REPUBLIC OF TURKEY

ACIBADEM MEHMET ALİ AYDINLAR UNIVERSITY

INSTITUTE OF HEALTH SCIENCE

EXPRESSION AND PURIFICATION OF RECOMBINANT MPT64 PROTEIN IN *ESCHERICHIA COLI* FOR SKIN TEST

ECE AKSOY

MASTER THESIS

SUPERVISOR

Assist. Prof. Erkan Mozioğlu

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DECLARATION

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

23.12.2020

Ece Aksoy

ACKNOWLEDGEMENT

I would like to express my gratitude to my advisor Assist. Prof. Dr. Erkan Mozioğlu and my co-advisor Prof. Dr. Zühtü Tanıl Kocagöz for their guidance and patience they showed throughout this process. Additionally, I am grateful to my family who constantly push me to do better in life. Furthermore, I feel lucky to have Ayşegül Ekmekçioğlu as a sidekick and a companion in this adventure. Also, I am grateful to have Sezgi Yalçınkaya and Gizem Şele. I always considered myself privileged to have them in my life. Lastly, I would like to thank to my friends I made during this period of time in lab. Even though we started separately, we conclude this era together.

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LIST OF ABBREVIATIONS AND SYMBOLS

BCG	Bacillus Calmette-Guérin
CFP10	Culture filtrate protein 10
CP	Cell pellet
D	Dialyzed pellet
DTH	Delayed-type hypersensitivity
DW	DTT wash
DTT	1,4-dithiothreitol
ESAT-6	Early secretory antigenic target 6
IGRA	Interferon gamma release assay
IMPACT	Intein Mediated Purification with an Affinity Chitin-binding Tag
IPTG	Isopropyl β d-1-thiogalactopyranoside
LAM	Lipoarabinomannan
LAMP	Loop-mediated isothermal amplification
LTBI	Latent tuberculosis infection
MDR	Multidrug-resistance
MOTT	Mycobacterium other than tuberculosis
MPT64	Mycobacterium Protein Tuberculosis 64
NAAT	Nucleic Acid Amplification Test
OT	Old Tuberculin
P	Pellet
PCR	Polymerase chain reaction
PPD	Purified Protein Derivative
POC	point-of-care
RD	Region of Difference
S	Supernatant
St	Stripped w/NaOH
TB	Tuberculosis
TST	Tuberculosis Skin Test

W Wash

WHO World Health Organization

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SUMMARY

MPT64 protein is found in Purified Protein Derivative (PPD) that is commonly used in Tuberculosis Skin Test (TST). TST gives false positive results frequently for the patients who had Bacillus Calmette-Guérin (BCG) vaccine before. Using purified MPT64 proteins instead of a protein mixture in PPD holds a great potential to eliminate false positive results due to BCG vaccination. So, MPT64 protein provides a better and more reliable results with this diagnostic test by increasing the sensitivity. The aim of this study was to produce and purify MPT64 protein. It was aimed to purify protein in a single chromatographic step to bring a new perspective and a more practical way to obtain MPT64 protein. This thesis explains how expression of recombinant MPT64 protein inserted in pTXB1 and pTYB21 plasmids with intein tag in *Escherichia coli* ER2566 separately done and purification of MPT64 protein by self-cleavage due to thiol reagents in chitin column feature that Intein Mediated Purification with an Affinity Chitin-binding Tag (IMPACT) system offers.

Key words: Intein Tag, MPT64 Protein, *Mycobacterium tuberculosis,* Purified Protein Derivative, Tuberculin Skin Test

ÖZET

Deri Testi İçin *Escherichia coli* Bakterisinde Recombinant MPT64 Proteininin İfadesi ve Saflaştırılması

MPT64 proteini, Tüberküloz Deri Testinde (TDT) yaygın olarak kullanılan Saflaştırılmış Protein Türevinde (PPD) bulunur. Daha önce Bacillus Calmette-