA

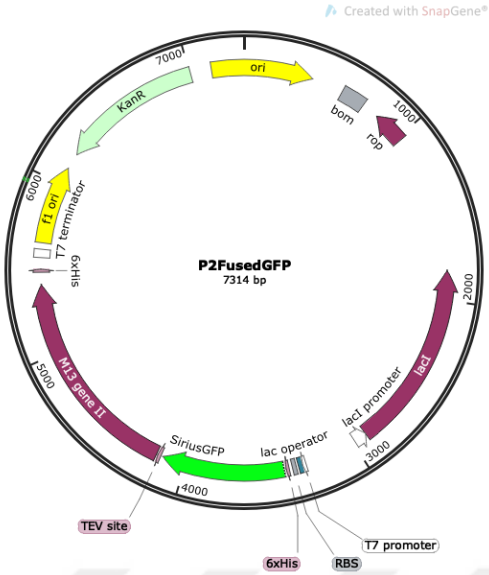


Figure 4.2: Gene cassette of recombinant plasmid. It is carrying M13KE gene II (Snapgene Viewer, version 4.2.11).

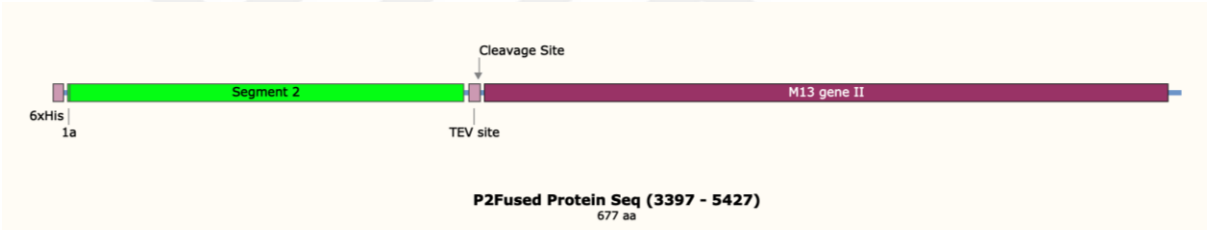


Figure 4.3: Representing of protein translation sequence. P2 protein was expressed with GFP (Segment 2) and 6×His Tag (Snapgene Viewer, version 4.2.11).

4.3 Cloning

4.3.1 Preparation of vector

An agarose gel image of the SspI restriction enzyme digestion sample (150 ng, 6.072 bp) is shown in Figure 4.4.

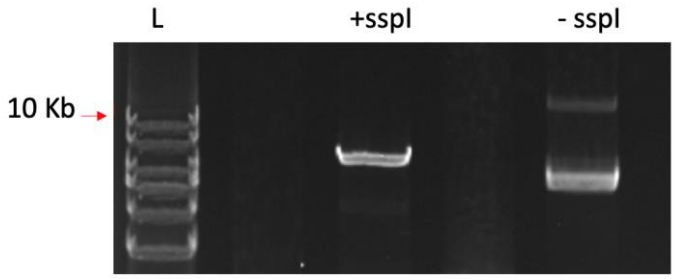


Figure 4.4: Digestion of vector DNA with SspI. L, 1kb DNA Ladder; +sspI, digested p1GFP; -sspI, non-digested p1GFP in 0.8% agarose gel with EtBr.

4.3.2 Amplification of inserts

4.3.2.1 Polymerase chain reaction

Based on the gradient PCR for annealing temperature optimization between 63-72°C, 64°C was selected as the optimal annealing temperature for *gene II* amplification primers. The concentration of the insert DNA was determined to be 10.13 ng/µL according to A_{260} value. The A_{260}/A_{280} and $A_{260}/A2_{30}$ ratios were 1.4 and 1.8, respectively. An agarose gel image of 60 ng PCR products is shown in Figure 4.5.

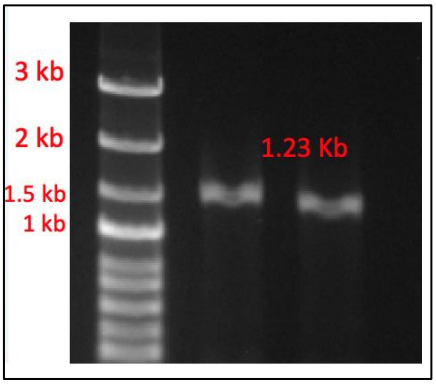


Figure 4.5. Agarose gel image of P2 insert. The amplified PCR products of insert DNA (*gene II*) from M13KE replicative form on 1% agarose gel with EtBr.

4.3.3 Transformation

Transformed bacterial cells were selected on kanamycin-selective LBA plates, as shown in Figure 4.6. The 1:2 vector:insert molar ratio was more efficient than other ratios. No colonies were obtained on the negative control petri dish where competent cells plated. Transformation efficiency was high for the control plate, which included the transformation of the non-digested vector plasmid.

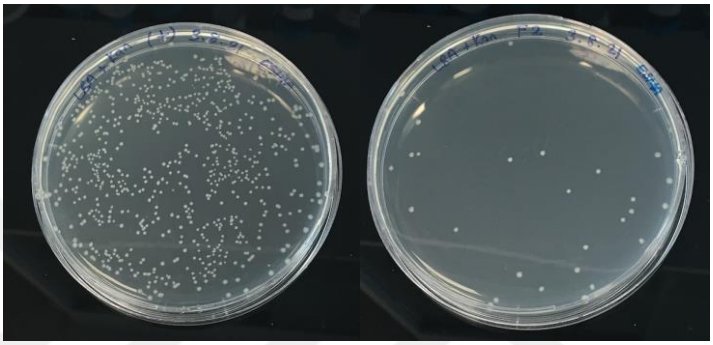


Figure 4.6: Kanamycin-selective LBA plate of transformant bacteria. The left and right petri dishes represent the positive control of the transformation method, where the vector plasmid was used, and the transformation of the recombinant plasmid with a 1:2 vector: insert molar ratio, respectively. 30 colonies were counted for the transformation of the recombinant plasmid on the right.

4.3.4 Colony screening

4.3.4.1 Colony PCR

The analysis of the agarose gel electrophoresis image of colony PCR products is shown in Figure 4.7. The colony containing the backbone vector was used as a control for cloning due to the absence of the targeted DNA sequences.

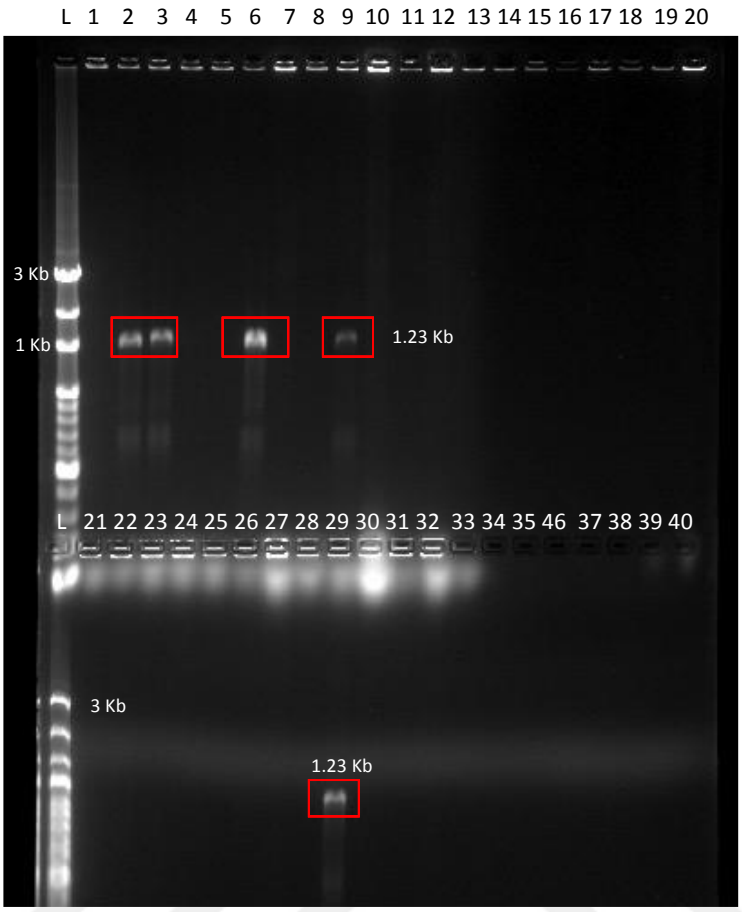


Figure 4.7: Agarose gel Image of colony PCR samples. Each well represents a unique colony. 6th colony was selected for expression.

4.3.5 Plasmid isolation and DNA purification

The recombinant plasmid and vector DNA backbone were compared on agarose gel electrophoresis, as shown in Figure 4.8. The recombinant plasmid appeared heavier than the backbone DNA due to the insert DNA, as expected.

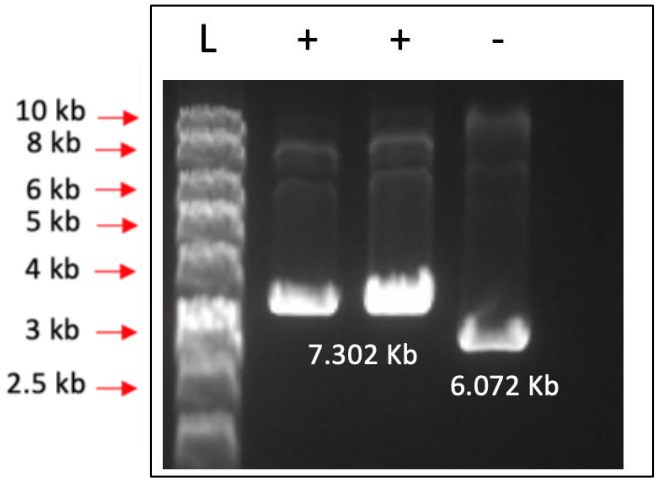


Figure 4.8: Agarose gel image of plasmids. Lane 1, Cloning vector plasmid (p1GFP); Lane 2, Recombinant plasmids (pP2); Lane 3, 1 kb DNA Ladder in %0.8 agarose gel with Sybr Gold.

4.4 Protein Expression

4.4.1 Optimization of expression conditions

The bright green color was observed when GFP-P2 was expressed through the fluorescent light of GFP. Cell pellets could be seen by the naked eye in a bright green, and fluorescence could be observed under blue light, as shown in Figure 4.9. Florescence of cell pellets and supernatants under different expression conditions is shown in Figure 4.10.

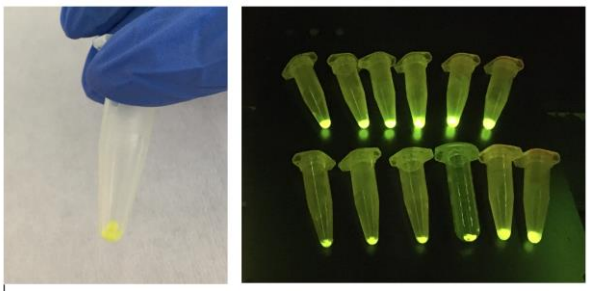


Figure 4.9: Cell pellets of P2-GFP expressed culture. Cell pellets could be seen by the naked eye in a light green color, and fluorescence could be observed under blue light due to the GFP.

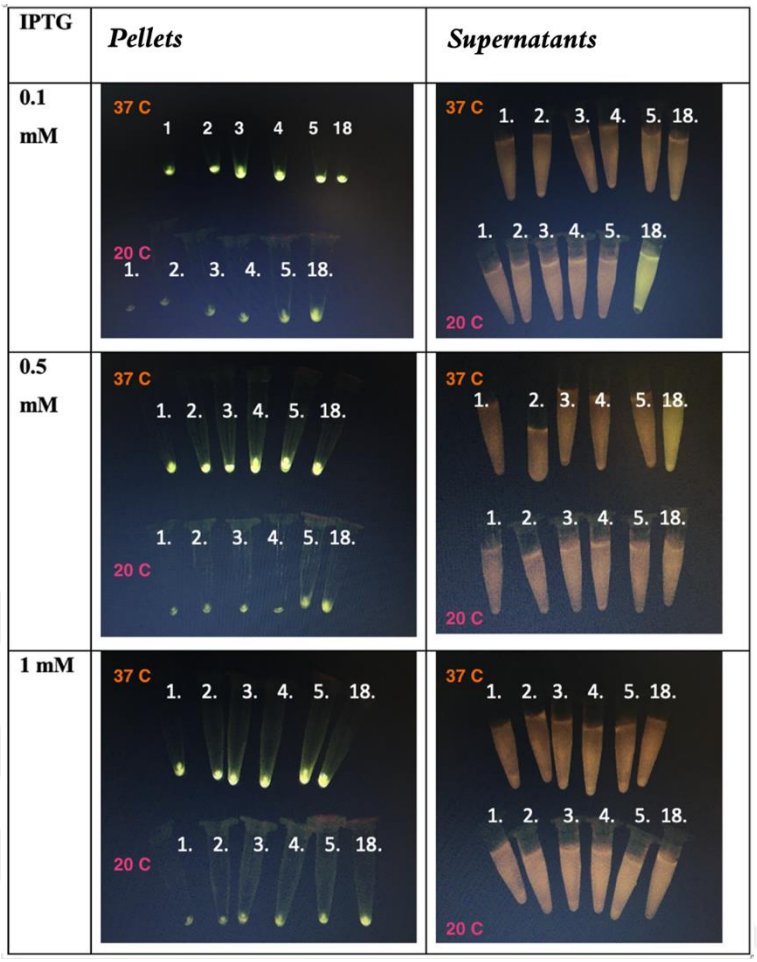


Figure 4.10: Fluorescent lightening of different expression samples under blue light. 37 °C, 20 °C; 0.1 mM, 0.5 mM, 1 mM IPTG inductions; 1., 2., 3., 4., 5., 18. hours incubation times were shown.

4.4.2 Detection of protein localization

SDS-PAGE analysis of cell pellets and supernatant samples under different expression conditions is shown in Figure 4.11-13. When induced and non-induced samples were compared, P2-GFP was observed only in the induced culture samples (Figure 4.12 and 4.13). The optimal expression condition was determined to be incubation at 37°C with 0.5 mM IPTG induction for 5 hours, based on the band intensities of P2-GFP (Figure 4.11 and 4.13).

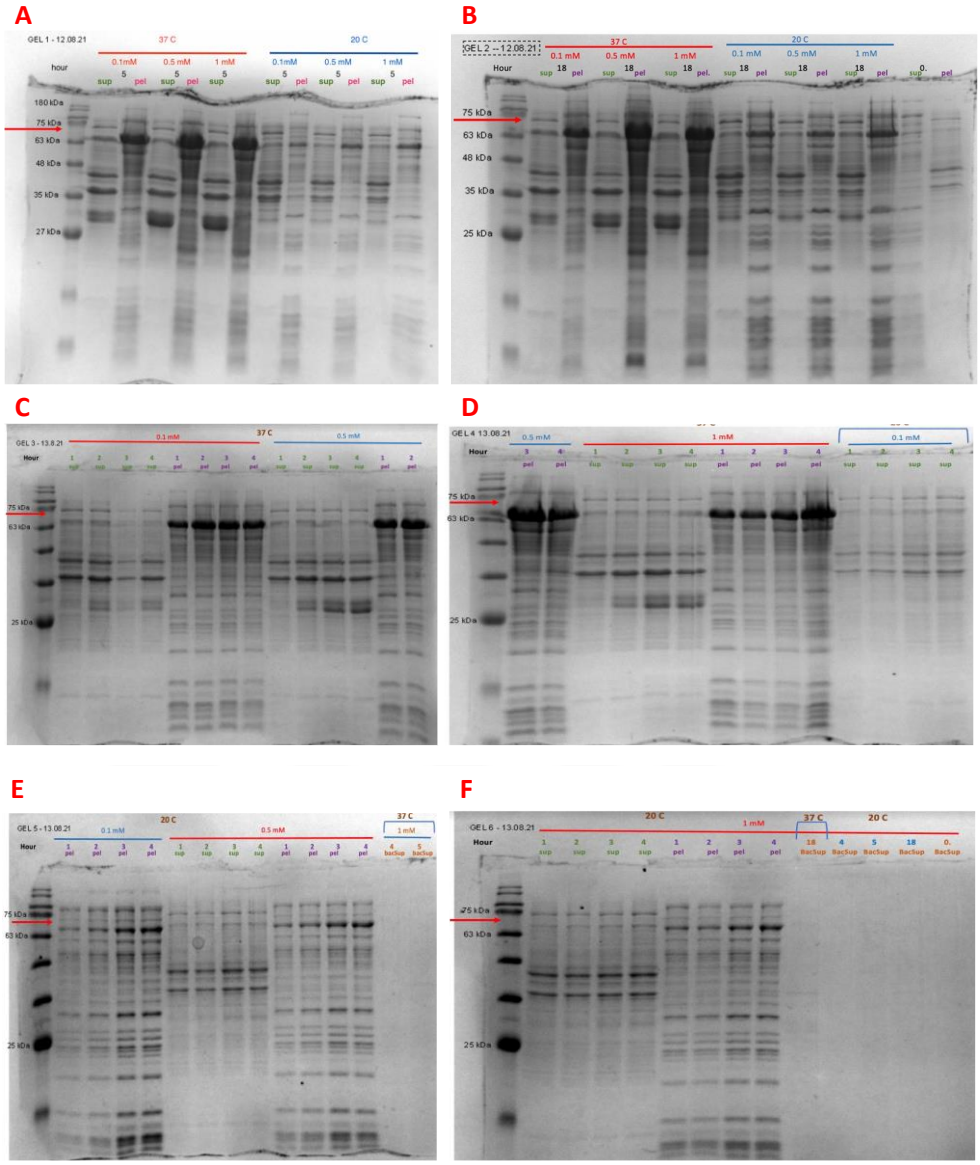


Figure 4.11: SDS-PAGE images of cell cultures at different expression conditions. 37°C, 20°C temperatures and 0.1 mM, 0.5 mM, 1 mM IPTG inductions with 1., 2., 3., 4., 5., 18. hours incubation times for expression are shown. “BacSup”, “pel” and “sup” represent the bacterial culture supernatant media, pellets, and supernatants of the B-PER treated sample respectively. The desired band size is indicated with a red arrow.

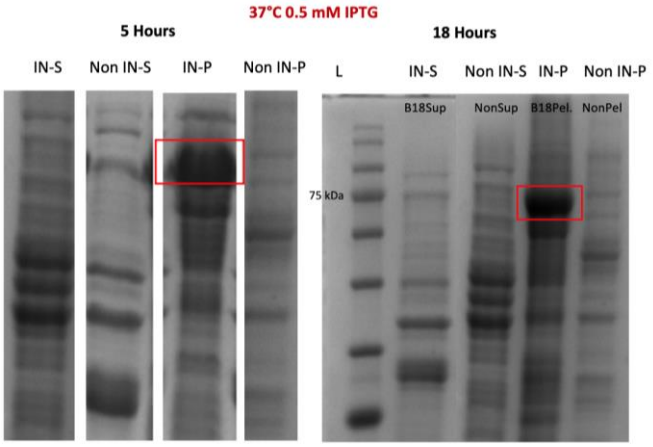


Figure 4.12: SDS-PAGE images of induced (IN-S and IN-P) and non-induced (Non-IN-S and Non-IN-P) cell culture. The supernatant and pellet samples of cultures incubated at 37 °C with 0.5 mM IPTG for 5 and 18 hours are shown. The desired band size is indicated with a red box.

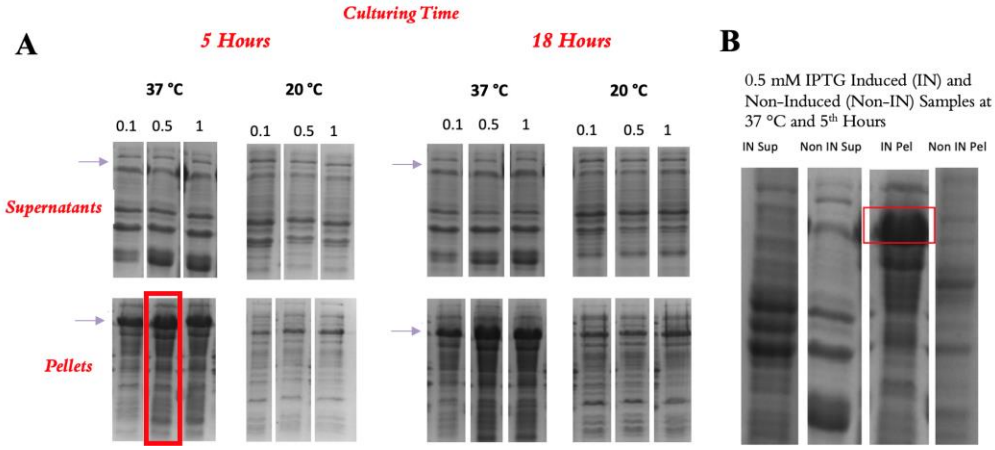


Figure 4.13: SDS-PAGE gel images of expression cultures. **A.** Expected band size of P2 was observed in the cell pellet, and the band of protein sample expression condition at 37 °C with 0.5 mM IPTG induced for 5 hours incubation time was more intense than other cell fractions. **B.** Comparison of 0.5 mM IPTG induced and non-induced protein samples of bacterial culture at 37 °C and 5 hours incubated. The desired band size is indicated with a red box, and it is not seen in non-induced samples.

4.4.2.1 Western blotting

Western Blotting analysis of the cell pellet and supernatant samples of different expression conditions is given in Figure 4.14.

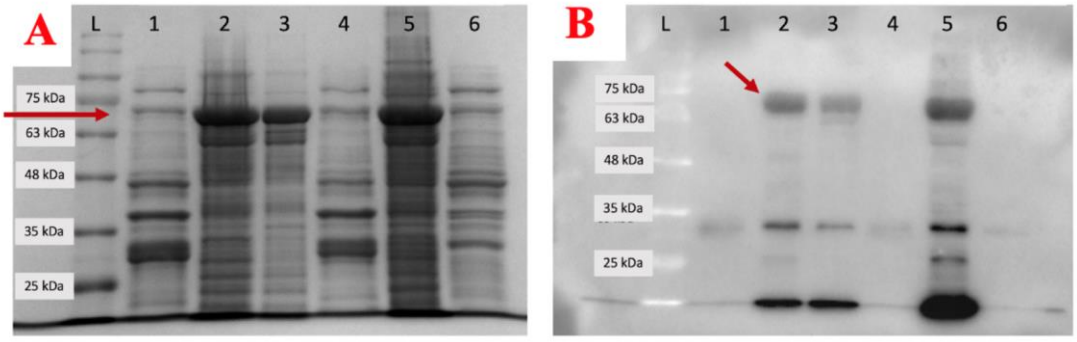


Figure 4.14: SDS-PAGE and Western Blotting images of expression**. A.** SDS-PAGE Gel Image- Gel with Coomassie Brilliant blue. L is protein marker; 3,1 and 4 are the supernatant sample belonging to 20°C, 37 °C with 0.5 mM IPTG induced for 5 hours and 18 hours, respectively; 6, 2 and 5 are the pellet sample belonging to 20°C, 37 °C with 0.5 mM IPTG induced for 5 hours and 18 hours respectively. Expected band is indicated with red arrow. **B.** Membrane Image of Western Blotting- Protein samples were tagged with 1:1000 Histag Rabbit mAB Antibody and 1:3000 anti-rabbit IgG Antibody on membrane.

4.4.2.2 Non-denaturing polyacrylamide gel electrophoresis

Non-Denaturing PAGE gel images are given in Figure 4.15-17. The brightness of the band dependent on GFP expression for optimal condition (incubation at 37°C with 0.5 mM IPTG induction for 5 hours) was brighter than other conditions (Figure 4.15-16). When cell pellets and supernatants were compared, band formation was obtained for only supernatant samples as shown in Figure 4.17. SDS-PAGE gel image of the protein sample which was gel-purified from different band samples from native PAGE is given in Figure 4.18. The band size was equal to GFP's molecular weight, and this suggested that the culture supernatant included the soluble GFP protein fraction (Figure 4.18). SDS-PAGE results of Figure 4.19 reinforced this result.

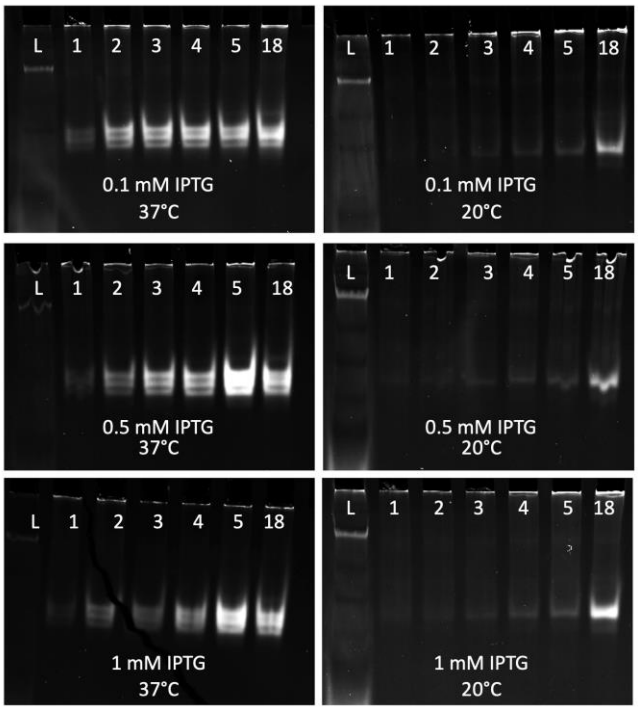


Figure 4.15: Native-PAGE gel images. The Native-PAGE gel images of supernatant samples of the B-PER treated cell pellets (soluble protein fraction) after 18 hours of incubation. The brightness of the band dependent on GFP expression for optimal conditions was observed to be brighter than for other conditions. L is a protein marker.

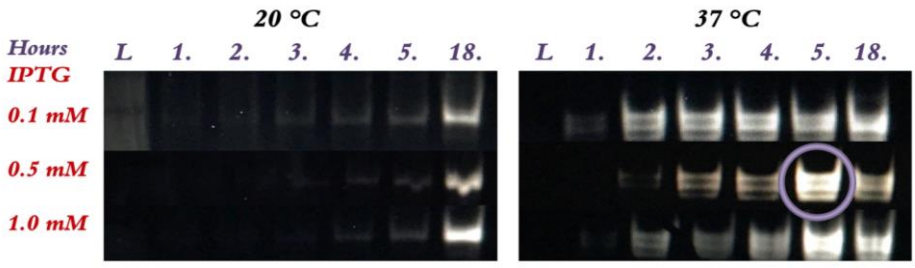


Figure 4.16: Native-PAGE gel image comparison. The Native-PAGE gel images of supernatant samples of the B-PER treated cell pellets (soluble protein fraction). The brightness of the band dependent on GFP expression for optimal conditions was observed to be brighter than for other conditions (shown in a circle). L is a protein marker.

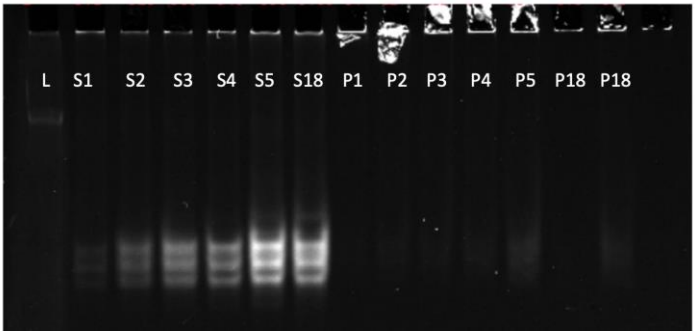


Figure 4.17: Native-PAGE gel image. The supernatant (S1-S18) and pellet samples (P1-P18) of 0.5 mM IPTG-induced fast induction samples at different time courses (1h-18h) after B-PER treatment were shown. L is a protein marker.

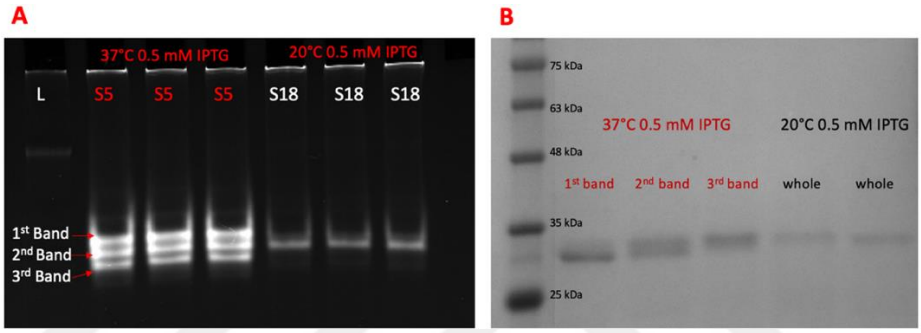


Figure 4.18: Native and Denaturant PAGE images. **A.** The Native-PAGE gel image of soluble fraction samples of different conditions at 5-hour and 18-hour incubation time points. Each band is indicated with an arrow. **B.** SDS-PAGE gel image of each band from the Native Gel.

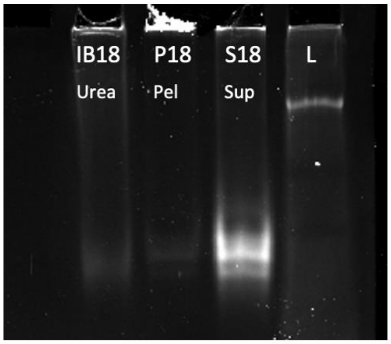


Figure 4.19: Native-PAGE gel image of 18h expression. The samples of 18-hours incubated with 0.5 mM IPTG at 37°C are shown. L is a protein marker. IB18, P18, and S18 were inclusion body dissolved sample, inclusion body pellet, and soluble protein supernatant, respectively.

4.5 Protein Purification

4.5.1 Protein purification by dyna-beads

SDS-PAGE gel image of different protein fraction samples from protein purification steps of Dyna-Bead method is given in Figure 4.20 and 4.21.

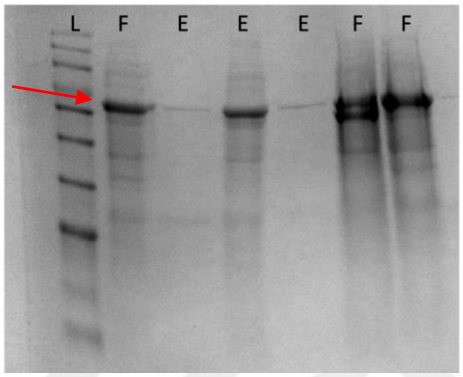


Figure 4.20: SDS-PAGE gel image of the dyna bead purification. The flow-through (F) and elution (E) samples are given. L is a protein ladder. P2-GFP is indicated by red arrow.

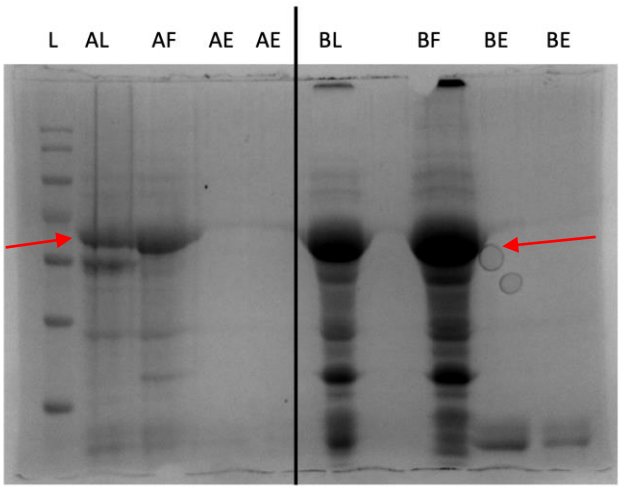


Figure 4.21: SDS-PAGE gel image of dyna bead purified samples. The samples are given with (right side) or without (left side) sonication step. Lysate (AL, BL), Flow-through (AF, BF) and elution (AE, BE) samples are shown. L is a protein ladder. P2-GFP is indicated by red arrow.

4.5.2 Protein purification by immobilized metal affinity chromatography

SDS-PAGE gel image of different protein fraction samples from protein purification steps of IMAC method is given in Figure 4.22 and 4.23.

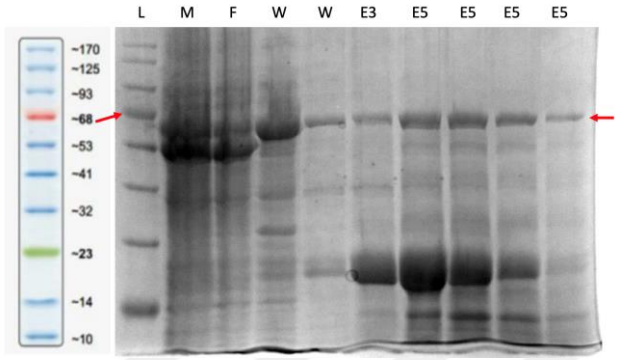


Figure 4.22: SDS-PAGE gel image of nickel agarose bead purified samples. Protein ladder (L), Lysate (M), flow-through (F), washing (W), elution with 300 mM imidazole (E3) and 500 mM imidazole (E5) samples are shown. The expected band is shown by a red arrow.

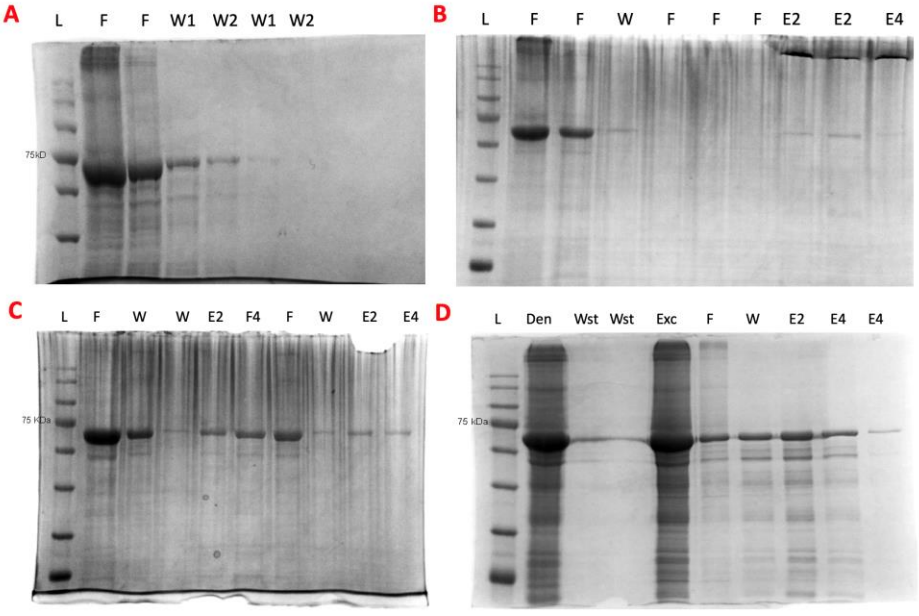


Figure 4.23: SDS-PAGE gel image of IMAC purified samples. Protein ladder (L), Lysate (den), flow-through (F), washing (W), waste fraction of filter tube (Wst), buffer exchanged purified sample (Exc), elution with 200 mM imidazole (E2) and 400 mM imidazole (E4) samples are shown.

4.5.3 Protein purification by passive gel elution method

SDS-PAGE gel image of purified protein samples of passive gel elution method is given in Figure 4.24.

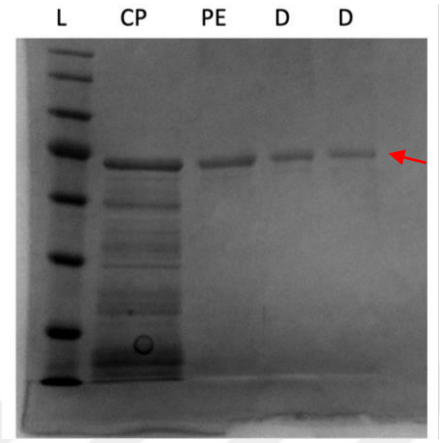


Figure 4.24: SDS-PAGE gel image of passive gel elution method. Protein ladder (L), filter purified (CP), Passive eluted sample (PE), and after dialyzing (D) sample of passive eluted. P2-GFP is indicated by red arrow.

4.5.4 Protein purification by size exclusion chromatography

SDS-PAGE and Western Blotting gel images of protein purification samples of SEC method are given in Figure 4.25. Lyophilized sample results are given in Figure 4.26. The concentration of all SEC sample fractions was calculated by using equivalation that resulted in reference points (y= (973.4x-171.63); R^2: 0.9691) and it is given in Table 4.2.

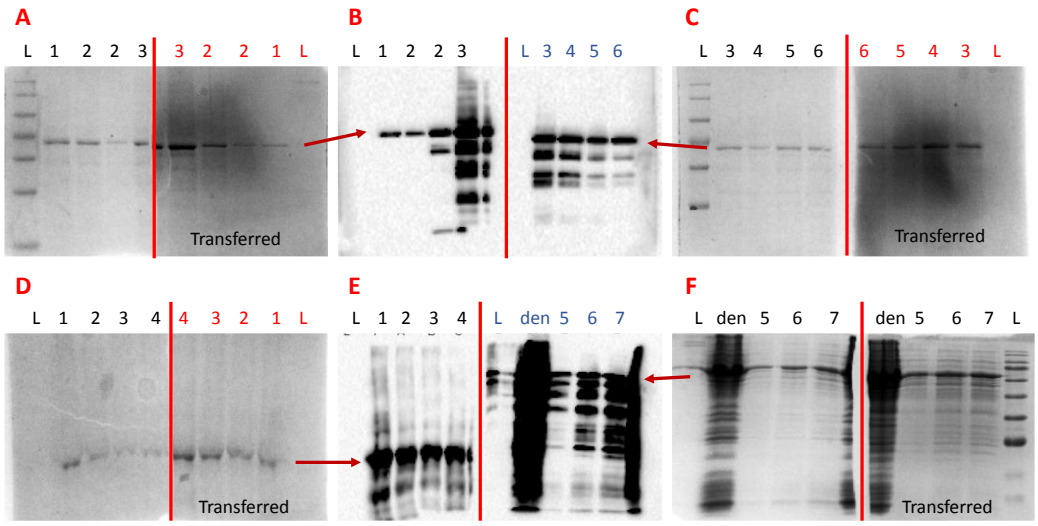


Figure 4.25: SDS-PAGE gel and Western Blotting images of the SEC. There are Sephadex G-100 **(A, B, C)** and G-50 **(D, E, F)** purified samples. The left side is shown before the membrane transferring image with Coomassie and the right side is shown after transferring. Western results of A and C are given on B. Western results of D and F are given on E. The expected band is indicated by a red arrow.

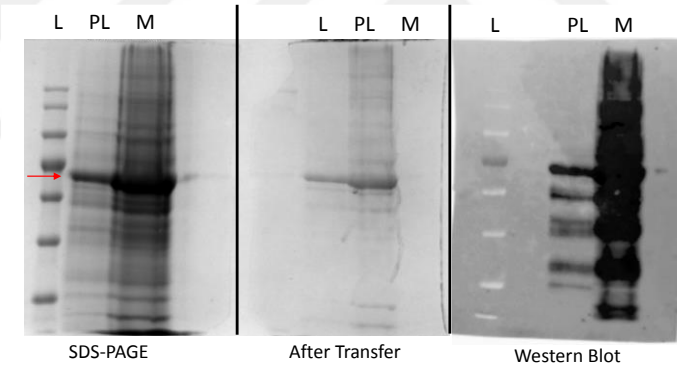


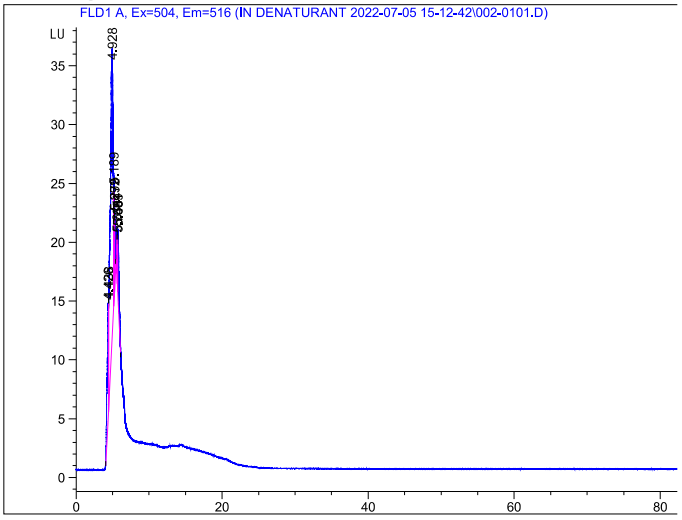
Figure 4.26: SDS-PAGE gel and Western Blotting images of lyophilized SEC sample (PL). Lysate and Protein Ladder are shown as M and L respectively. The expected band is indicated by a red arrow.

Table 4.2: Protein Concentration of SEC Fractions

Sample (G-100, **G-50**)	**Concentration (mg/mL)**
1	2
2	0.54
3	0.56
4	1.17
5	2.7
6	8.3
1	0.178
2	0.852
3	4.7
4	1.3
5	23.52
6	35.3
7	overflow
Lyophilized	29

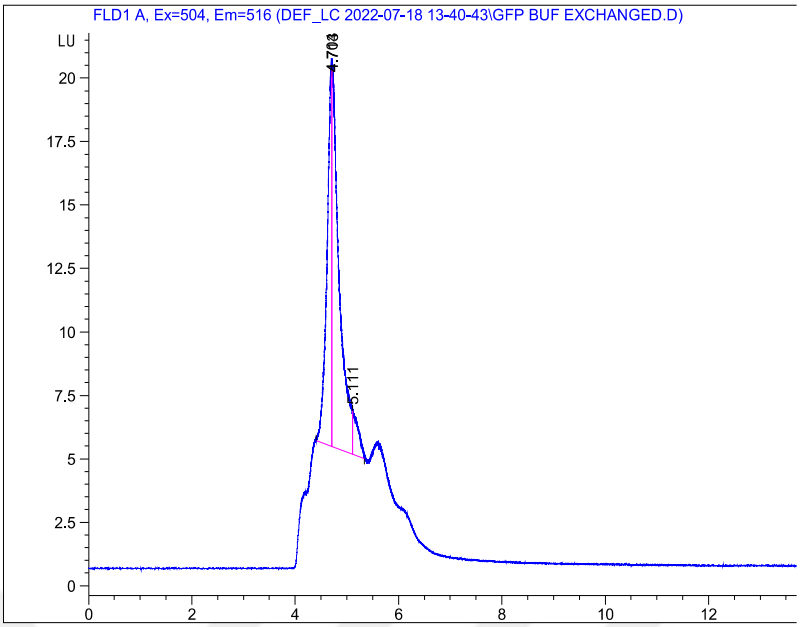
4.5.5 Protein purification by HPLC

Chromatograms of the denatured sample and buffer-exchanged samples are given in Figure 4.27, and Figure 4.28, respectively.




```
=====================================================================
                        Area Percent Report
=====================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: FLD1 A, Ex=504, Em=516

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [LU*s]      [LU]        %
----|-------|----|-------|----------|----------|--------|
   1   4.426 BV    0.1317  103.85677    9.27675  10.5734
   2   4.438 VV    0.0101    6.53098    9.26382   0.6649
   3   4.928 VV    0.3300  697.91730   24.75428  71.0534
   4   5.189 VV    0.0830   49.42551    9.92911   5.0319
   5   5.279 VV    0.0107    4.69125    6.64850   0.4776
   6   5.292 VV    0.0605   32.67638    6.41629   3.3267
   7   5.614 BV    0.0340    8.72216    3.08771   0.8880
   8   5.661 VV    0.0397   14.16927    4.28176   1.4425

   9   5.689 VV    0.0352   13.56532    4.67892   1.3811
  10   5.740 VB    0.1233   50.68810    4.83908   5.1604

Totals :                   982.24303   83.17622
```

Figure 4.27: HPLC chromatogram of denature sample.




```
=====================================================================
                      Area Percent Report
=====================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: FLD1 A, Ex=504, Em=516

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [LU*s]      [LU]        %
----|-------|----|-------|----------|----------|--------|
   1   4.704 BV    0.0728   93.09732   15.14334  36.6987
   2   4.713 VV    0.1144  146.56793   15.08779  57.7767
   3   5.111 VB    0.1316   14.01460    1.77461   5.5245

Totals :                   253.67986   32.00573
```

Figure 4.28: HPLC chromatogram of buffer exchanged sample.

SDS-PAGE gel image of the HPLC purified protein samples is given in Figure 4.29. The concentration of the injection sample (M) was measured 480 mg/mL and the purified peak pool was measured 1.5 µg/mL by BCA kit according to the reference equation ($y = 856.35x - 20.019$; $R^2 = 0.983$). While OD504 of the injection sample was 5.742, HPLC peak samples were ~0.06.

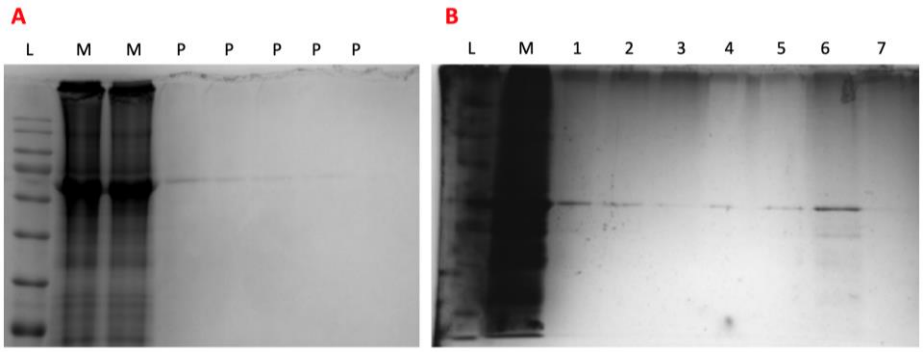


Figure 4.29: SDS-PAGE gel of HPLC purified peak samples. The samples are (P and 1-7) at pH 7.4 **(A, B-1,2)**, pH 6.8 **(B-3,4)** and pH 8.0 **(B-5,6,7)**. A protein Ladder, and the injection sample are shown as L and M respectively.

4.5.6 Protein purification by FPLC

A chromatogram of the GFP-P2 purification by using the IMAC-FPLC system is given in Figure 4.30 and sample analysis by SDS-PAGE and Western Blot are given in Figure 4.31 and 4.32. Fluorescent imaging of the sample is given in Figure 4.33. The fluorescent signal was observed for elution sample when it was compared with control sample, as given in Figure 4.33.

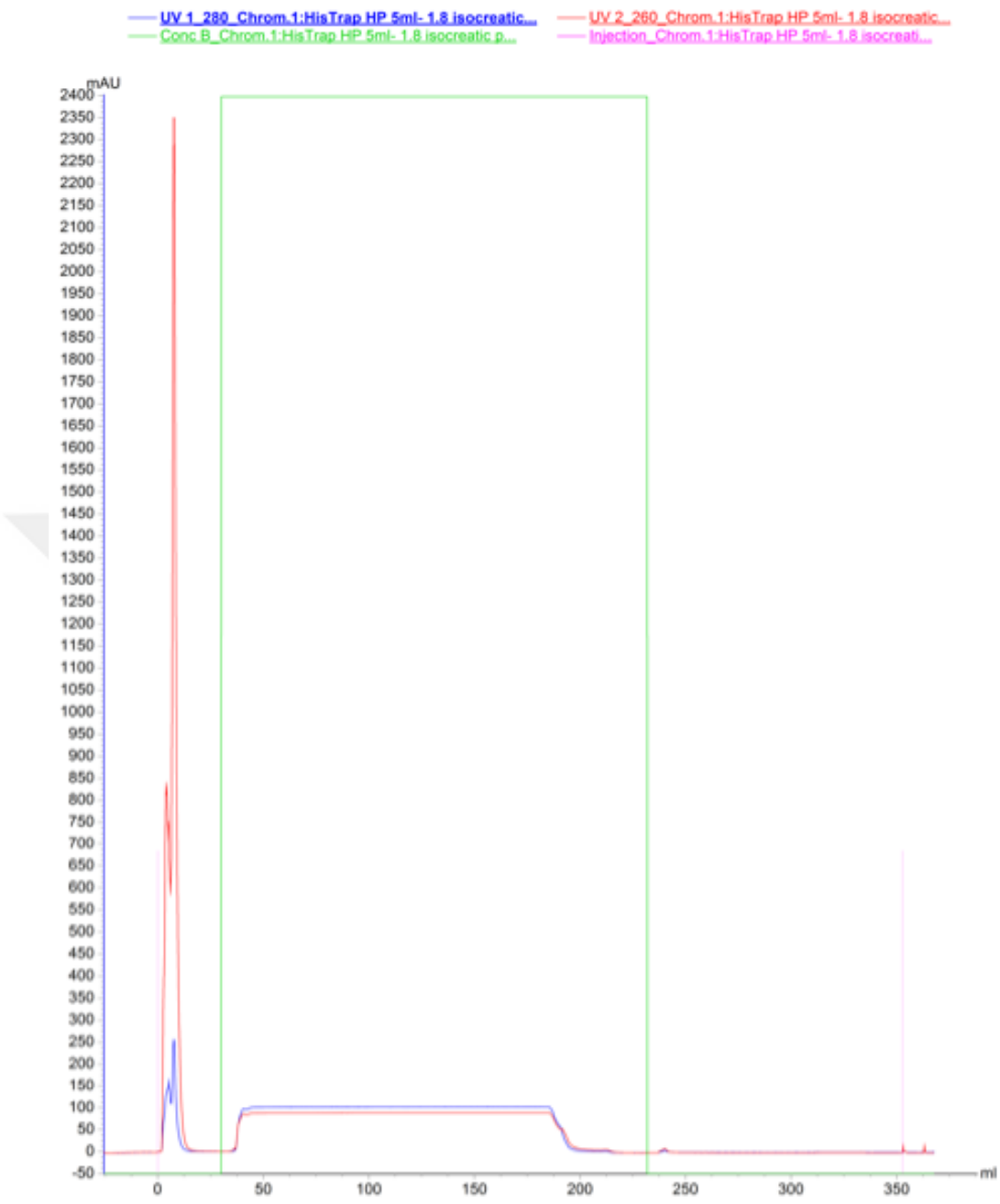


Figure 4.30: FPLC chromatogram. The isocratic elution method with His-Trap FF column was performed.

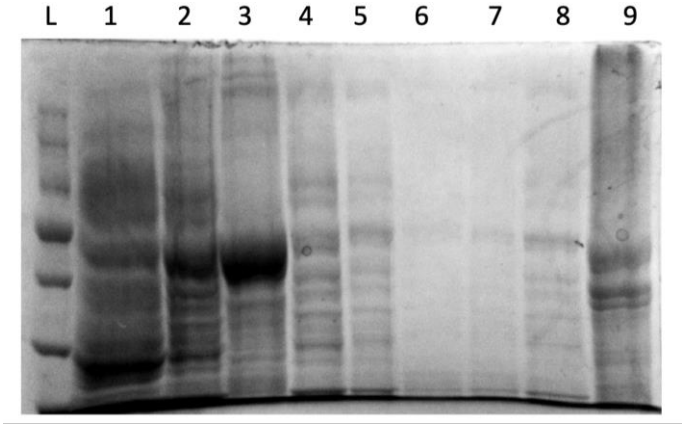


Figure 4.31: SDS-PAGE gel of FPLC purified peak samples. L is protein ladder prestained, 2 is first soluble fraction of B-PER treated sample, 3 is inclusion body, 4 is inclusion body in inclusion body dissolving solution including urea, 5-8 are supernatant samples of washing steps, 9 is diluted inclusion body resuspension.

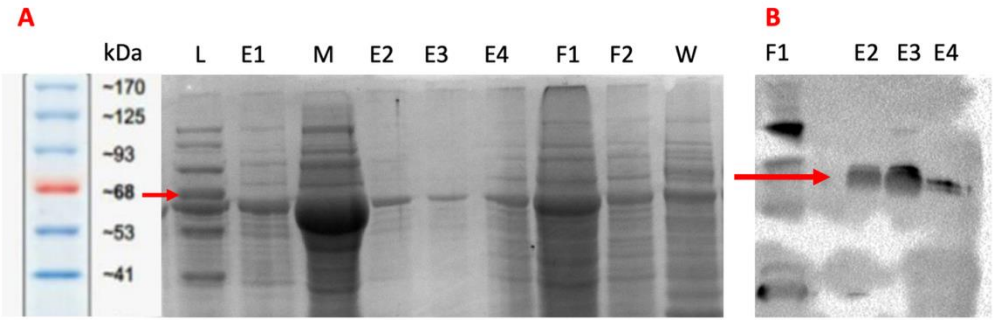


Figure 4.32: SDS-PAGE gel and Western Blotting images of FPLC purification samples. Protein ladder (L), Lysate (Mnflow-through (F), washing (W) and elution (E) samples are shown. The expected band is shown by a red arrow.

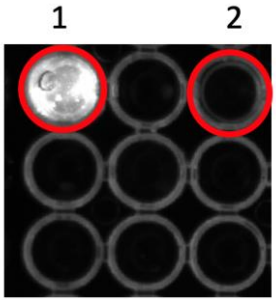


Figure 4.33: Fluorescent image of FPLC purified sample. Purified sample (1) and nuclease-free ddH_2O as a control (2) are shown.

4.6 TEV Digestion

Fluorescent imaging of TEV digestion reaction samples is given in Figure 4.34. The removed GFP fraction is shown with bright fluorescence, while only the P2 protein part is not visible.

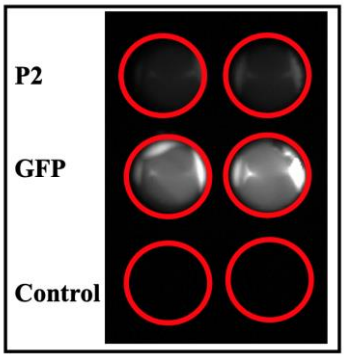


Figure 4.34: Fluorescent analysis of TEV cleavage P2 (P2) and the removed GFP (GFP). The removed GFP sample gives brighter fluorescence than the P2-GFP sample. The control includes nuclease-free water.

4.7 Residual DNA Control of Purified P2

Q-PCR amplification curve of residual host genomic DNA control in FPLC-purified protein sample is given in Figure 4.35. The Cq value of positive control (PTC) was 25.53, while control sample (C1) and negative control (NTC) did not show any amplification signal (N/A). This result suggests that the purified protein sample did not contain any significant amount of residual host DNA.

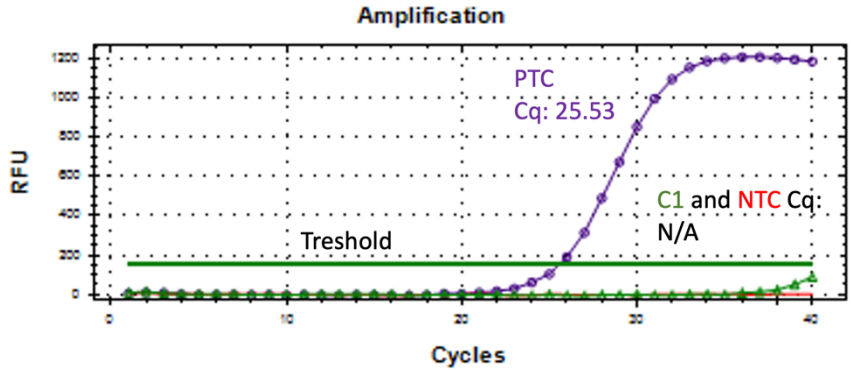


Figure 4.35: Q-PCR result of purified P2 with 16S rRNA primers as control of residual host DNA. Quantification signal data are shown for the positive control (PTC-purple), negative PCR control (NTC-red), and test sample which is P2 enzyme stock (C1-green). The Cq value of PTC was 25.53 while C1 and NTC are not given any amplification signal (N/A).

4.8 Activity Tests

4.8.1 DNA nicking

4.8.1.1 Linear DNA nicking

4.8.1.1.1 Preparation of substrates

Agarose gel images of different linear DNA substrates are given in Figure 4.36. The optimal annealing temperature for PCR amplification of substrate I, II and III, IV was selected as 62 °C, 61 °C, and 55 °C, respectively. Agarose gel image of gel-cleaned PCR products is given in Figure 4.37. Urea gel image of substrate VII is given in Figure 4.38.

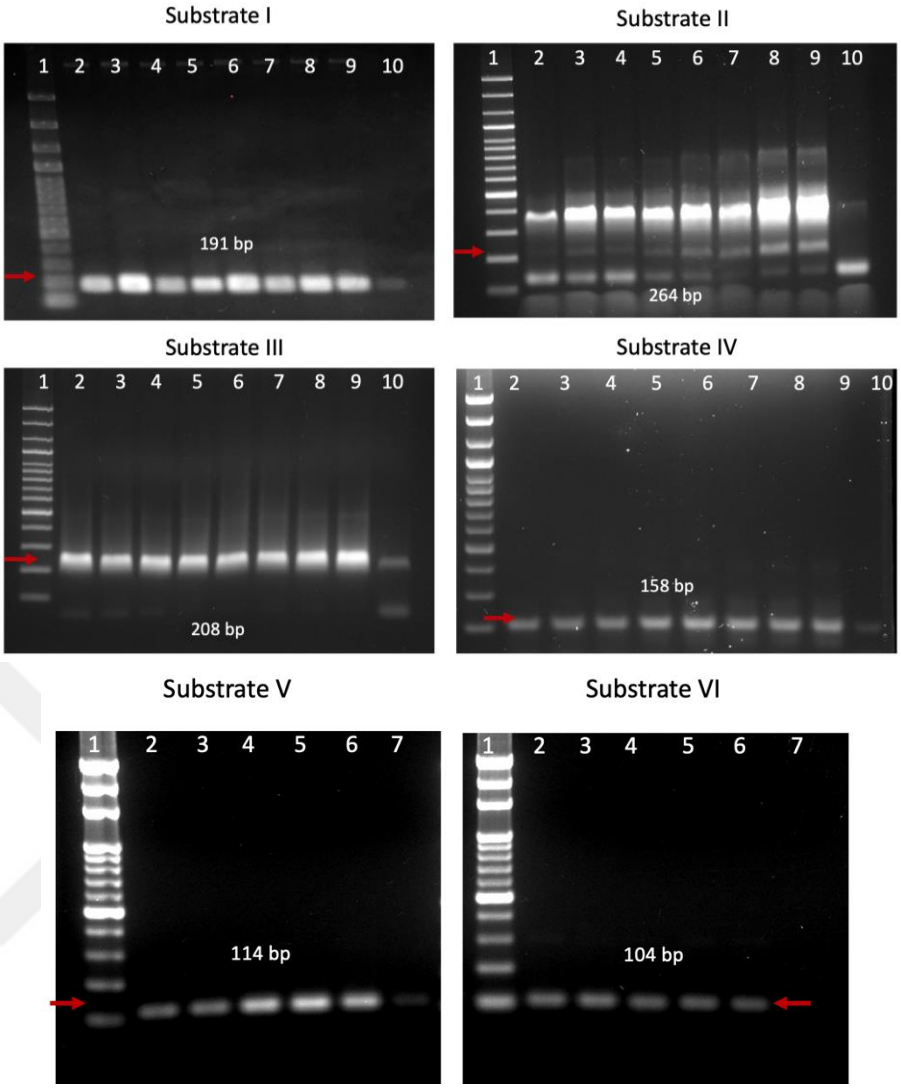


Figure 4.36: Agarose gel images of different substrates. Gradient PCR products were obtained for substrates I, II, III, IV, V and VI, respectively. Lane 1 contains the 100 bp DNA Ladder. Lanes 2 to 9 and 2 to 7 (for substrate V and VI) show the temperatures with a decreasing gradient. For substrate I, lanes 2-9 correspond to 62.0°C, 61.4°C, 60.4°C, 58.9°C, 57.1°C, 55.5°C, 54.6°C, and 54°C, respectively. Lane 10 of substrates I, II, III, IV and lane 7 of substrate V, VI represent the negative control of PCRs, which were performed at 62.0°C. For substrates II, III, IV, V and VI , lanes 2-9 correspond to 64.0°C, 63.3°C, 62.2°C, 60.5°C, 58.4°C, 56.7°C, 55.6°C, and 55.0°C, respectively. Lane 10 represents the negative control of PCR, which was performed at 58.4°C. Red arrows indicate the expected band sizes for each substrate. Gels were dyed with EtBr.

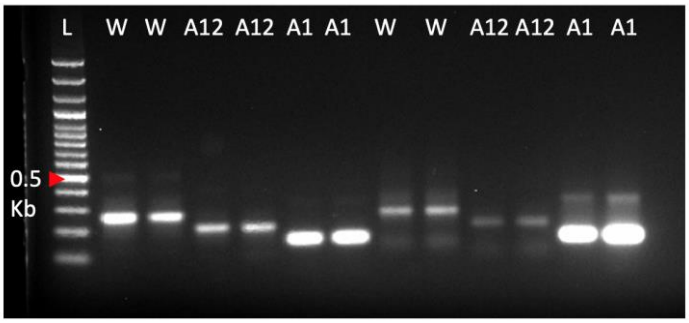


Figure 4.37: Agarose gel image of gel cleaned substrates. W (whole part) is representing to substrate II, A12 (A1 and A2 loops) to substrate III and A1 (only A1 loop) to substrate IV.

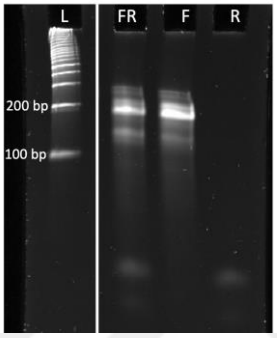


Figure 4.38: Urea gel image of substrate VII. L is 100 bp DNA Ladder, FR is reaction sample, F and R are only forward (oligomer template) and reverse primer controls, respectively.

4.8.1.1.2 Nicking reaction

4.8.1.1.3 Product analysis by urea gel

Urea gel images of substrate II and substrate I nick reaction products with SEC-purified P2-GFP are given in Figure 4.39 and Figure 4.40, respectively. Urea gel images of substrate I and substrate IV nicking reaction products with HPLC-purified and passive gel elution method-purified P2-GFP are given in Figure 4.41. Band differences are shown between control and non-purified P2-GFP (crude extract) samples when they are compared in Figure 4.41. Urea gel image of different substrates and their products with FPLC-purified P2-GFP in nicking reaction is given in Figure 4.42.

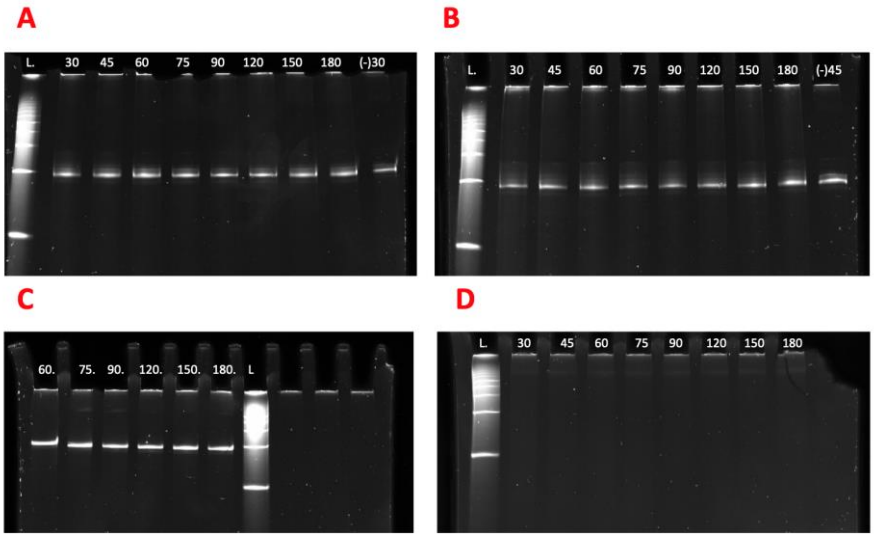


Figure 4.39: Urea gel image of substrate II nicking reaction at different time courses (30-180 minutes). L is a 100 bp DNA Ladder, "(-)30" and "(-)45" are reaction controls without P2-GFP at 30th and 45th minutes, respectively. 29 mg/mL (A) and 145 mg/mL (B) concentrations of lyophilized P2-GFP were tried. Other reaction controls without P2-GFP are given at C and without substrate given at D. Gels are 1X Sybr Green stained.

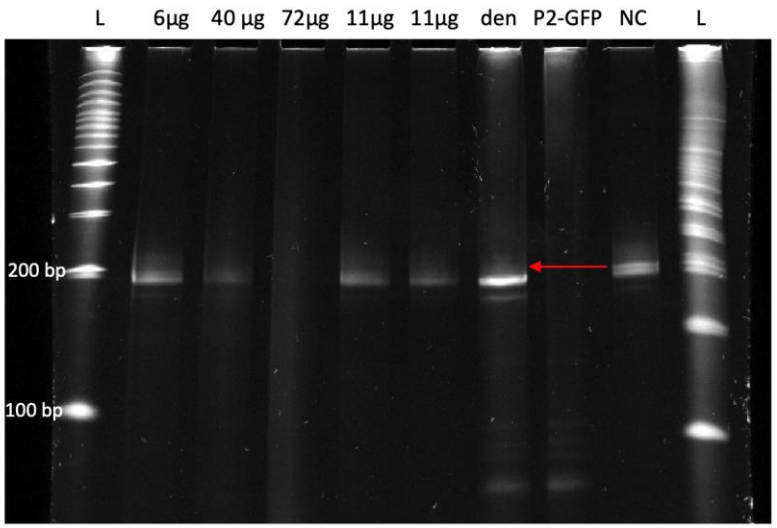


Figure 4.40: Urea gel image of nicking reaction with substrate I and SEC purified P2-GFP. The reaction samples that included different concentrations of P2-GFP are shown in each line as labeled. The reaction sample of the non-purified P2-GFP is shown as "den". Reaction controls are the only purified protein sample without substrate or only the substrate DNA without enzyme labeled as "P2-GFP" and "NC" respectively. L is a 100 bp DNA Ladder. The red arrow indicates the band differences between the control.

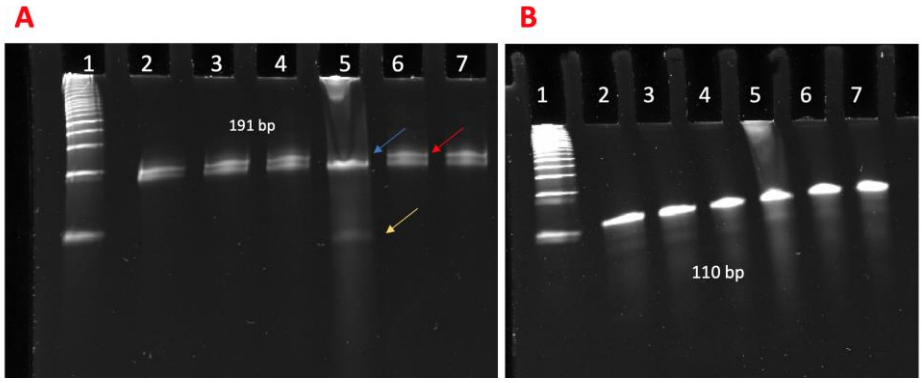


Figure 4.41: Urea gel images of nicking reaction of different substrates. Reaction samples of Substrate I **(A)** and Substrate VIII **(B)** with HPLC or passive gel elution method purified-P2-GFP were shown. The reaction samples that include pH 7.0, 6.8 and 7.4 of purified P2-GFP are labeled 2,3 and 4, respectively. The reaction sample of non-purified P2-GFP labeled as 5. Reaction control was only substrate DNA without enzyme and is labeled as 6. The reaction sample of the passive gel elution method purified-P2-GFP is labeled as 7. 1 represents the 100 bp DNA Ladder. Arrows indicate the band differences between the control.

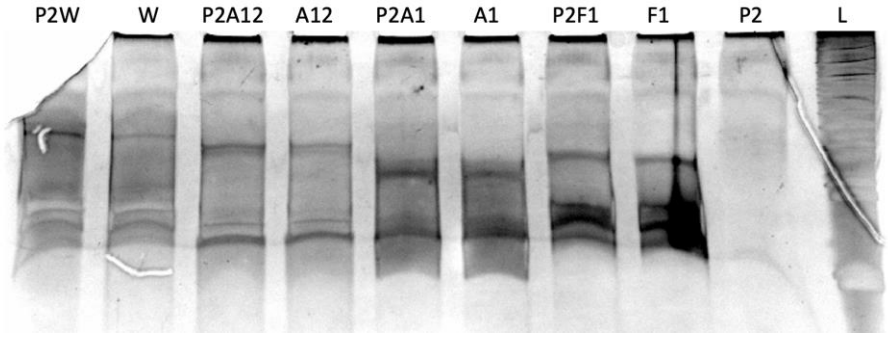


Figure 4.42: Urea gel image of nicking reaction of substrate I (P2F1), II (P2W), III (P2A12), IV (P2A1) with FPLC purified P2-GFP. Reaction controls were only purified protein samples without substrate (labeled P2) and only substrate I, II, III, and IV DNA without enzyme labeled F, W, A12, and A1 respectively. L represents 100 bp DNA Ladder. The gel was stained with silver staining.

4.8.1.2 Circular DNA nicking

Nicking reaction activity on circular plasmid DNA was followed as shown in Figure 4.43. Agarose gel images and analyses of different conditions are given in Figure 4.44-46. The greatest nicking activity was observed at concentrations of 10 mM MgCl2, 80 mM KCl, and 10 mM β-mercaptoethanol (BME) at 30 °C (Figure 4.44). The comparison between the nicking activity at different temperatures is shown

in Figure 4.44, and room temperature (20–25 °C) and 30 °C are compared in Figure 4.45, 4.46. No difference in enzymatic activity was observed between the incubation at room temperature and that at 30 °C; the OC-DNA percentage increased at the same rate at both temperatures (Figure 4.46). Reaction incubation time differences are given in Figure 4.46, 4.47 and 4.48. The product amount increased over time. No difference in enzymatic activity was observed between the p1GFP plasmid DNA substrate and M13KE RF DNA as the wild-type substrate (Figure 4.47). When the nicking activity of GFP-P2 and GFP-cleaved P2 were compared, they showed the same enzyme activity level (Figure 4.49). When the stability of the P2 enzyme over time compared, the results showed that the activity of the enzyme decreased by 27.58% and 29.5% (Figure 4.52).

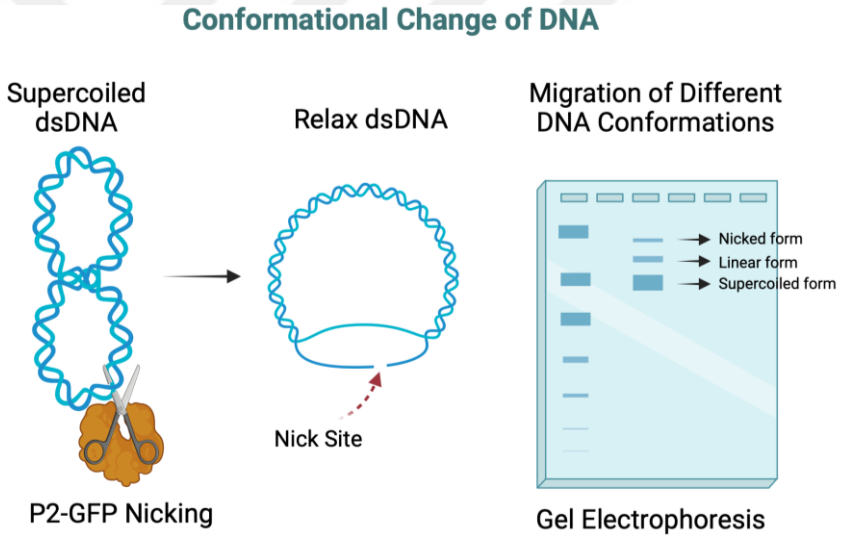


Figure 4.43: Representation of nicking enzyme reaction of plasmid DNA products (Created in BioRender).

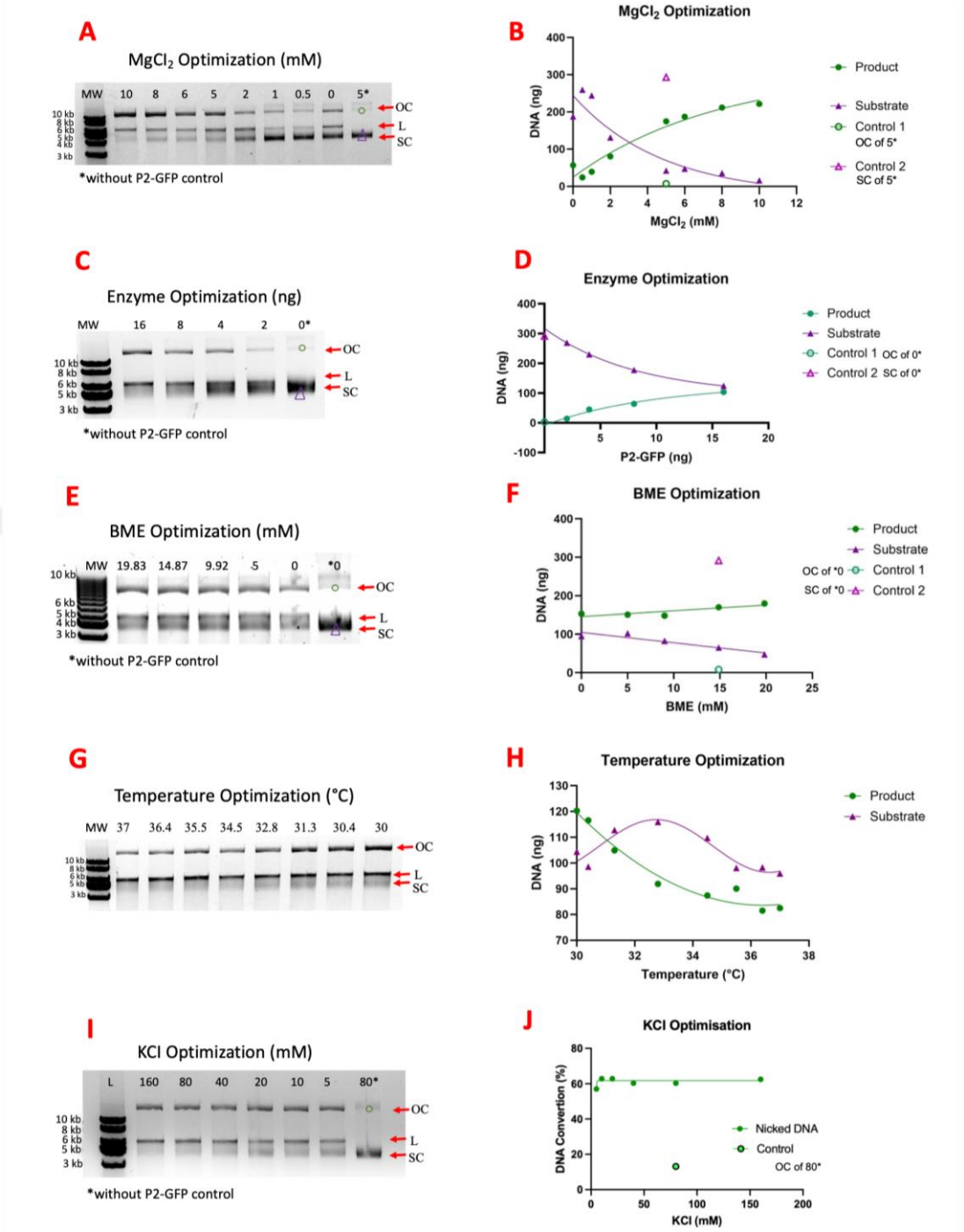


Figure 4.44: Agarose gel images of substrate–product analysis with different conditions (**A**,**C**,**E**,**G**,**I**) and quantitative band analysis (**B**,**D**,**F**,**H**,**J**). OC, L, and SC are open circular, linear, and supercoiled DNA, respectively. OC-DNA was used as a product, and SC-DNA was used as a substrate. "Control 1", "Control", and "Control 2" in the graphics show OC-DNA and SC-DNA amounts of control reaction samples, respectively. MW is 1 kb DNA Ladder (Genemark, Taichung, TP).

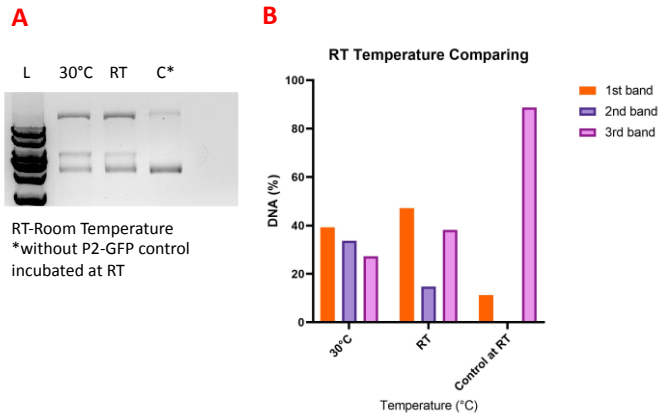


Figure 4.45: Agarose gel image (A) and band analysis (B) of substrate-product percentage. (**A)** DNA bands belong to the reaction which was performed at 30°C and room temperature. (**B)** Agarose gel bands were analyzed by Image J and the graph is shown. 1st band and 3rd band represent the product and substrate, respectively.

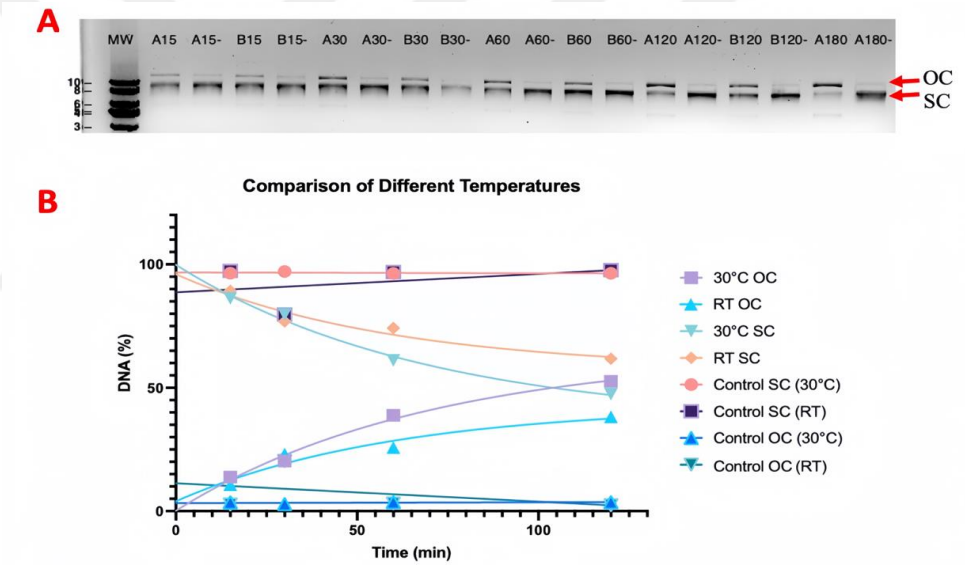


Figure 4.46. Temperature comparison of nicking reaction. (**A**) Agarose gel image of time course samples at 30 °C is labeled as "A" and room temperature (RT) is labeled as "B". Time points are given from the 15th to 120th minute. Each time point is labeled with a number and temperature letter tag (A15 and B15 belong to 15th minute of the reaction sample at 30 °C and RT, respectively). Without P2-GFP enzyme control reactions are represented as "–" for each condition and time course. (**B**) Non-linear regression analysis of gel image. OC and SC represent open circular and supercoiled DNA, respectively. Controls show the band intensity of control samples. MW is 1 kb DNA Ladder, and each band is sized as kb.

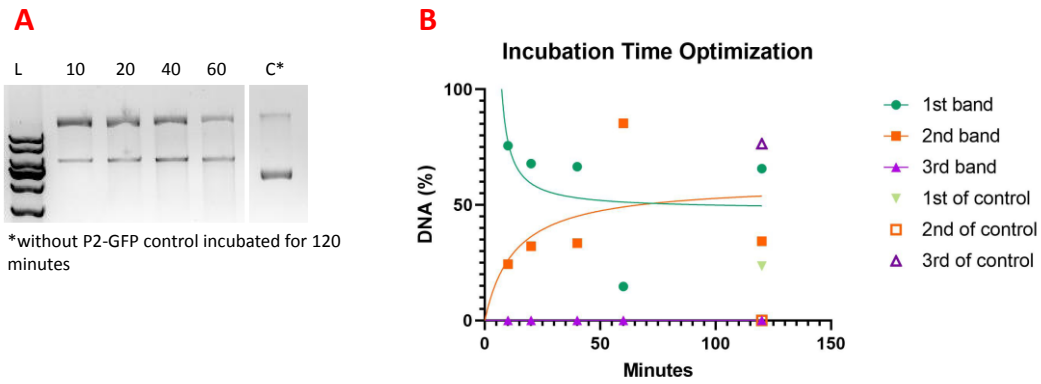


Figure 4.47: Agarose gel image (A) and band analysis (B) of substrate-product percentage. (**A)** DNA bands belong to different incubation times of the nicking reaction. (**B)** Agarose gel bands were analyzed by Image J and graphic is shown. 1st band and 3rd band represent product and substrate, respectively.

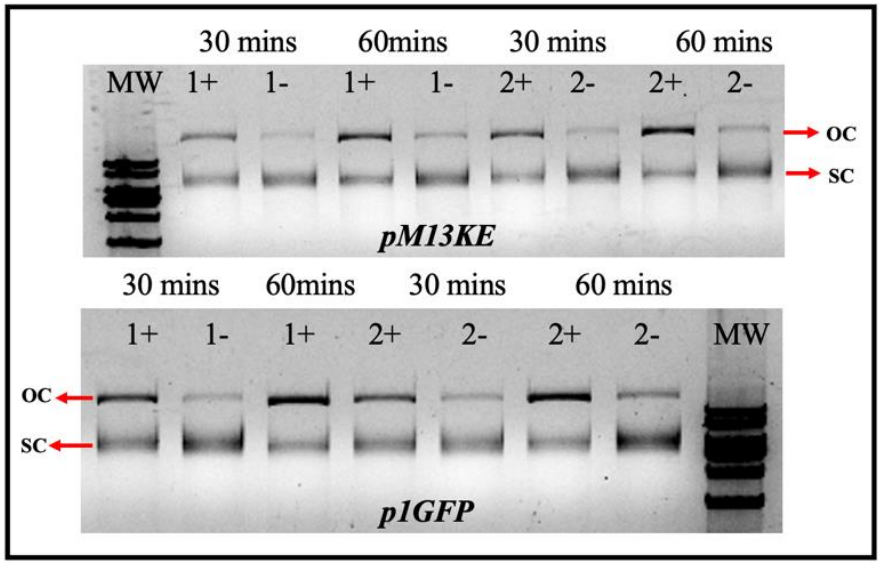


Figure 4.48: Agarose gel images of pM13KE and p1GFP nicking reaction samples at different time courses. Duplicate reactions with and without P2-GFP enzyme samples are labeled as "1+", "2+"; and "1-","-2" respectively. **OC**, and **SC** are open circular, and supercoiled DNA, respectively.

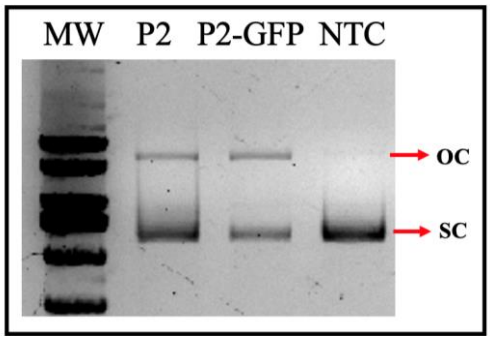


Figure 4.49: Agarose gel image of P2 and P2-GFP nicking reaction samples. Nicking reaction samples of P2-GFP and GFP fusion tag removed P2 (P2) give the same product as open circular DNA (OC). SC is supercoiled DNA as the substrate. Control (NTC) includes only substate in the reaction without enzyme. p1GFP was used as a substrate.

4.9 Stability Control

Agarose gel image and analysis of P2 nicking reaction after different time periods is given in Figure 4.50-52.

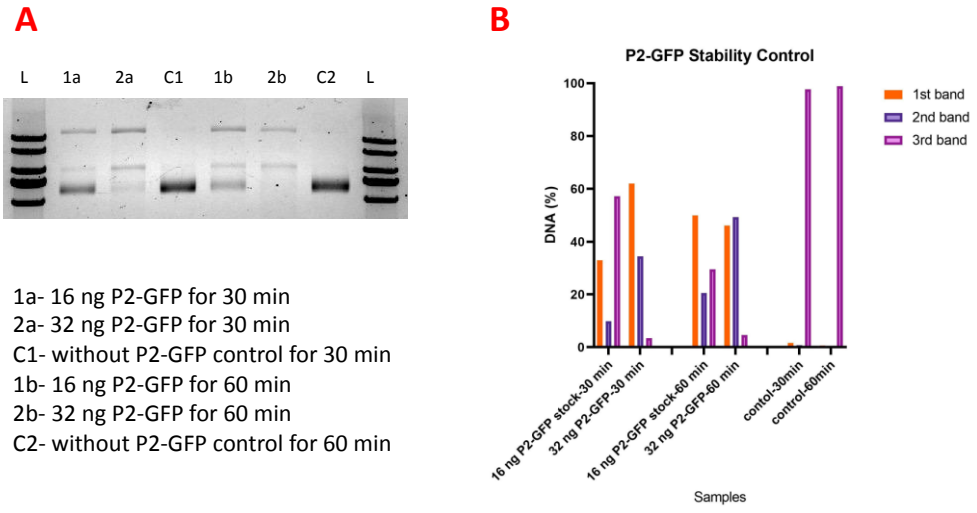


Figure 4.50: Agarose gel image (A) and band analysis (B) of substrate-product percentage. (**A)** DNA bands belong to two different amounts of P2-GFP enzyme that were incubated for 30 minutes and 60 minutes in the reaction. (**B)** Agarose gel bands were analyzed by Image J and graphic is shown. 1st band and 3rd band represent the product and substrate, respectively.

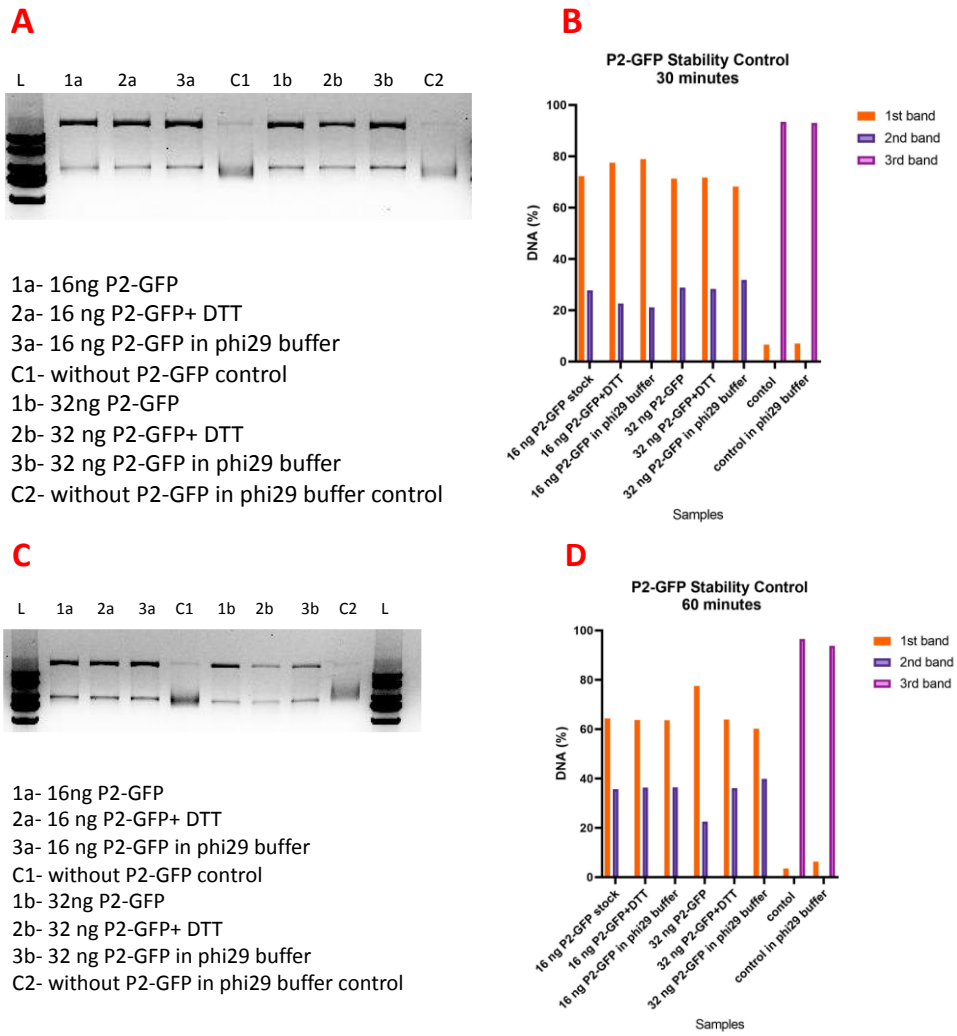


Figure 4.51: Agarose gel image (A, C) and band analysis (B, D) of substrate-product percentage. (**A, C)** DNA bands belong to two different amounts of P2-GFP enzyme that were incubated for 30 and 60 minutes, respectively. (**B, D)** Agarose gel bands were analyzed by Image J and graphic is shown. 1st band and 3rd band represent product and substrate, respectively.

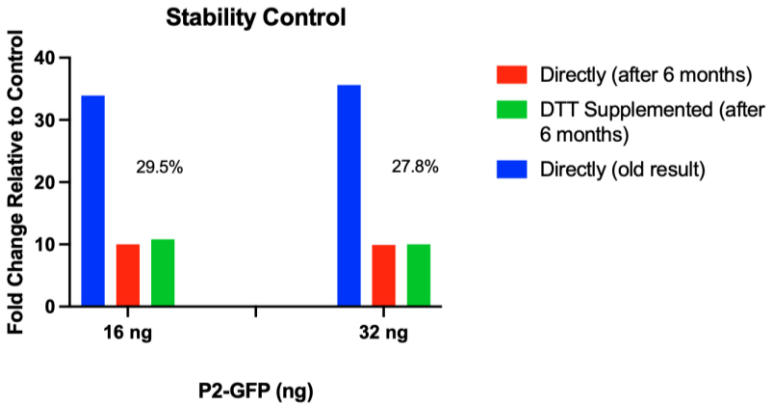


Figure 4.52: Stability analysis of P2 enzyme. Substrate-product conversions by P2 are given for 16 ng and 32 ng of P2 enzyme concentration after 6 months. Previous relative fold change to control of P2 data and the same reaction data of P2 after 6 months at –20°C are shown by blue and red boxes. DTT supplemented old P2 stock results are shown by green box.

4.10. Determination of Minimal Recognition Sequence for P2 Nicking

P2 was able to recognize and cut both strands of pA2L, which carries the smallest fragment of the P2 recognition site in supercoiled form. Nicking reaction activity on circular plasmid DNA was followed as given Figure 4.53. When the f1 origin DNA part (F1) and insert DNAs (W, A12, A1), which are cloned into pW, pA12, and pA1, were obtained by PCR and treated as linear form DNA substrate with P2-GFP, no cutting activity was observed as given in Figure 4.54.

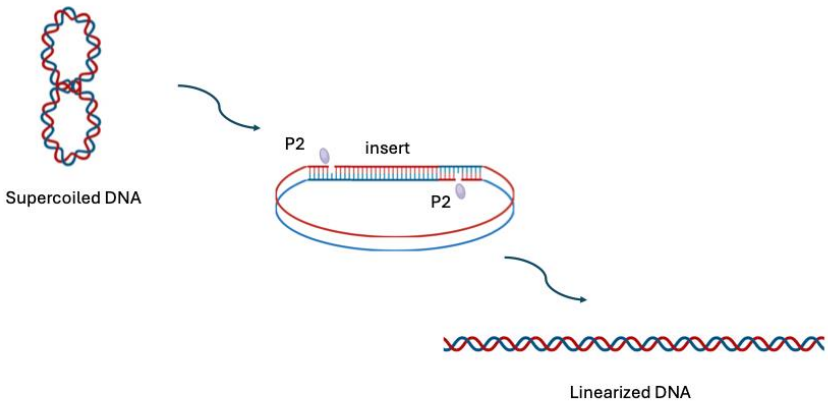


Figure 4.53: Representation of nicking enzyme reaction of recombinant plasmid DNA. When plasmid DNA carries different P2 recognition site on both strands oppositely, it is nicked by P2 enzyme, DNA linearizes (Created in BioRender).

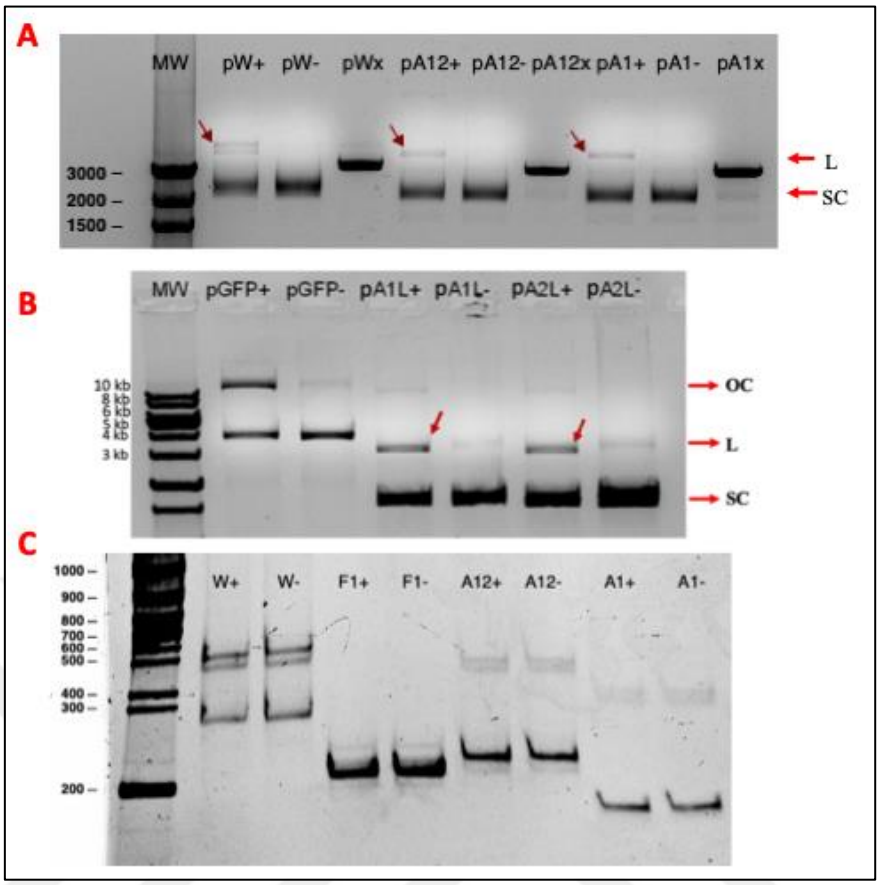


Figure 4.54: Gel images of different plasmid nicking products. **(A)** Agarose gel image of pW, pA12, and pA1 nicking reactions. "+" labeled plasmids represent P2-GFP-added reactions; "−label represents control samples (without P2-GFP). "x" represents the digested plasmid that was treated with the XhoI restriction enzyme as a conformational control of linearized DNA on the gel. P2-GFP cut both strands of supercoiled plasmids through carrying P2 recognition sites at both. Red arrows indicate linearized DNAs by P2-GFP. **(B)** Agarose gel image of pGFP, pA1L, and pA2L nicking reactions. "+" labeled plasmids represent P2-GFP-added reactions; "−label represents control samples (without P2-GFP). Red arrows indicate linearized DNAs by P2-GFP. **(C)** DNA-PAGE image of linear DNA substrates amplified by PCR. While "+" labeled F1, W, A12, and A1 DNA fragments represent P2-added reactions, "−" represents control samples of reactions which include F1, W, A12, and A1 DNA fragments without P2-GFP. "F1+" and "F1−" belong to PCR products of f1 origin as a natural substrate. OC, L and SC are open circular, linear and supercoiled DNA, respectively.

4.11. Binding Assay

Polyacrylamide DNA gel image of binding assay is given in Figure 4.55. Results are shown that P2 did not stay on DNA molecule according to migration of the molecule complex.

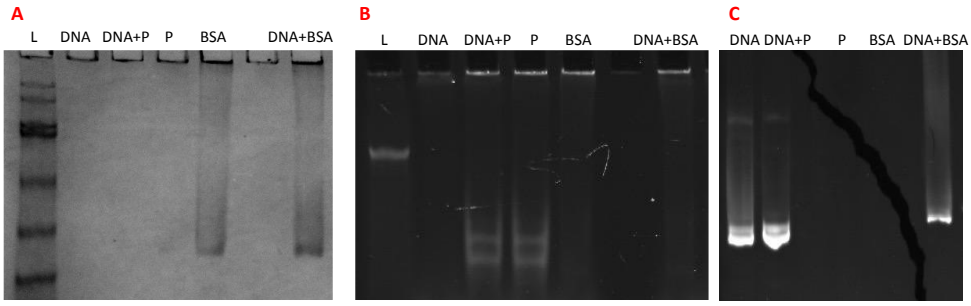


Figure 4.55: Gel images of binding assay. Coomassie Brillant Blue R-250 (A), Fluorescent Imaging (B), EtBr (C). "DNA" is only substrate DNA and "DNA+P2" is the P2-GFP and substrate together in the reaction sample, "BSA" is bovine serum albumin, "P" is only P2-GFP control, "DNA+BSA" is substrate DNA with BSA instead of P2-GFP.

4.12. Enzyme Kinetics

Michaelis–Menten equation analysis is given in Figure 4.56. The Vmax of the enzyme was 0.06 ng/µL·min^{-1}, and the Km was 34.17 ng/µL (Figure 4.55).

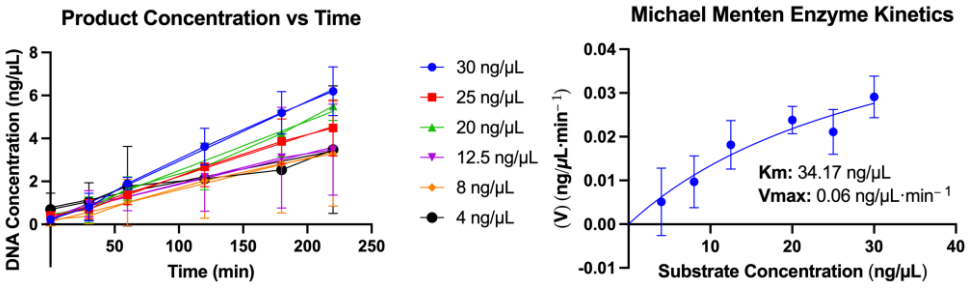


Figure 4.56: Kinetic analysis of P2-GFP. Open circular DNA intensity of different substrate concentrations and Michaelis–Menten equation for enzyme kinetics.

4.13 P2 Mediated DNA Amplification at Room Temperature

DNA amplification mechanism from plasmid DNA carrying P2 recognition sequence by using the P2 enzyme is given in Figure 4.57. Q-PCR amplification of PMA1 (trial 1) diagram and baseline-matched version of peaks are given in Figure 4.58. Triplicated Q-PCR results and melt-curve diagrams of PMA2 (trial 2) for different test samples are given in Figure 4.59-63. The signal of PMA2 reaction control tubes, which include only template DNA with other reagents except for P2 enzyme, were used to arrange amplification threshold (Figure 4.59). The effect of DMSO addition and using denaturated-P2 (inactive P2 enzyme) in the PMA reaction are shown in Figure 4.60. Results showed that denaturated-P2 did not give any amplification signal as expected (Figure 4.60). DMSO addition did not significantly change the signal intensity (Figure 4.60). The signal of PMA reaction control tubes, which include only active P2 enzyme with other reagents except for template DNA, and (+) test samples, were given a signal, contrary to the inactive P2 included version (Figure 4.61).

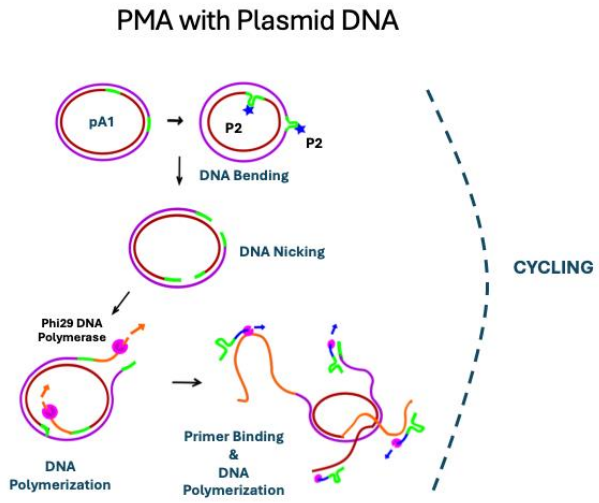


Figure 4.57: PMA mechanism from plasmid DNA. When P2 enzyme nicks one of the DNA strands at both sites, phi29 DNA polymerase binds to the 3'OH end and starts new DNA synthesis. While new DNA strand polymerization continue, the other strand displaced by the same polymerase enzyme, and primer pairs carrying the P2 recognition site specifically bind to these strands. When the recognition site is created again by DNA polymerase enzyme, P2 cuts the strand again at the same position. DNA amplification is achieves by cycling through these steps.

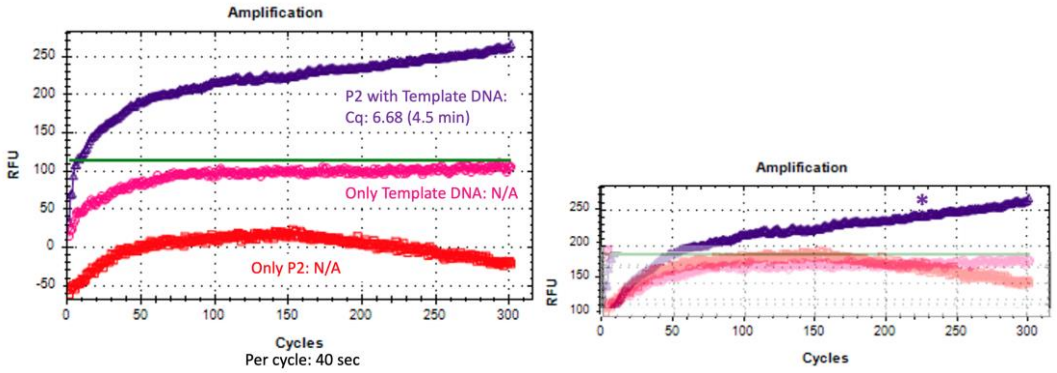


Figure 4.58: Q-PCR result of PMA1. Quantification signal data (left) are shown for P2 and template DNA together with the test sample (purple); only template DNA including the sample (pink) and only P2 including the sample (red) as controls for the test. The Cq value of P2 with template DNA was 6.68 (~4.5 min), while test controls did not give any amplification signal (N/A). Baseline overlapped signals from the first reading are given on the right side.

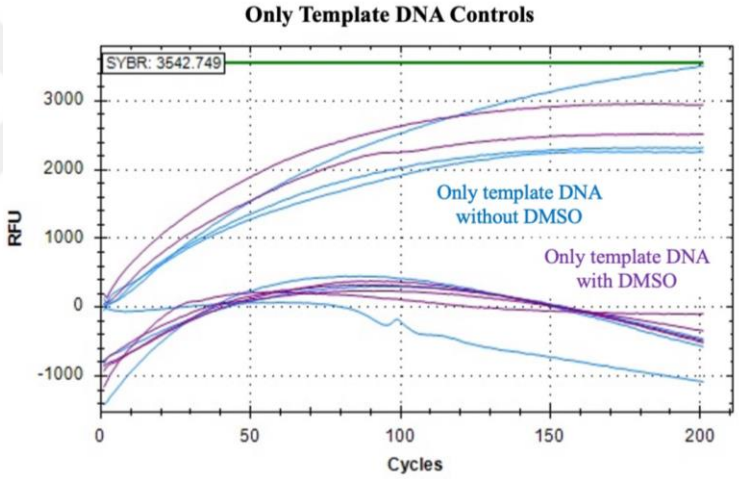


Figure 4.59: Q-PCR result of only template DNA control of PMA2. PMA reaction control tubes, which include only template DNA with other reagents except for P2 enzyme, are shown. Signals of DMSO-added samples are shown in purple, while control samples without DMSO are shown in blue. All signals are under the threshold (N/A).

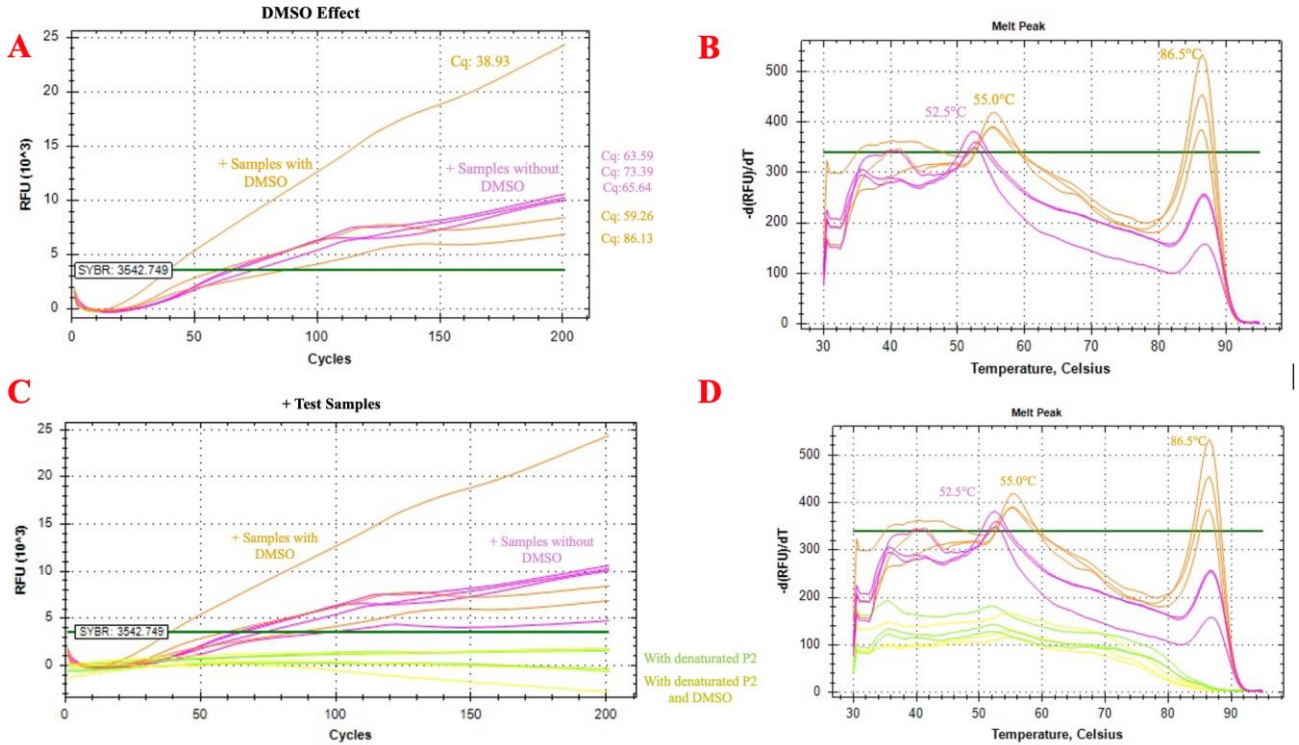


Figure 4.60: Q-PCR results of PMA2 with DMSO and denaturated-P2 effect. **(A)** Orange and pink signals are shown test sample which include every reagent for PMA reaction (+) with or without DMSO addition respectively. **(B)** Melt curve analysis of A. **(C)** Comparison of active and inactive P2 (denaturated-P2) enzyme activity in PMA. Amplification signal of active P2 added (+) test tube is shown in green. **(D)** Melt curve analysis of C.

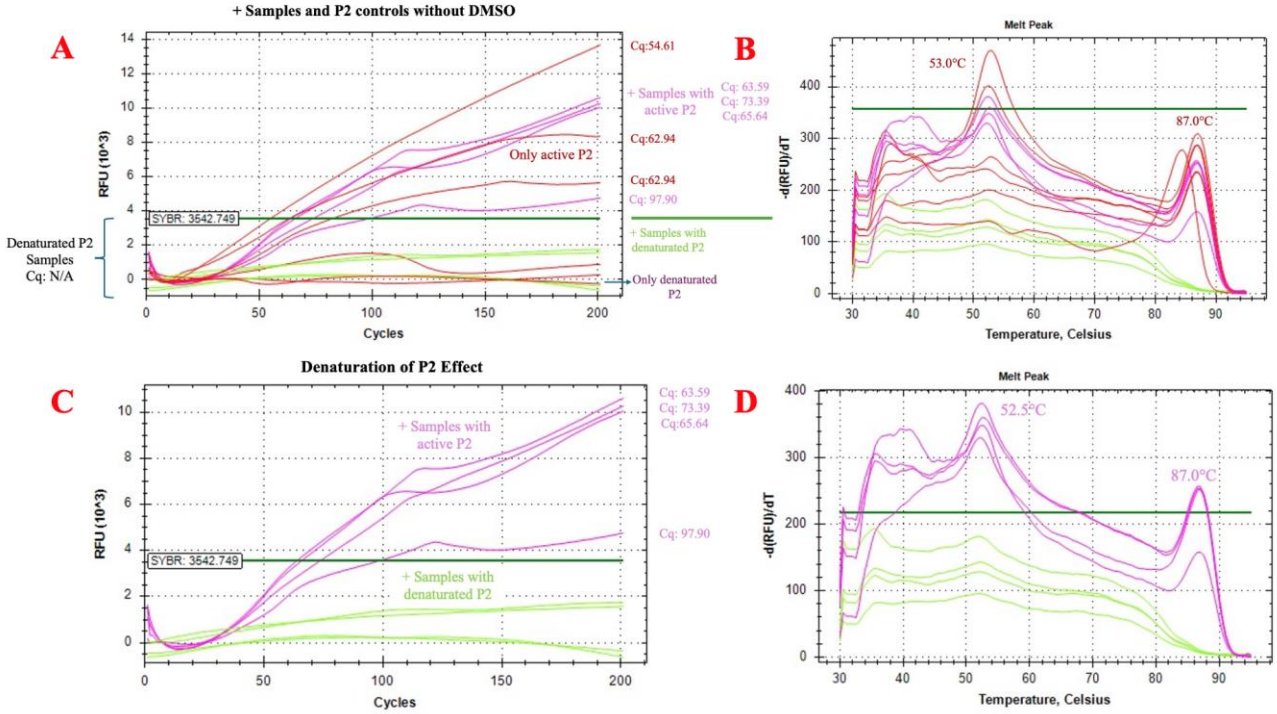


Figure 4.61: Comparing of enzyme activity in PMA2 without DMSO. **(A)** Pink signals are shown test sample which include every reagent for PMA reaction (+) with active P2 without DMSO addition. Amplification signal of inactive P2 added (+) test tube is shown by green. Signal of PMA reaction control tube which include only active or inactive P2 enzyme with other reagents except for template DNA is shown by red and purple, respectively. **(B)** Melt curve analysis of A. **(C)** Comparison of active (pink) and inactive P2 (denaturated-P2) enzyme (green) activity in PMA (+) test samples. **(D)** Melt curve analysis of C.

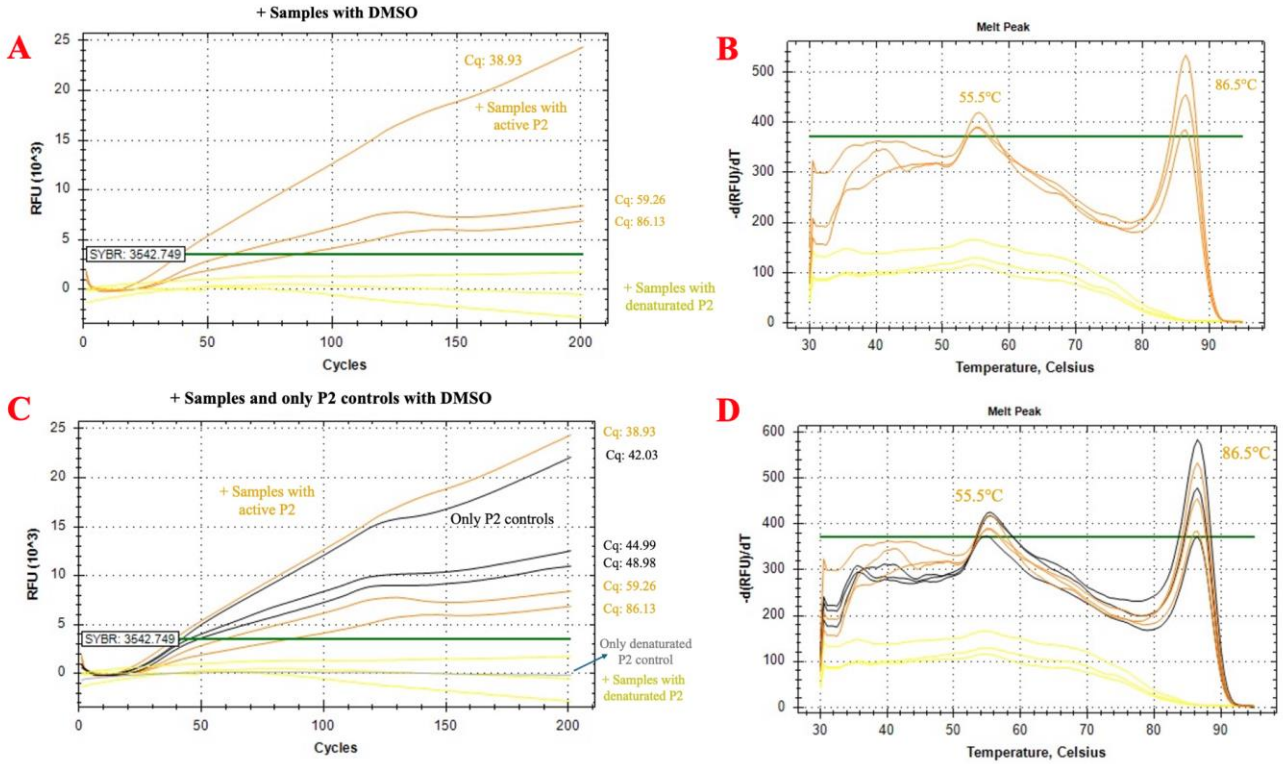


Figure 4.62: Comparing of enzyme activity in PMA2 with DMSO. **(A)** Orange and yellow signals are shown test sample which include every reagent for PMA reaction (+) with active or inactive P2 with DMSO addition respectively. **(B)** Melt curve analysis of A. **(C)** Signal of PMA reaction control tube which include only active or inactive P2 enzyme with other reagents except for template DNA is shown by black and gray, respectively. **(D)** Melt curve analysis of C.

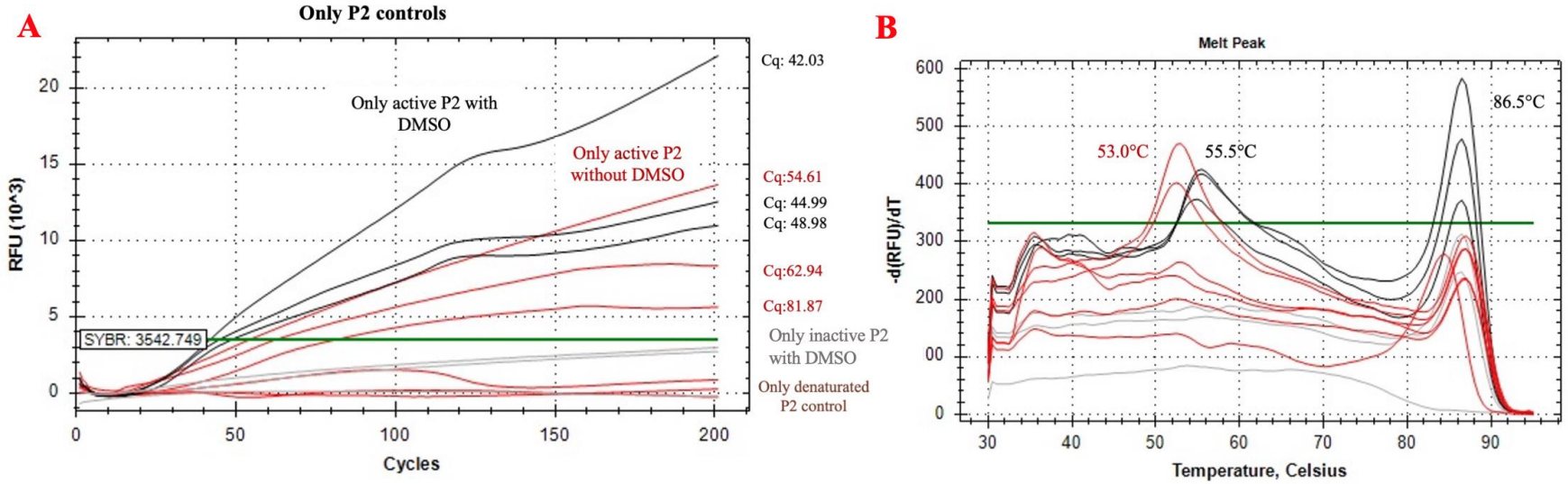


Figure 4.63: Comparing of enzyme activity in PMA2. **(A)** Signal of PMA reaction control tube which include only active (black and red) or inactive P2 enzyme (gray and brown) with other reagents except for template DNA. DMSO included ones are shown by black and gray. **(B)** Melt curve analysis of A.

5 DISCUSSION

In this study, we investigated the enzymatic activity of the P2 protein from the M13 bacteriophage, which we obtained by cloning the P2 gene into *E. coli* and using it in a new isothermal nucleic acid amplification method. We aimed to utilize P2 for development of a new isothermal nicking enzyme-mediated nucleic acid amplification technique at room temperature, using designed primers that include a minimal recognition site. This study will lead to the development of new signal amplification methods-based point care diagnostic tests for patients in the future.

5.1 Cloning

Using the LIC method is an easy way to clone genes. On the other hand, attaching the insert to the prepared vector was more challenging than expected due to the secondary structure of DNA samples at room temperature. To overcome this problem, the prepared insert and vector samples were treated with dCTP/dGTP and then heated to high temperatures to break any secondary structures in the DNA. The temperature was then gradually decreased to room temperature. This additional step resolved the annealing reaction problem in this study, and we this information can also help other researchers.

5.2 Protein Expression

The best expression condition for P2 was determined to be 0.5 mM IPTG induction at 37°C for 5 hours incubation. Although it was expected that using the GFP fusion tag would make the recombinant protein soluble, P2-GFP was observed in inclusion bodies. Despite the denaturation of protein in denaturant solution, GFP maintained its green fluorescence. This suggests that the GFP was very resistant to denaturants, similar to the results published by Saeed and Ashraf (61). However, it helps to observe the protein expression by the naked eye through the GFP part of the protein as a reporter, which may be disturbing the wild-type of P2 structure. A

structural study was not performed in this study. But P2-GFP demonstrated enzymatic nicking activity, which suggests that GFP does not disturb the P2 structure as much as it interferes with the activity. When lysate and His-tag purified samples were analyzed, there was additional GFP expression in the soluble protein fraction. It was thought that this problem might be caused by the cloning plasmid construct.

5.3 Protein Purification

The high concentration of urea (8M) was not sufficient to solubilize the inclusion bodies. However, the addition of thiourea and SDS enabled the solubilization of the inclusion bodies. Removing the denaturants is very important to obtain active protein, as they disrupt protein conformation. Different protein purification methods such as IMAC, HPLC, passive gel elution, SEC and IMAC-FPLC were used to obtain pure and high-quality P2-GFP. When gravity-based Nickel affinity column chromatography was used, purification was not sufficient, and it resulted in more nonspecific binding products. SEC removed these non-specific proteins from the elution fractions, but it also decreased the yield of the protein. The passive gel elution method was more efficient for obtaining a pure protein sample, despite the protein quantification being low. The HPLC method did not have high resolution for efficient separation of the different protein peaks. IMAC-FPLC was the best method for purification of P2, due to its efficiency, purity, and yield, as it allows the system to be fully automated, provides real-time observations, and enables the collection of peaks separately.

5.5 Nicking Reaction

P2-GFP's nicking activity was monitored via conformational changes in the plasmid DNA. The reaction was observed for all recombinant plasmids carrying truncated versions, including the smallest part (pA2L), which is the loop where the nicking site is present. When these different DNA fragments were used as linear substrates for the nicking reaction, there were no differences between the test and control groups. This suggests that P2 requires the supercoiled DNA structure for the

nicking reaction, as previously reported by Meyer and Geider (1979) (7,30). However, because the DNA fragments obtained by P2's nicking activity can be very small, it may be possible that these fragments went undetected in the electrophoresis gels. When plasmid DNA was treated with P2, it was converted into an OC form, indicating a nick in one of the strands created by P2. Different parts of the P2 recognition site, including the whole recognition site (W), only A1-A2 loops (A12), only A1 loop (A1) and truncated A1 loop parts (A1L and A2L), were used to investigate the region essential for nicking activity. These truncated recognition site fragments were cloned into both strands of a vector to create supercoiled DNA including these fragments. Nicking of the dissolved crude extract in dissolving solution was used to nick the linear DNA substrate, and there were band differences between the reaction sample and control (Figure 4.40 and Figure 4.41). This suggests that the unpurified lysate can work under denaturant conditions with other cell fractions. When nicking activity of P2 and P2-GFP was compared on SC DNA, results showed that there were not any significant differences between them. Also, an important thing about the nicking reaction is the use of freshly transformed and purified plasmid DNA to maintain the supercoiled conformational form. When old plasmid DNA was used, 2^{nd} and 3^{rd} band formations were not observed onto the gel.

When DNA binding features of P2 were analyzed, results suggested that P2 was not tightly bound to DNA, and this strengthens the previously published results about P2 binding (22).

We calculated the Km as 34.17 ng/μL and the Vmax as 0.06 ng/μL.min^{-1} for P2 using gel images showing the conversion of supercoiled plasmid DNA to OC form (Figure 4.56). However, the error rate may be high for making predictions from the measured intensity of plasmid DNA. Stabilization results show that P2 remains active in stock solution after 6 months at -20°C.

The most recent study on the features of P2 that we could find in the literature was published in 1987 (6). Therefore, the current study is the first in almost 40 years to add more information about the recognition site of P2 (40–48,62). The first studies on *gene*

II products that P2 started with Geider K. and Meyer T. F. in 1979 (63). In these first studies, isolated P2 from M13 phages infecting *E. coli* cells were used to study the enzymatic activity of P2. This activity was examined for phage supercoiled dsDNA cleavage in-vitro by examining the relaxed DNA amount (21). (64). Horiuchi K. and colleagues determined the nicking activity signals of P2 (7,22,40,41).(17). All these studies have shown that ~110 bp sized DNA sequence is important for binding as a recognition sequence and nicking of P2. We have used different recognition sites of varying sizes and cloned them into a cloning vector, because, P2 has nicking activity on only supercoiled dsDNA, as mentioned in Greenstein D. and Horiuchi K. 1987 (17). This helped to determine the minimal recognition sequence of P2 by utilizing the truncations of this long sequence.

Some single-stranded DNA viruses can cause infections in humans and animals, some of which have the same replication mechanisms as M13. The human pathogen, parvovirus B19, causes inflammatory and bone marrow diseases (67). The treatment of parvovirus B19 infections using anti-sense oligonucleotides (ASOs) has been investigated (68,69). In the lifecycle of B19, its replication is similar to M13 bacteriophage replication: in both virus types, viral ssDNA converts into double-stranded DNA after infection into a host cell. In B19 replication, non-structural protein 1 (NS1) specifically binds to its recognition sequence in the replication origin and creates a nick in the DNA strand. Replication continues according to the rolling hairpin replication model (70). ASOs are single-stranded DNA oligomers that are chemically modified to be able to bind to specific RNA sequence targets (71). In recent years, ASOs have been developed as antiviral drugs; Formivirsen and Bepirovirsen, which are used for the treatment of Cytomegalovirus (CMV) retinitis and hepatitis B (HBV), respectively, are successful examples (68,71). ASOs are also under development for the treatment of influenza A and SARS-CoV-2 (71). In addition, other studies have investigated the development of ASOs for the treatment of B19 infections [43,44]. The nicking site of NS1 may be a good target for this purpose. M13 infections in E. coli may be a practical model for the development of drugs against single-stranded viruses that cause infections in humans.

5.6. P2 Mediated DNA Amplification

Nowadays, researchers are focused on different signal amplification systems for diagnostic tests and NAATs, which are used for bio-detection methods with high specificity. Generally, PCR is used as a non-isothermal method for these purposes. On the other hand, isothermal methods are based on using only one constant temperature for the DNA amplification reaction. PCR-based based methods may have some disadvantages such as time retention for working with large sample size, difficulties in sending the sample to another laboratory for the testing, and requiring expensive thermal cycling machines and a well-trained personnel for analysis (31). Also, it requires expensive thermal-stable enzymes, making it less accessible in low-cost laboratories. That's why nowadays, researchers are focused on developing the isothermal techniques for NAATs (32).

LAMP, RCA, HDA, CRISPR mediated methods are examples of some INAAMs (33–35). Despite these methods successfully denaturing the double-stranded DNA strands without high temperatures, as conventional PCR methods do, there is still a need for an initial temperature or a special chemical for the reaction. Therefore, developing simple, specific, low-cost, and accessible new techniques might be very important in this field (36). NEAR, as an INAAM, is based on the use of a nicking enzyme. The enzyme can create a nick site on one of the DNA strands as an endonuclease. DNA Polymerase, which has strand displacement activity, can synthesize the new strand from the created 3'OH prime while displacing the old strand in front of it. While DNA synthesis continues, the nicking enzyme's specific target sequences will be created again, and the reaction steps will cycle. On the other hand, the displaced strands come together with overlapping sequences, creating a new template with free primes for DNA Polymerase. The targeted DNA site can be amplified by cycling these reactions (37–39).

For optimization of the nicking enzyme-based amplification method, Nb. BtsI endonuclease enzyme (NEB) and specific primers were purchased. Primers for Sars-CoV2 cDNA were designed with an enzyme recognition site on one strand of DNA that is 3'-CGTCAC/NN-5' tag. The forward primer, which carries the nicking site (**bold**), is 5'-**CTATCAC**ACTGCGACCCCAAAATCAGCGAAATG-3', and the reverse primer is 5'-**TTA**CCCCACTGCGTTCTCCATTCTGGTTACTG-3', used for amplifying the PCR product of 105 bp in size from Sars-CoV2 cDNA. However, the method did not work well, and it needs more optimization. That is why this method, and the results are not given in the thesis.

The first trial of the DNA amplification reaction was performed with purified P2-GFP, Substrate I (f1 origin sequence), and Klenow Fragment as Q-PCR. While P2 nicked one of the DNA strands at the P2 recognition site, which is in the middle of the substrate, Klenow uses the free 3'OH end, displaces the front strand, and synthesizes the new strand according to the complementary strand. When the results were compared with the negative controls, such as only nicking enzyme or substrate DNA-included reactions, signal amplification was obtained for all samples at different levels. Also, the negative control tube that includes only the P2-GFP enzyme showed an increased signal. When an enzyme sample was screened by using universal 16S rRNA primers, it produced the PCR product, meaning that it still contained residual genomic *E. coli* DNA from the protein purification step. To remove this DNA, the sample was treated with DNase I enzyme and then purified by IMAC-FPLC. This method successfully removed the residual DNA from the sample. The negative results of using Klenow enzyme in PMA suggested that Klenow Fragment may be replaced with a different isothermal DNA polymerase that has more strand displacement activity than Klenow, and it was replaced with phi29 DNA Polymerase in further PMA reactions.

Sybr Green I concentration in the DNA amplification reaction was optimized by comparing the purchased Q-PCR master mix performance by using the same sample amplification. 1× Sybr Green I gave a signal of 6000 RFU, while 0.5× and commercial Q-PCR master mix gave an average signal of 3000 RFU. This suggests

that 0.5× concentration of Sybr Green I can be used for observing the DNA amplification signal in the designed reaction.

We thought that, when P2 recognizes the A1 loop sequence on the pA1 plasmid DNA, it creates a nick site on one of the strands and releases free 3' prime end for DNA polymerase. Phi29 DNA polymerase uses the 3'OH end and starts DNA polymerization according to the complementary strand, while displacing the strand in front of it. This nicking, displacement and DNA polymerization cycle reactions occur for the opposite strand at the same time, because of carrying the same recognition site on the complementary strand. Displaced strands from both sides bind together by overlapping complementary sequences, and the non-overlapped strand parts (complex I) are turned to double stranded according to the sequence of the opposite complementary strand by phi29 DNA polymerase. Meanwhile, primers that carry the same A1 loop sequence bind to some of the displaced strands, and phi29 DNA polymerase synthesizes the new strands according to this template DNA by using the 3' end. If this DNA amplification step by primer binding occurs on overlapped complex I, phi29 DNA polymerase displaces the strand in front of it again. Also, when A1 loop sequence as a P2 recognition site is created, P2 nicks the newly generated strand again, and all nicking reaction-triggered amplification reaction steps start again. DNA amplification is achieved by the cycling of these tandem reactions.

The DNA amplification signal increase was observed in PMA reaction even when using the smallest part of P2 recognition site as a substrate of P2. DMSO addition in PMA reactions did not cause any significant difference. All (+) test samples, which include P2 enzyme and template DNA together in PMA Q-PCR reaction mixture, gave a signal as intended. The control sample, which includes template DNA without P2 enzyme (control I) did not give any significant signal as intended. The low signals come from this control sample were background signals, and that's why they were set as the threshold value of the PMA Q-PCR. On the other hand, the control sample, which includes P2 enzyme without template DNA (control II), gave a signal. It was thought that there may have been nonspecific DNA amplification due to residual host genomic DNA in the P2 stock as a substrate for P2. Because genomic DNA of *E. coli*

may carry the P2 recognition site naturally. But an important point about this is the activity of P2 in the DNA amplification reaction. Therefore, P2 was denatured by heating to make inactive enzyme, and it was used in the same (+) and control II reactions. When the PMA reactions, which include active or inactive P2, were compared, there was no amplification signal for the included inactive P2, and even the (+) test sample of inactive P2. These results show that P2 plays a role in PMA efficiently as intended in this thesis goal. Nevertheless, PMA needs further optimization studies about eliminating non-specific amplification, as mentioned about control II. P2 can be purified by a second purification step like ion-exchange chromatography to remove any residual DNA more efficiently. Also, PMA reaction optimization studies can be improved with further studies. But all these studies suggest that P2 can be used in a nicking reaction-based DNA amplification method as development of new INAAM technology at room temperature.

Developing novel diagnostic tests that are able to work at room temperature may provide access to much-needed diagnostic methods in low-resource, high-disease burden areas. The amplification signals from different targets with specific primers can be used to detect many pathogens by PMA with further studies. This method can be combined with a lateral flow assay to design a rapid diagnostic test with low cost, high specificity, sensitivity, and accessibility while improving cost-effectiveness with further technologies.

6 CONCLUSION

In this study, we investigated the enzymatic activity of the P2 protein of the M13 bacteriophage, which we obtained by cloning the P2 gene into *E. coli* and using it in a new isothermal nucleic acid amplification method at low temperatures, called PMA. The best expression condition for P2 was determined to be 0.5 mM IPTG induction with a 37°C and 5 hours incubation. P2-GFP's nicking activity was monitored via conformational changes in the plasmid DNA from the SC form to the OC form. Different parts of the P2 recognition site, including the whole recognition site (W), only A1-A2 loops (A12), only the A1 loop (A1) and truncated A1 loop segments (A1L and A2L), were used to investigate the region essential for nicking activity. The nicking reaction was observed for all recombinant plasmids carrying truncated versions, including the smallest part (pA2L), which is the loop where the nicking site is present. When these different DNA fragments were used as linear substrates for the nicking reaction, there were no differences between the test and control groups. We calculated the Km as 34.17 ng/µL and the Vmax as 0.06 ng/µL.min^{-1} for P2. The current study is the first in almost 40 years to add more information about the recognition site of P2 (40–48,62).

Some single-stranded DNA viruses can cause infections in humans and animals, some of which have the same replication mechanisms as M13. The lifecycle of some of them is similar to that of M13 bacteriophage replication, such as parvovirus B19. In recent years, antisense oligomers (ASOs) have been developed as antiviral drugs, even for these viruses [43,44]. M13 infections in *E. coli* may be a practical model for the development of drugs against single-stranded viruses that cause infections in humans.

Nucleic acid amplification method-based tests are commonly used to detect different pathogens as a point-of-care test. Isothermal NAATs are an easy way to access much-needed diagnostic methods in low-resource, high-disease burden areas, while non-isothermal NAATs have some disadvantages, such as requiring expensive thermostable enzymes, thermal cyclers and well-trained personnel. Also, current

isothermal methods have disadvantages as well, such as requiring an initial temperature or a chemical for the reaction. That's why researchers are focused on developing new, simple isothermal NAAT technologies for patients. We suggest that P2 can be used as a nicking enzyme for isothermal nucleic acid amplification methods at room temperature. Our results show that P2 plays a role in PMA efficiently at room temperature, as intended in this thesis objective. This idea will lead to the development of new isothermal signal amplification methods for room-temperature-based technologies for point-of-care diagnosis.

7 REFERENCES

1. Kasman L, Porter L. StatPearls. 2019. Bacteriophages. Available from: https://www.ncbi.nlm.nih.gov/books/NBK493185/
2. Kutter E. Bacteriophages. In: Encyclopedia of Genetics. Sydney: Academic Press; 2001. p. 179–86.
3. Suggs S V., Ray DS. Nucleotide sequence of the origin for bacteriophage M13 DNA replication. Cold Spring Harb Symp Quant Biol. 1979;43(1):379–88.
4. Sambrook JF, Russell D. M13 Bacteriophages. In: Molecular Cloning: A Laboratory Manual. 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2001.
5. Brasino M. Engineering of Filamentous Bacteriophage for Protein Sensing. 2016;1–85.
6. John R.Giudicessi, BA.Michael J.Ackerman. 2013. Bacteriophages and their Genomes Graham. Bone. 2008;23(1):1–7.
7. Russel M. Moving through the membrane with filamentous phages. Trends Microbiol. 1995;3(6):223–8.
8. Smeal SW, Schmitt MA, Pereira RR, Prasad A, Fisk JD. Simulation of the M13 life cycle II: Investigation of the control mechanisms of M13 infection and establishment of the carrier state. Virology. 2017;500:275–84.
9. II - Replication-associated protein G2P - Enterobacteria phage M13 (Bacteriophage M13) | UniProtKB | UniProt [Internet]. [cited 2024 Apr 26]. Available from: https://www.uniprot.org/uniprotkb/P69547/entry
10. X - Replication-associated protein G2P - Enterobacteria phage M13 (Bacteriophage M13) | UniProtKB | UniProt [Internet]. [cited 2024 Apr 26]. Available from: https://www.uniprot.org/uniprotkb/J7I4I2/entry#structure
11. Dotto GP, Horiuchi K, Zinder ND. The functional origin of bacteriophage f1 DNA replication. Its signals and domains. J Mol Biol. 1984;172(4):507–21.
12. Dotto GP, Enea V, Zinderi ND. Functional analysis of bacteriophage f1 intergenic region. Virology. 1981;114(2):463–73.
13. Smeal SW, Schmitt MA, Rodrigues R, Prasad A, Fisk JD. Simulation of the M13 life cycle I : Assembly of a genetically-structured deterministic chemical kinetic simulation. Virology [Internet]. 2016; Available from: http://dx.doi.org/10.1016/j.virol.2016.08.017
14. Rakonjac J, Russel M, Khanum S, Brooke SJ, Raji M. Filamentous Phage: Structure and Biology. 2017;
15. Slonczewski J, Foster J. Viral Molecular Biology. In: Microbiology an Evolving Science. 2nd ed. W. W. Norton and Norton Company, Inc.; 2017.
16. Dotto GP, Enea V, Zinder ND. Gene II of phage f1: Its functions and its products. Proc Natl Acad Sci U S A. 1981;78(9 II):5421–4.
17. Greenstein D, Horiuchi K. Interaction between the replication origin and the initiator protein of the filamentous phage f1. Binding occurs in two steps. J Mol Biol. 1987;197(2):157–74.

18. Smeal SW, Schmitt MA, Rodrigues R, Prasad A, Fisk JD. Simulation of the M13 life cycle I : Assembly of a genetically-structured deterministic chemical kinetic simulation. Virology. 2017;500:259–74.

19. Morag O, Sgourakis G N, Abramov G, Goldbourt A. Filamentous Bacteriophage Viruses: Preparation, Magic-Angle Spinning Solid-State NMR Experiments, and Structure Determination. In: Ghose R, editor. Protein NMR. New York: Humana Press; 2018. p. 67–97.

20. Azzazy HME, Highsmith WE. Phage display technology: Clinical applications and recent innovations. Clin Biochem. 2002;35(6):425–45.

21. Geider K, Meyer T. Gene-II protein of bacteriophage fd in enzymatic replication of viral duplex DNA. Cold Spring Harb Symp Quant Biol [Internet]. 1979;43 Pt 1:59–62. Available from: https://www.semanticscholar.org/paper/3e0781a727a5636eeb641eba90a6b980452a7d7e

22. HARTH G, Bäumel I, MEYER TF, GEIDER K. Bacteriophage fd Gene- 2 Protein: Processing of Phage fd Viral Straands Replicated by Phage T7 Enzymes. Eur J Biochem. 1981;119(3):663–8.

23. Meyer TF, Geider K, Kurz C, Schaller H. Cleavage site of bacteriophage fd gene II-protein in the origin of viral strand replication [21]. Vol. 278, Nature. 1979. p. 365–7.

24. Suggs S V., Ray DS. Nucleotide sequence of the origin for bacteriophage M13 DNA replication. Cold Spring Harb Symp Quant Biol. 1979;43(1):379–88.

25. Peeters BP h., Schoenmakers JG g., Konings RN h. Comparison of the DNA Sequences Involved in Replication and Packaging of the Filamentous Phages IKe and Ff (M13, fd, and fl). Dna. 1987;6(2):139–47.

26. Baas PD, Jansz HS. Single-stranded DNA phage origins. Curr Top Microbiol Immunol. 1988;136(1959):31–70.

27. Higashitani N, Higashitani A, Guan ZW, Horiuchi K. Recognition mechanisms of the minus-strand origin of phage f1 by Escherichia coli RNA polymerase. Genes to Cells. 1996;1(9):829–41.

28. Horiuchi K. Initiation mechanisms in replication of filamentous phage DNA. Genes to Cells. 1997;2(7):425–32.

29. Higashitani N, Higashitani A, Guan ZW, Horiuchi K. Recognition mechanisms of the minus-strand origin of phage f1 by Escherichia coli RNA polymerase. Genes to Cells. 1996;1(9):829–41.

30. Dennehy JJ. Bacteriophages as model organisms for virus emergence research. Vol. 17, Trends in Microbiology. 2009. p. 450–7.

31. Ahmad F, Hashsham SA. Miniaturized nucleic acid amplification systems for rapid and point-of-care diagnostics: A review. Anal Chim Acta. 2012;733:1–15.

32. Alladin-Mustan BS, Mitran CJ, Gibbs-Davis JM. Achieving room temperature DNA amplification by dialling in destabilization. Chemical Communications. 2015;51(44):9101–4.

33. Ning L, Wang X, Xu K, Song S, Li Q, Yang X. A novel isothermal method using rolling circle reverse transcription for accurate amplification of small RNA sequences. Biochimie. 2019;163:137–41.

34. Wang T, Liu Y, Sun HH, Yin BC, Ye BC. An RNA-Guided Cas9 Nickase-Based Method for Universal Isothermal DNA Amplification. Angewandte Chemie - International Edition. 2019;58(16):5382–6.

35. Kang T, Lu J, Yu T, Long Y, Liu G. Advances in nucleic acid amplification techniques (NAATs): COVID-19 point-of-care diagnostics as an example. Biosens Bioelectron. 2022;206(September 2021):114109.

36. Qian C, Wang R, Wu H, Ji F, Wu J. Nicking enzyme-assisted amplification (NEAA) technology and its applications: A review. Anal Chim Acta. 2019;1050:1–15.

37. Niemz A, Ferguson TM, Boyle DS. Point-of-care nucleic acid testing for infectious diseases. Trends Biotechnol. 2011;29(5):240–50.

38. Shiju TM, Tripura C, Saha P, Mansingh A, Challa V, Bhatnagar I, et al. Ready-to-use vertical flow paper device for instrument-free room temperature reverse transcription. N Biotechnol. 2022;68:77–86.

39. Shi C, Liu Q, Zhou M, Zhao H, Yang T, Ma C. Nicking endonuclease-mediated isothermal exponential amplification for double-stranded DNA detection. Sens Actuators B Chem. 2016;222:221–5.

40. Geider K, Bäumel I, Meyer T. Intermediate stages in enzymatic replication of bacteriophage fd duplex DNA. J Biol Chem [Internet]. 1982;257 11:6488–93. Available from: https://www.semanticscholar.org/paper/de1a987174b9d112357661fce7ca2d1d7d9cacbb

41. Horiuchi K, Zinder N. Origin and direction of synthesis of bacteriophage fl DNA. Proc Natl Acad Sci U S A [Internet]. 1976;73 7:2341–5. Available from: https://www.semanticscholar.org/paper/9b6013c1f879e6ecda6c8106063ba0cc509587c5

42. Cleary JM, Ray D. Deletion analysis of the cloned replication origin region from bacteriophage M13. J Virol [Internet]. 1981;40:197–203. Available from: https://www.semanticscholar.org/paper/4aa7f80d5a4795dc11cf635664327547ac64ab7e

43. Johnston S, Ray D. Interference between M13 and oriM13 plasmids is mediated by a replication enhancer sequence near the viral strand origin. J Mol Biol [Internet]. 1984;177 4:685–700. Available from: https://www.semanticscholar.org/paper/34d2754ffc7deb5f3e2875cb3d40d1382adf52cb

44. Meyer TF, Geider K. Bacteriophage fd gene II-protein. II. Specific cleavage and relaxation of supercoiled RF from filamentous phages. J Biol Chem [Internet]. 1979;254 24:12642–6. Available from: https://www.semanticscholar.org/paper/5528690e46f4989ef3e23691191dd364659762ae

45. Meyer T, Geider K. Enzymatic synthesis of bacteriophage fd viral DNA. Nature [Internet]. 1982;296:828–32. Available from: https://www.semanticscholar.org/paper/5c9a2b4d7de58b5b7480062c33fca20e7d92bdb8

46. Lin N, Pratt D. Bacteriophage M 13 gene 2 protein: increasing its yield in infected cells, and identification and localization. Virology [Internet]. 1974;61 2:334–42. Available from: https://www.semanticscholar.org/paper/b840a6820955c3c2a8c93047bec4045fd1394dc8

47. Dotto G, Horiuchi K, Zinder N. The functional origin of bacteriophage f1 DNA replication. Its signals and domains. J Mol Biol [Internet]. 1984;172 4:507–21. Available from: https://www.semanticscholar.org/paper/996b8b4a474443cae3ca2eb6b9951e989aeaa2c6

48. Schaller H, Uhlmann A, Geider K. A DNA fragment from the origin of single-strand to double-strand DNA replication of bacteriophage fd. Proc Natl Acad Sci U S A [Internet]. 1976;73 1:49–53. Available from: https://www.semanticscholar.org/paper/eed3e1ca5899a4d60d0c8fd8ac6cf1f0a03c6eb1

49. Chung CT, Niemela SL, Miller RH. One-step preparation of competent Escherichia coli: Transformation and storage of bacterial cells in the same solution (recombinant DNA). Vol. 86. 1989.

50. Barbas C, Burton D, Scott J, Silverman G. Quantitation of DNA and RNA. In: Cold Spring Harbor Protocols. Cold Spring Harbor Laboratory Press; 2007.

51. OligoAnalyzer Tool - Primer analysis and Tm Calculator | IDT [Internet]. [cited 2024 Apr 23]. Available from: https://www.idtdna.com/pages/tools/oligoanalyzer

52. Primer designing tool [Internet]. [cited 2024 Apr 23]. Available from: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

53. Oligo Analyzer [Internet]. [cited 2020 Aug 26]. Available from: https://www.idtdna.com/calc/analyzer

54. LIC Cloning Protocol – QB3 Berkeley [Internet]. [cited 2024 Apr 23]. Available from: https://qb3.berkeley.edu/facility/qb3-macrolab/projects/lic-cloning-protocol/

55. NEB NEB. Ligation Calculator [Internet]. Available from: https://nebiocalculator.neb.com/#!/ligation

56. Sambrook JF, Russell D. M13 Bacteriophages. In: Molecular Cloning: A Laboratory Manual. 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2001.

57. Arndt C, Koristka S, Bartsch H, Bachmann M. Native Polyacrylamide Gels. In: Walker JM, Kurien BT, Scofield RH, editors. Methods in Molecular Biology. Humana Press; 2012. p. 49–53.

58. Zhong S, Rivera-Molina F, Rivetta A, Toomre D, Santos-Sacchi J, Navaratnam D. Seeing the long tail: A novel green fluorescent protein, SiriusGFP, for ultra long timelapse imaging. J Neurosci Methods. 2019;313(December 2018):68–76.

59. Meyer TF, Geider K. Bacteriophage fd gene II-protein. II. Specific cleavage and relaxation of supercoiled RF from filamentous phages. Journal of Biological Chemistry. 1979;254(24):12642–6.

60. Expasy - Translate tool [Internet]. [cited 2024 Apr 23]. Available from: https://web.expasy.org/translate/

61. Saeed IA, Ashraf SS. Denaturation studies reveal significant differences between GFP and blue fluorescent protein. Int J Biol Macromol. 2009;45(3):236–41.

62. Dotto G, Enea V, Zinder N. Functional analysis of bacteriophage f1 intergenic region. Virology [Internet]. 1981;114 2:463–73. Available from: https://www.semanticscholar.org/paper/9d393e15830c57fbb16dfac44639f0143d209d73

63. Geider K, Meyer TF. Gene-II protein of bacteriophage fd in enzymatic replication of viral duplex DNA. Cold Spring Harb Symp Quant Biol. 1979;43(1):59–62.

64. Meyer TF, Geider K. Cloning of the bacteriophage fd gene 2 and construction of a plasmid dependent on fd gene 2 protein. Proc Natl Acad Sci U S A. 1981;78(9 II):5416–20.

65. Dotto G, Horiuchi K, Jakes K, Zinder N. Replication origin of bacteriophage f1. Two signals required for its function. J Mol Biol [Internet]. 1982;162 2:335–43. Available from: https://www.semanticscholar.org/paper/41b7b422e82e28e8f2810907d23d972a17220ccf

66. Dotto GP, Horiuchi K, Zinder ND. The functional origin of bacteriophage f1 DNA replication. Its signals and domains. J Mol Biol. 1984;172(4):507–21.

67. Spurgers KB, Sharkey CM, Warfield KL, Bavari S. Oligonucleotide antiviral therapeutics: Antisense and RNA interference for highly pathogenic RNA viruses. Antiviral Res. 2008;78(1):26–36.

68. Yuen MF, Heo J, Jang JW, Yoon JH, Kweon YO, Park SJ, et al. Safety, tolerability and antiviral activity of the antisense oligonucleotide bepirovirsen in patients with chronic hepatitis B: a phase 2 randomized controlled trial. Nat Med [Internet]. 2021;27(10):1725–34. Available from: http://dx.doi.org/10.1038/s41591-021-01513-4

69. Guan W, Huang Q, Cheng F, Qiu J. Internal polyadenylation of the parvovirus B19 precursor mRNA is regulated by alternative splicing. Journal of Biological Chemistry. 2011;286(28):24793–805.

70. Ganaie SS, Qiu J. Recent advances in replication and infection of human parvovirus B19. Front Cell Infect Microbiol. 2018;8(JUN):1–12.

71. Quemener AM, Galibert MD. Antisense oligonucleotide: A promising therapeutic option to beat COVID-19. Wiley Interdiscip Rev RNA. 2022;13(4):1–12.

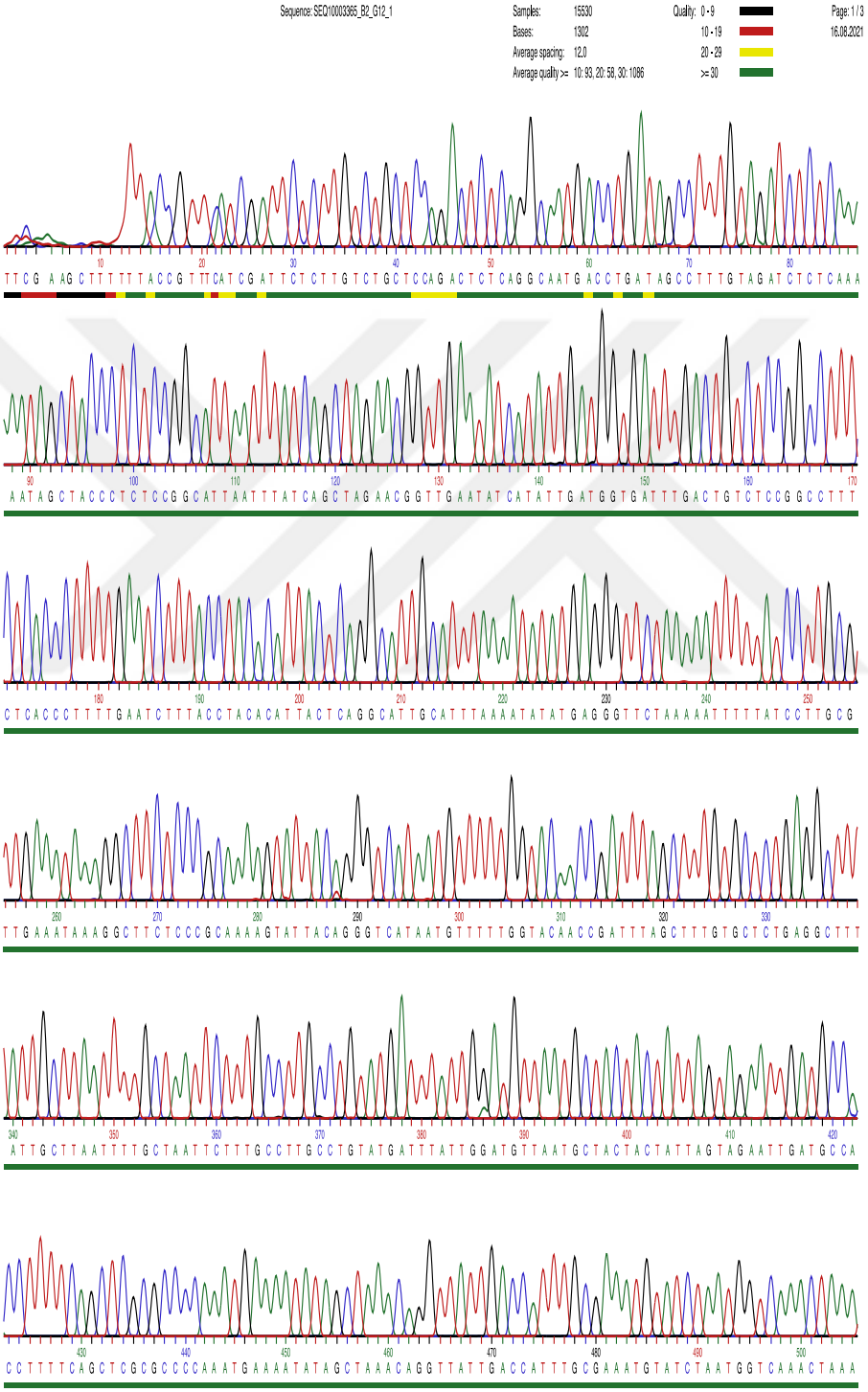
72. Meyer TF, Geider K. Bacteriophage fd gene II-protein. II. Specific cleavage and relaxation of supercoiled RF from filamentous phages. J Biol Chem. 1979;254 24:12642–6.

73. Meyer TF, Baeumel I, Geider K, Bedinger P. Replication of phage fd RF with fd gene 2 protein and phage T4 enzymes. Journal of Biological Chemistry. 1981;256(11):5810–3.

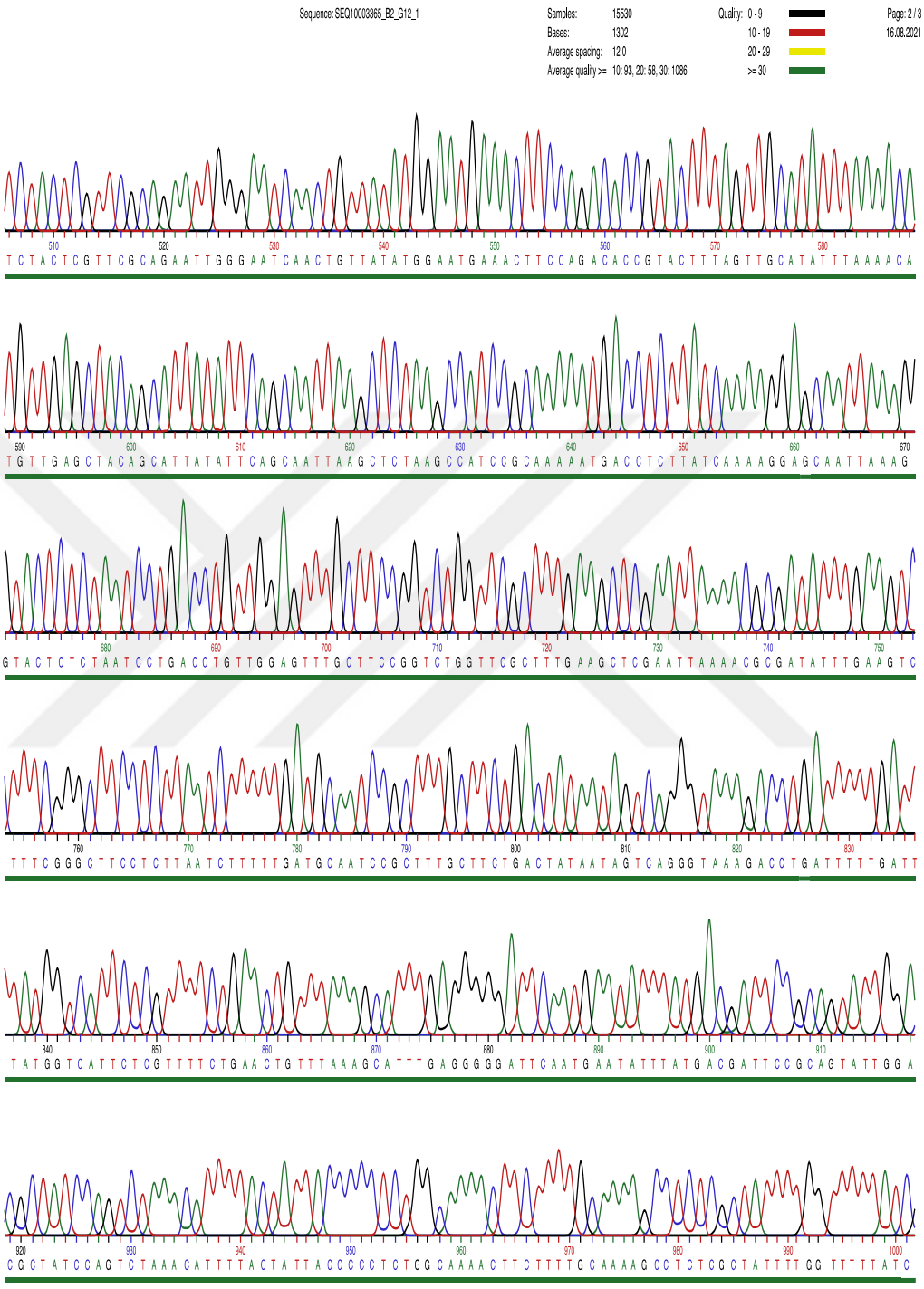
74. Geider K, Meyer T, Bäumel I, Reimann A. Proteins and nucleotide sequences involved in DNA replication of filamentous bacteriophage. Adv Exp Med Biol. 1984;179:45–54.

APPENDIX

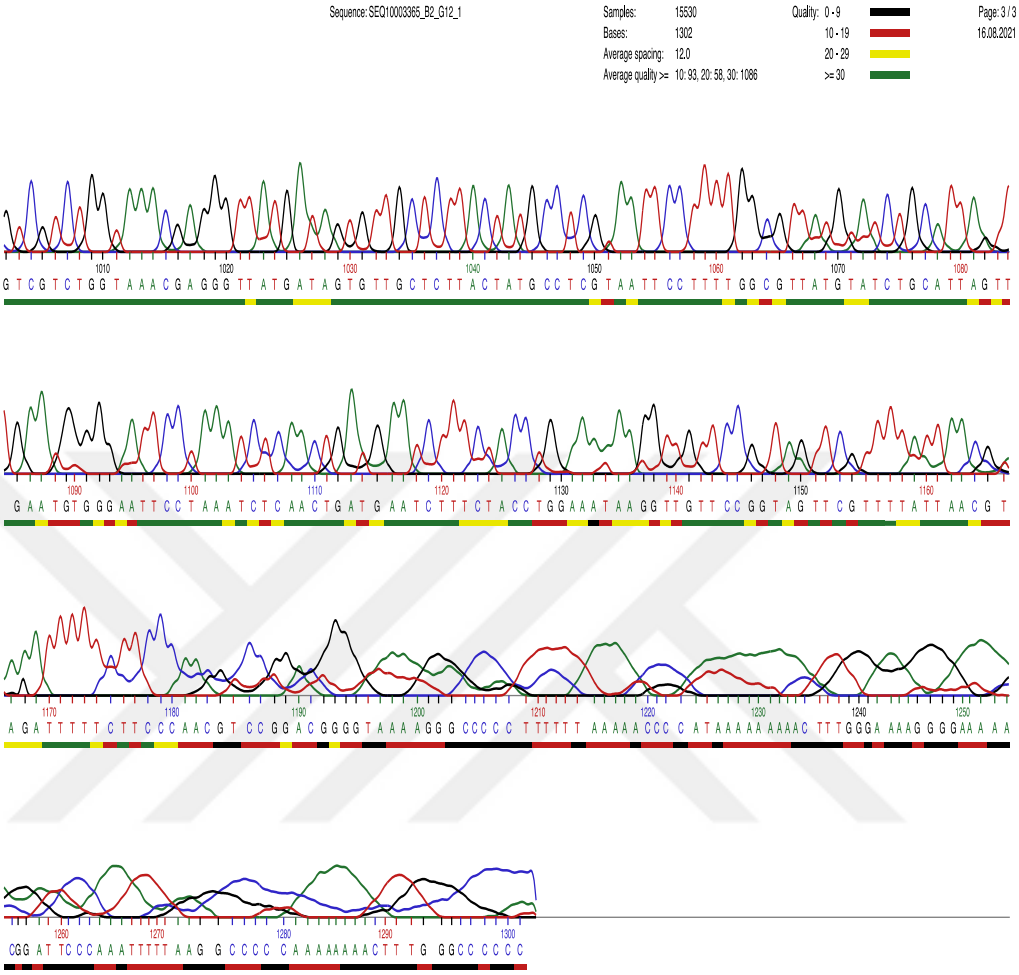
APPENDIX 1 –Forward Reading



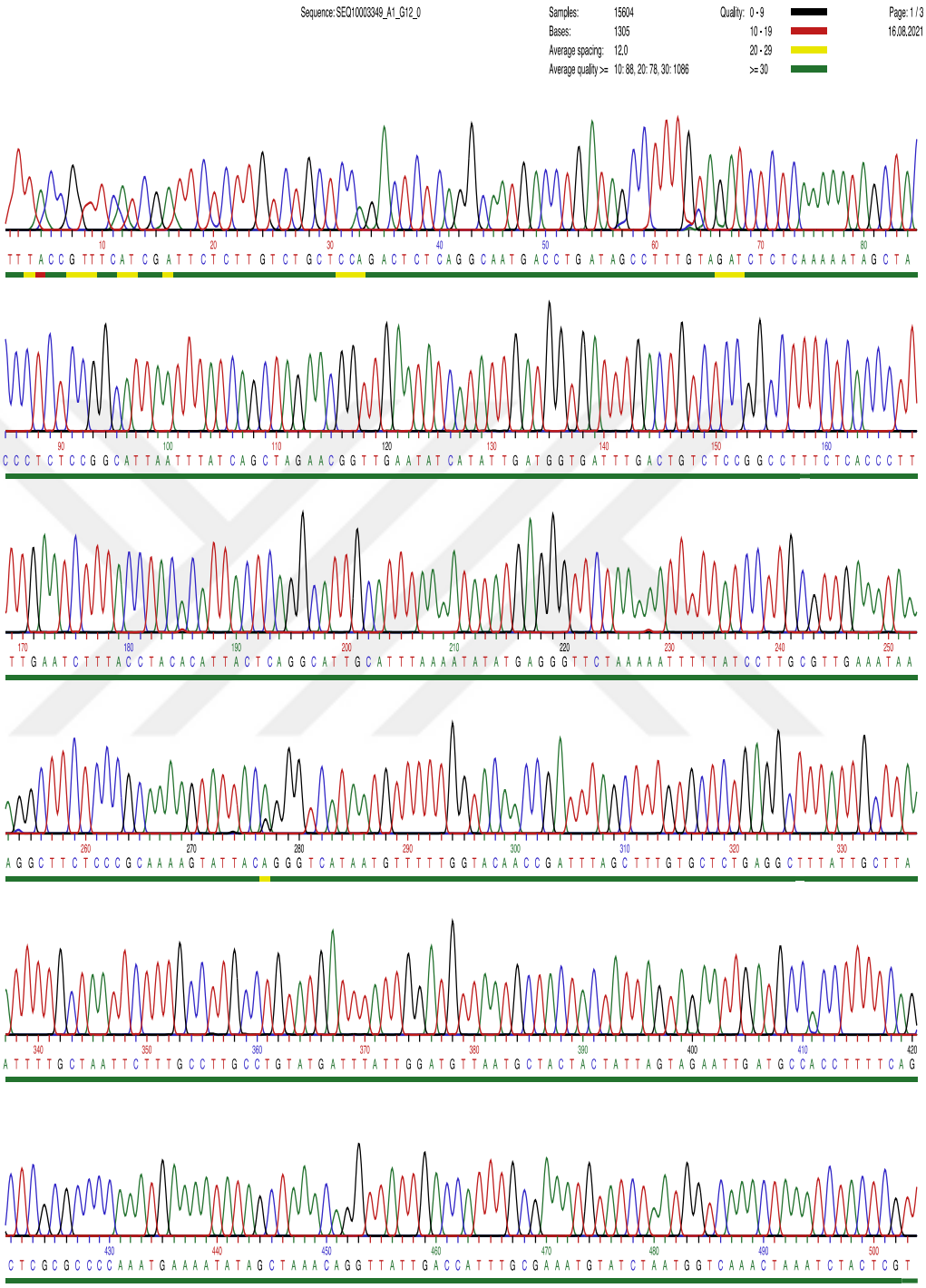
APPENDIX 1 –Forward Reading (Continue)



APPENDIX 1 –Forward Reading (Continue)



APPENDIX 2 – Reverse Reading



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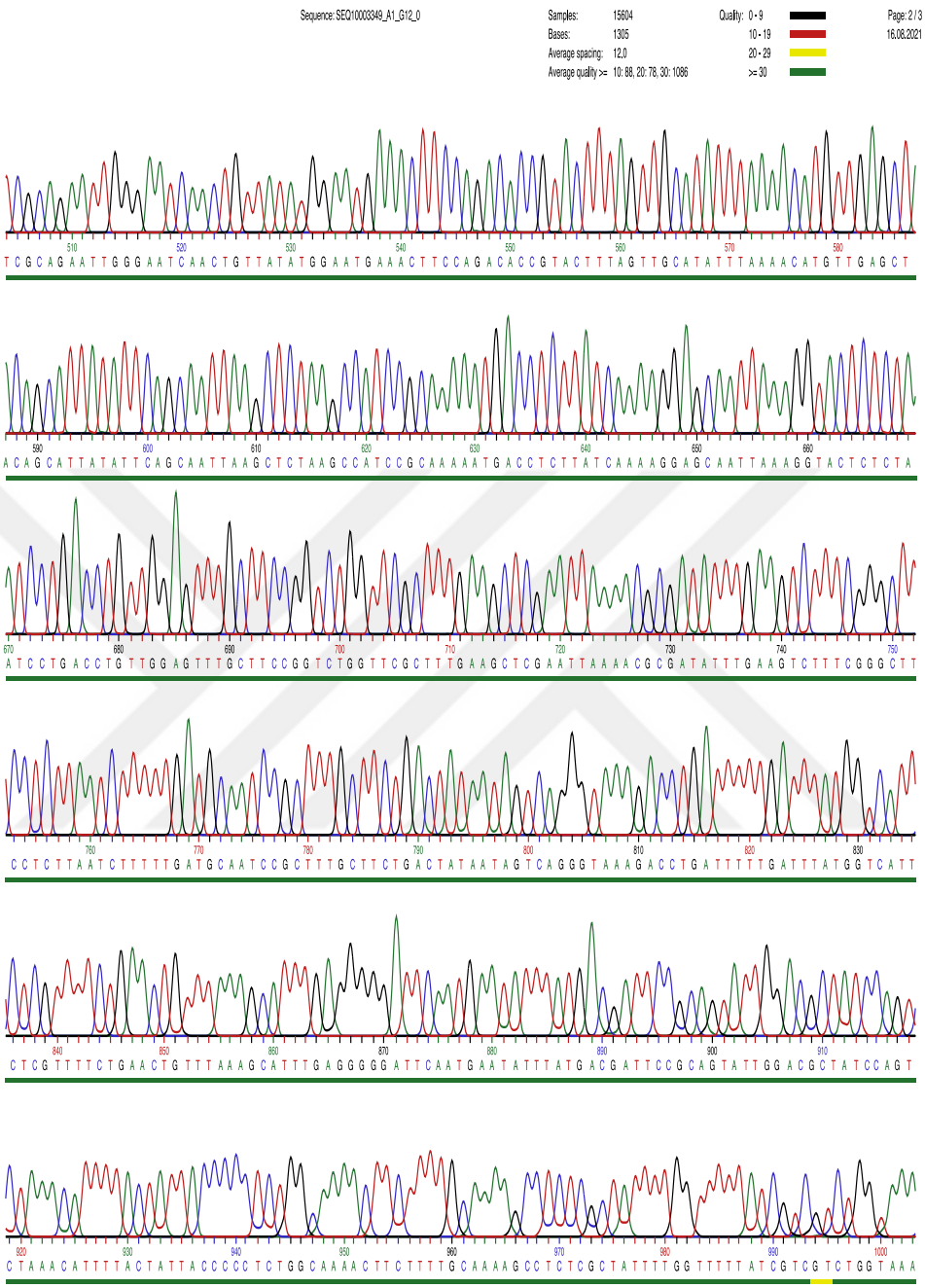

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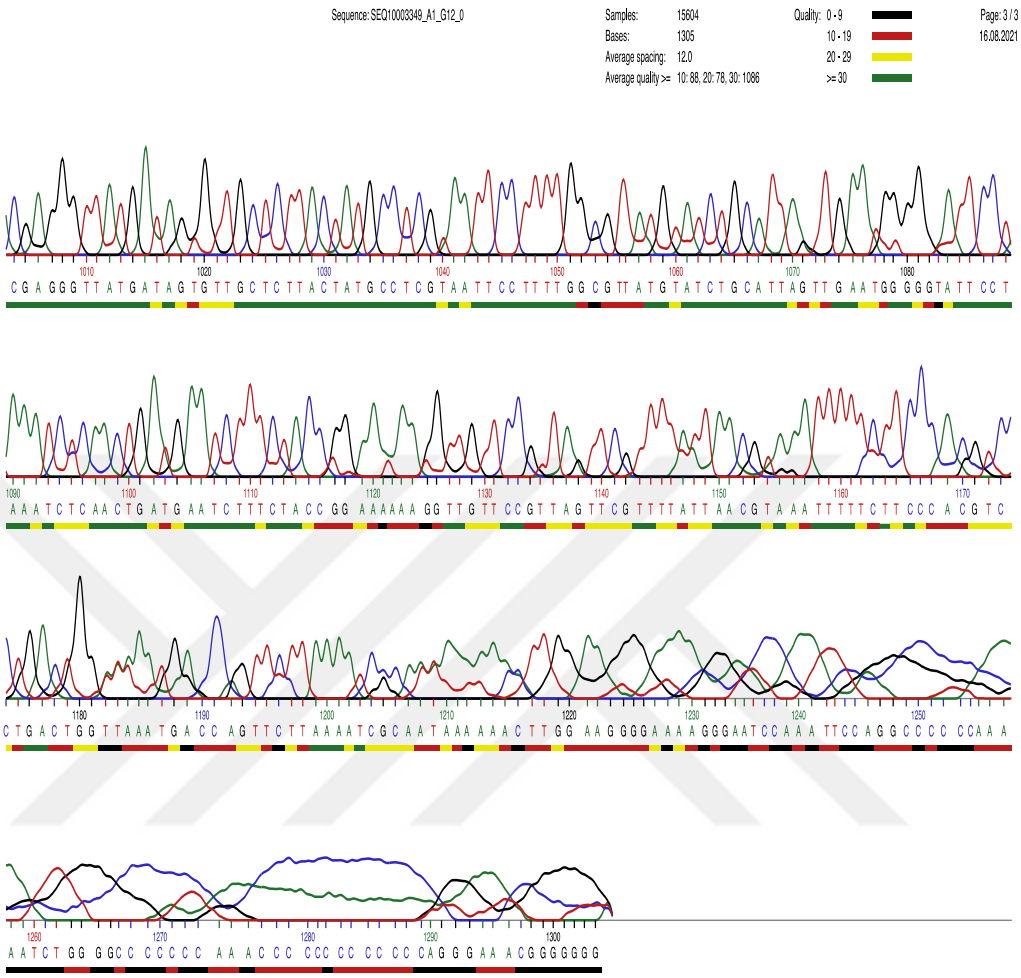
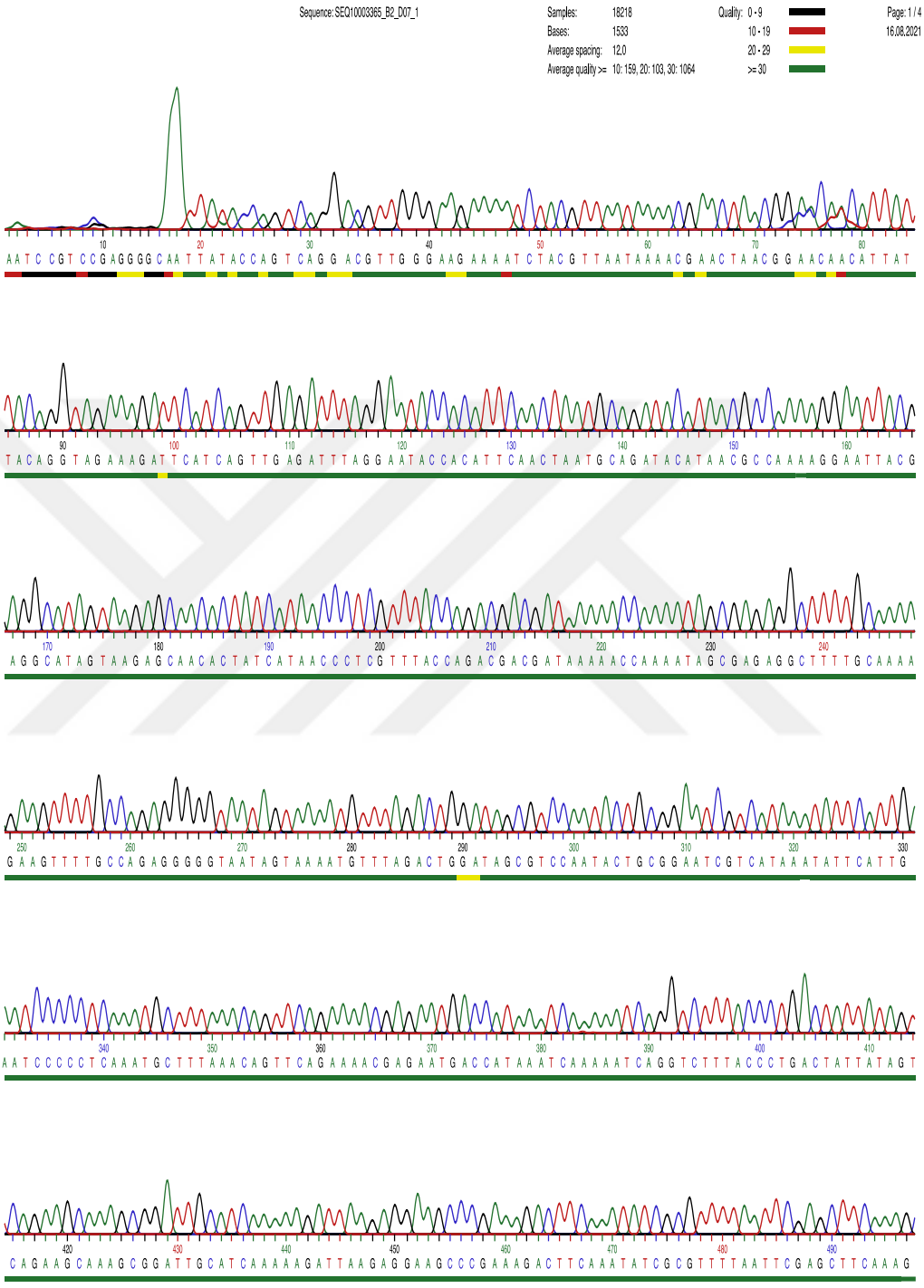


APPENDIX 3 – Forward Reading 2



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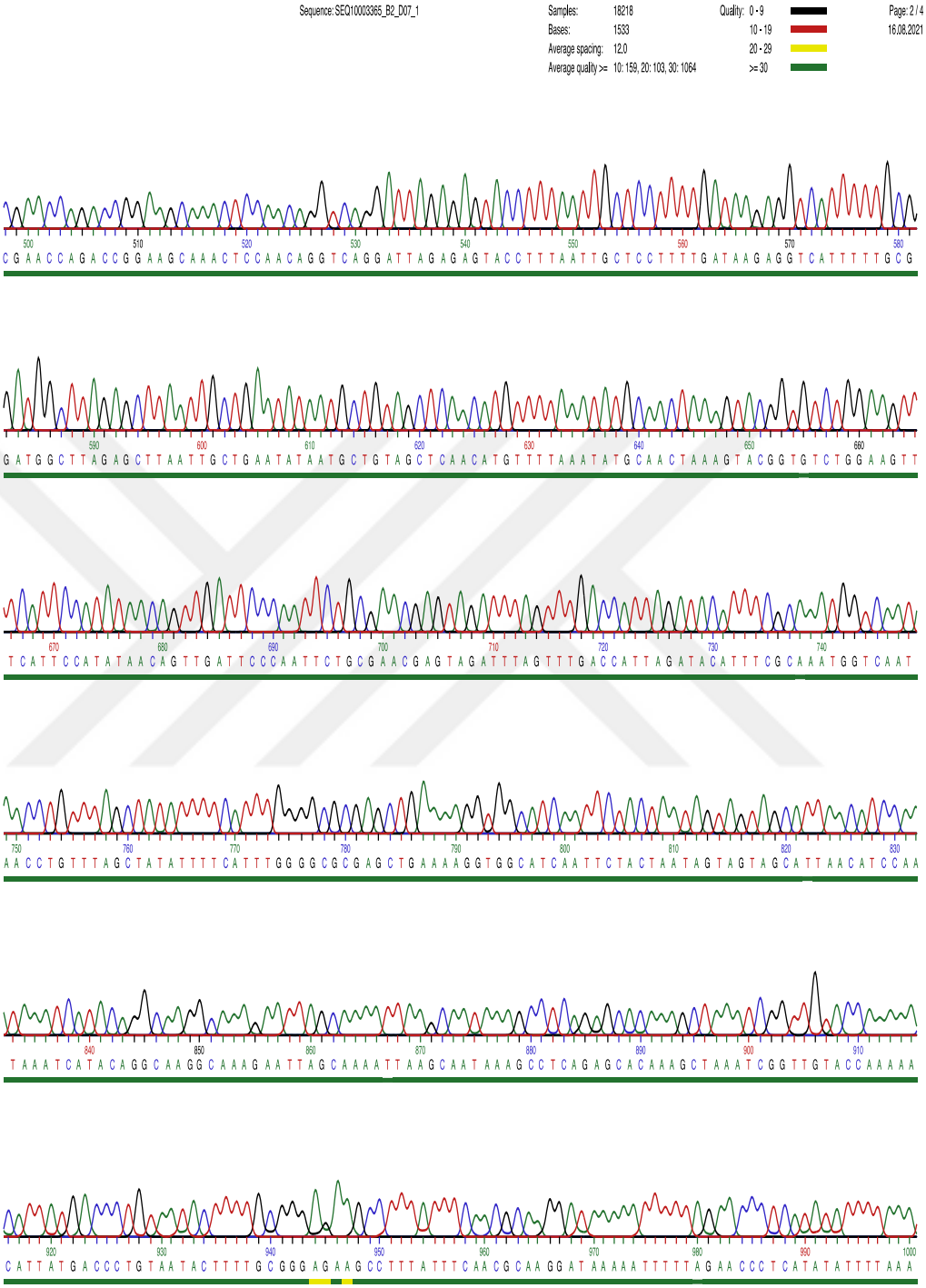

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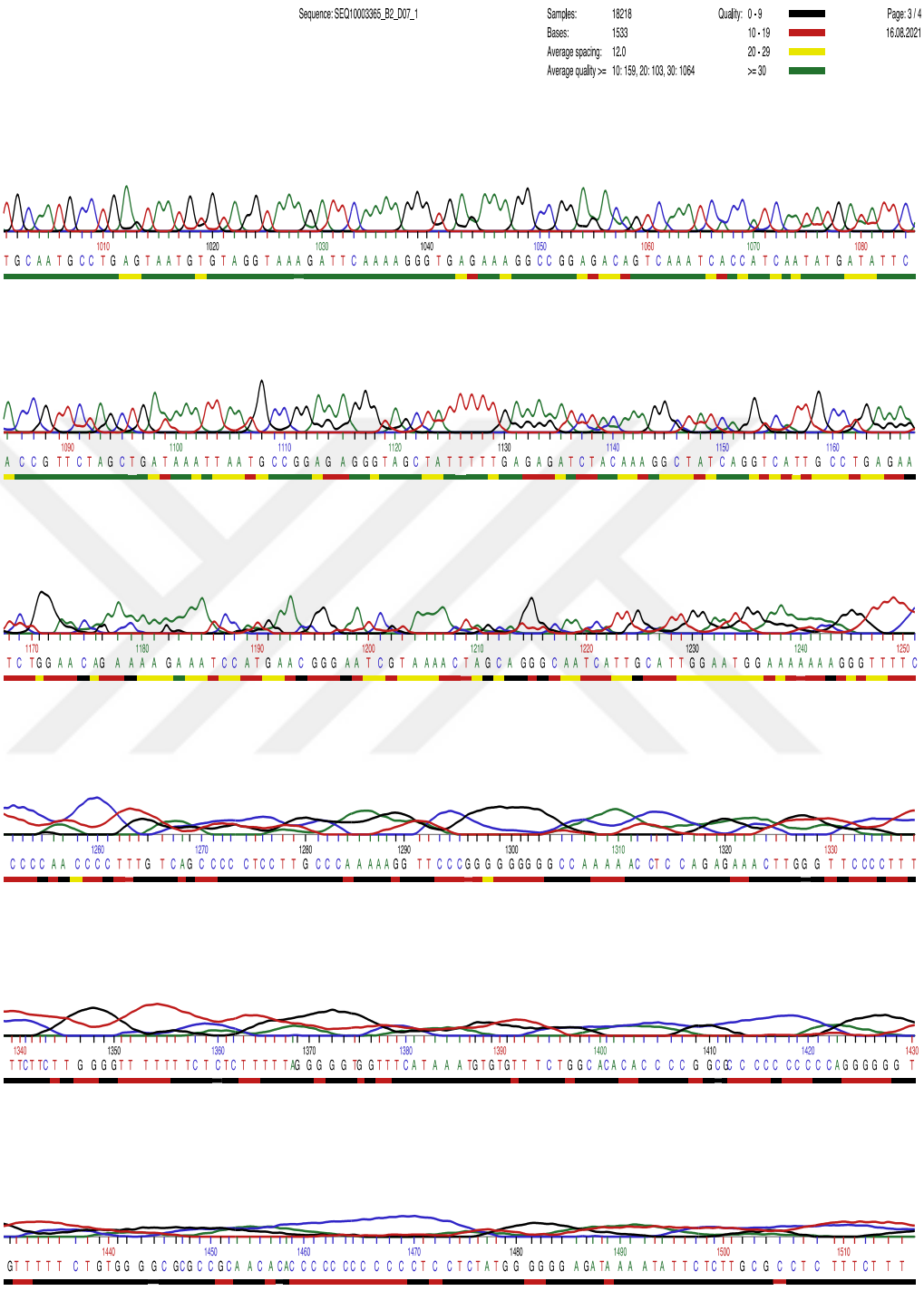

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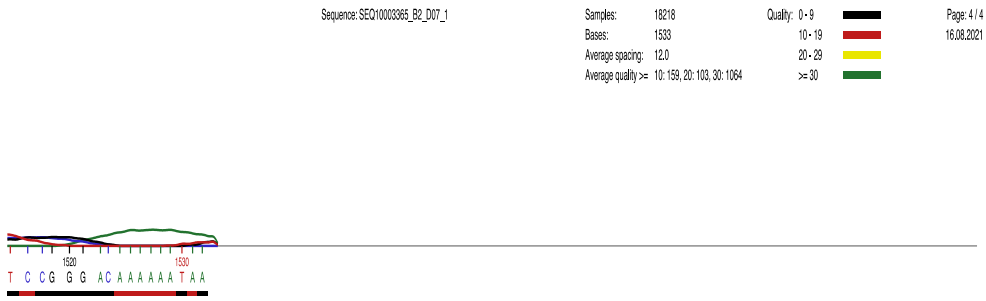
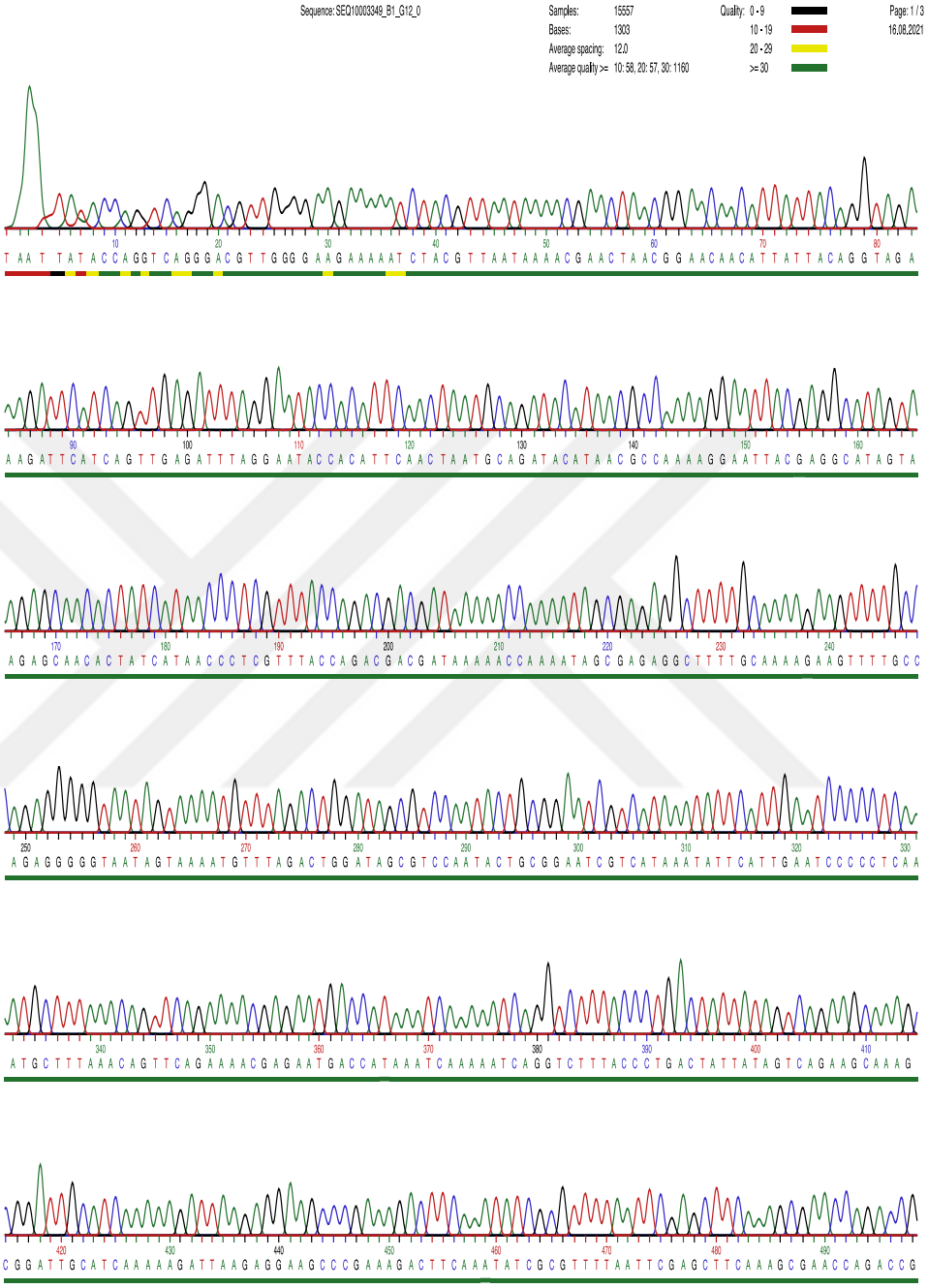


APPENDIX 4 – Reverse Reading 2



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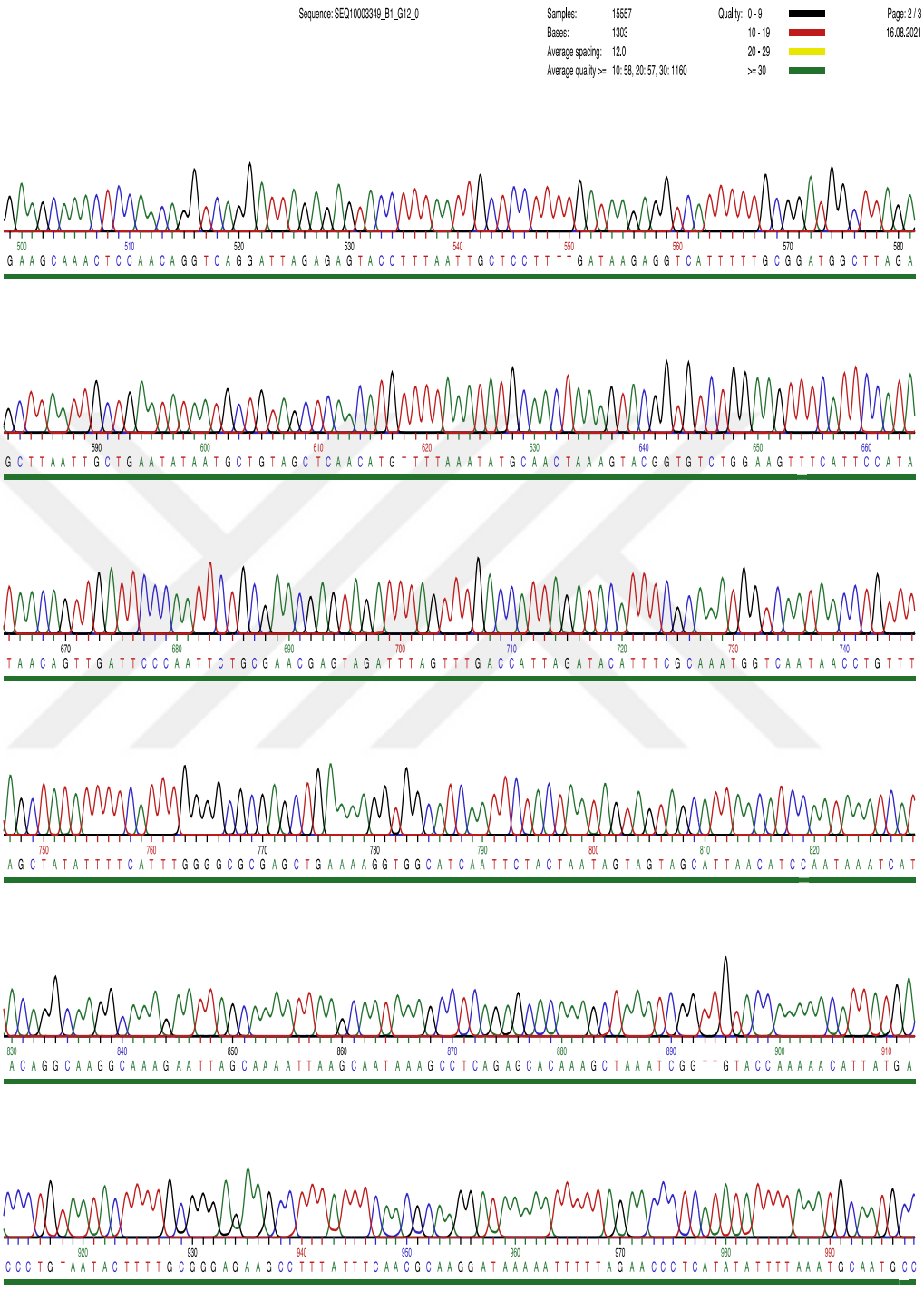

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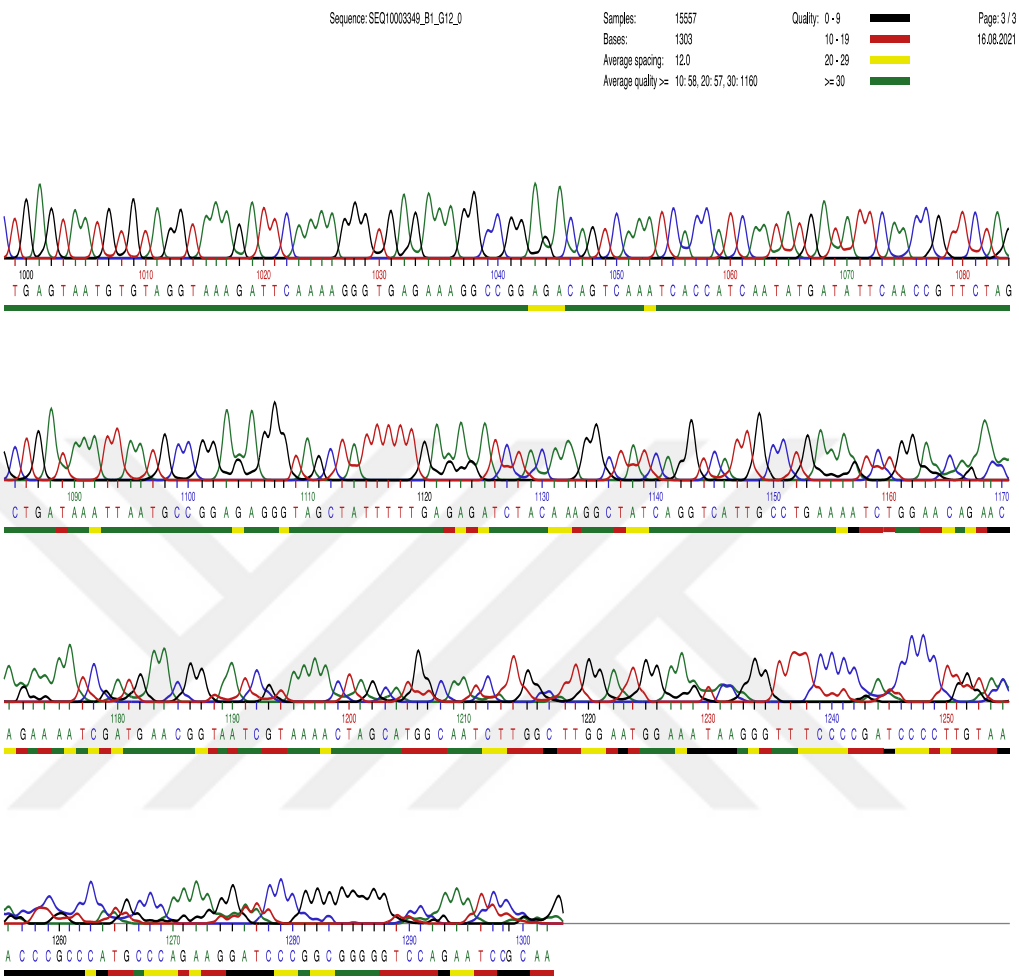
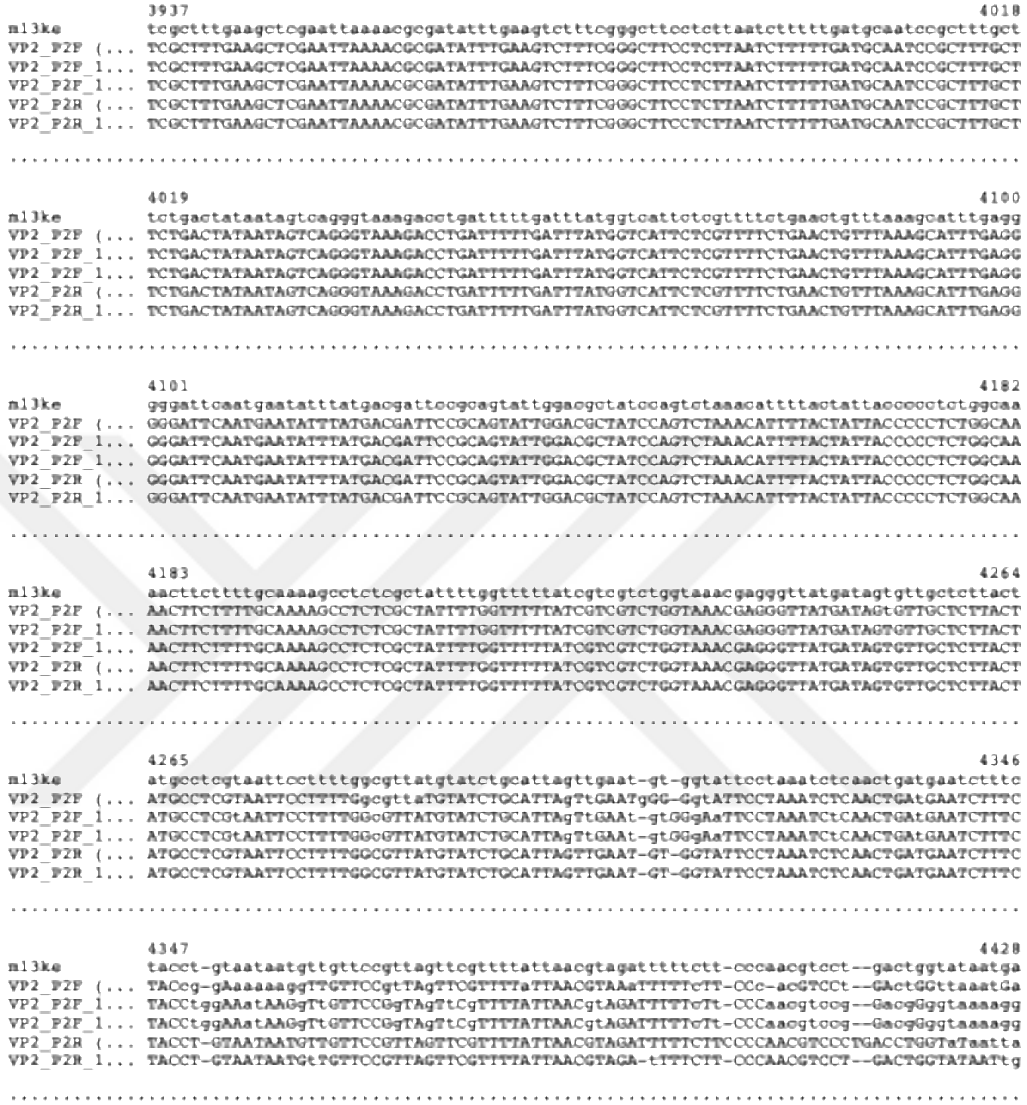


APPENDIX 5 – DNA Sequence Alignment

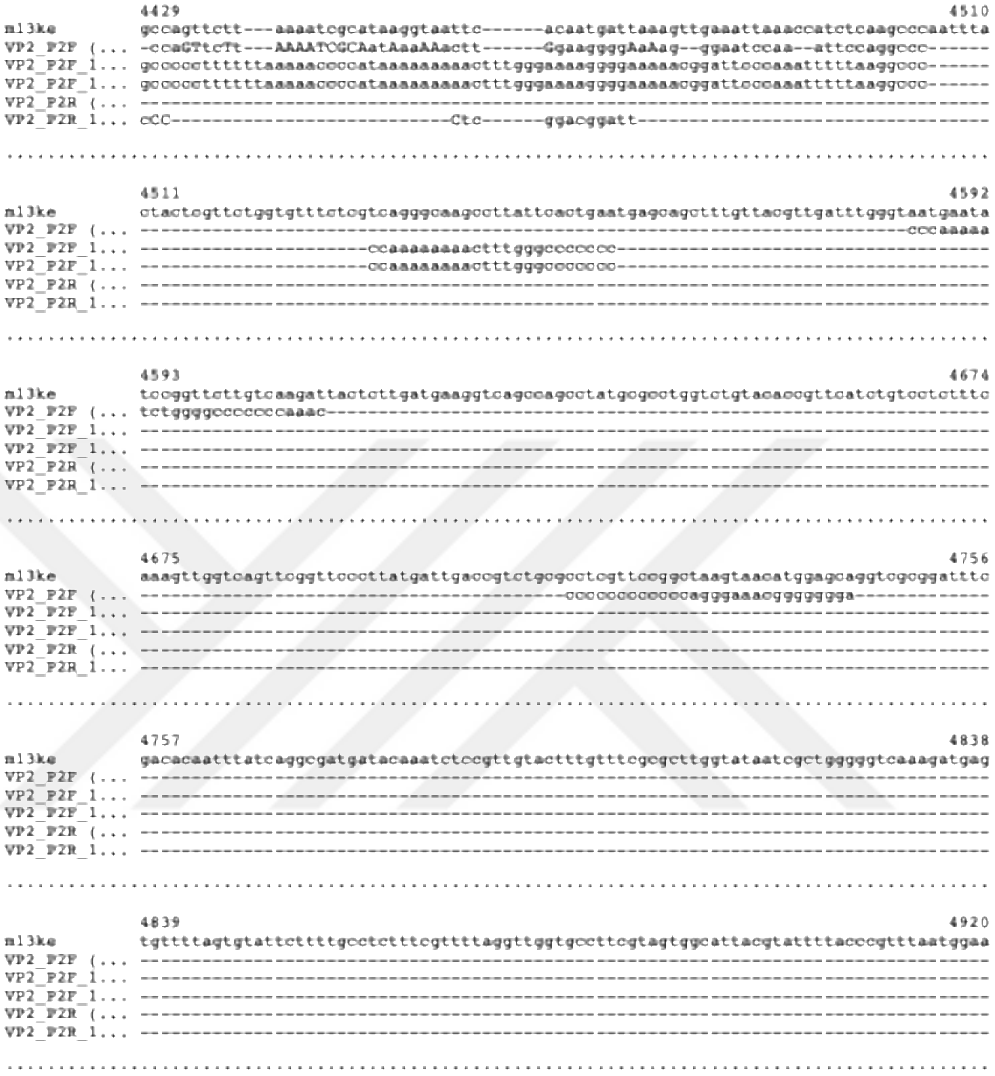
First sequence is the *gene II* of M13 phage DNA (M13KE), 2nd and 3rd ones belong to sequence reading by using of forward primer (F); 4th and 5th belong to sequence reading by using of reverse primer (R).

APPENDIX 5 – DNA Sequence Alignment (Continue)

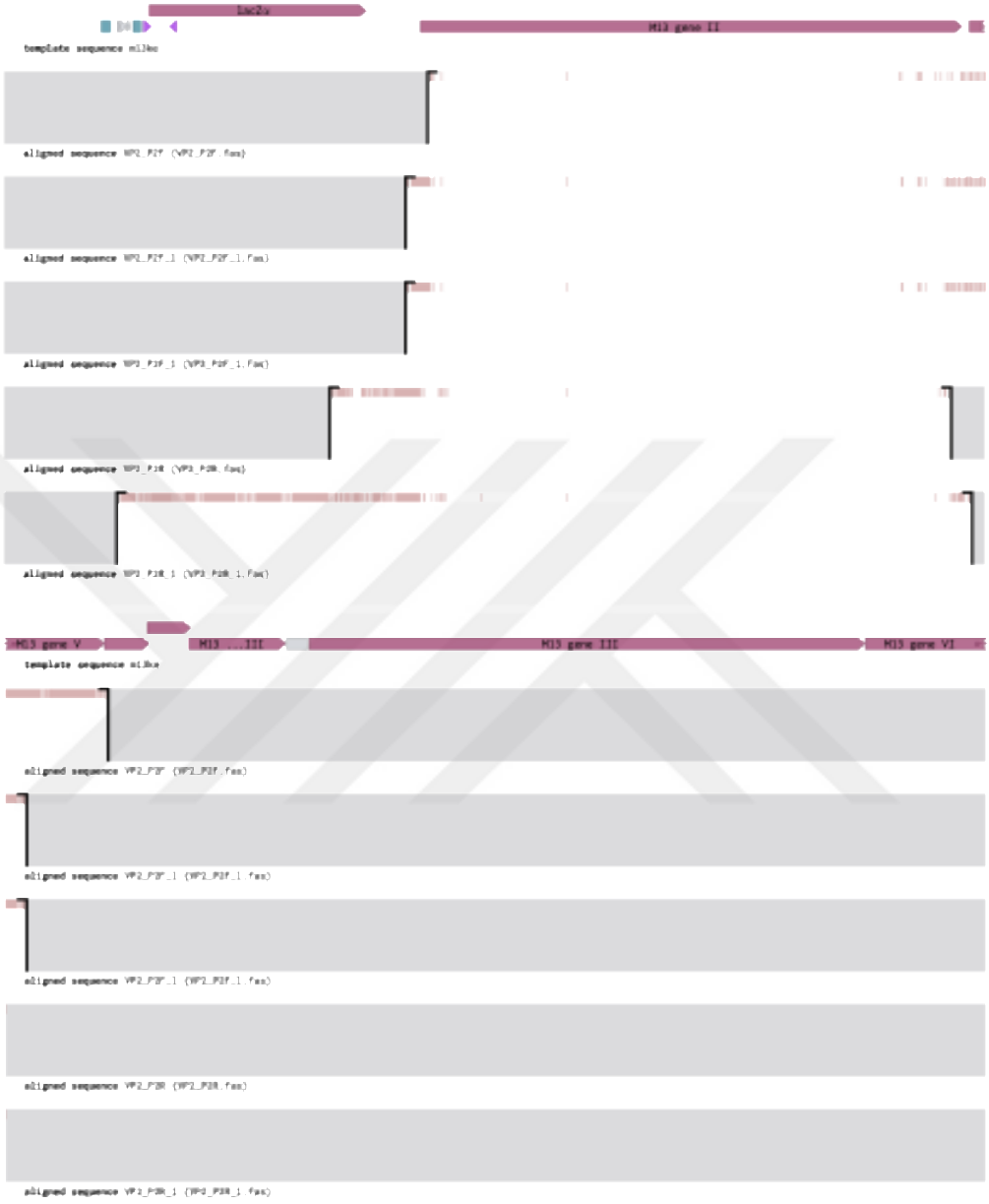
APPENDIX 5 – DNA Sequence Alignment (Continue)



APPENDIX 5 – DNA Sequence Alignment (Continue)



APPENDIX 5 – DNA Sequence Alignment (Continue)



8 CURRICULUM VITAE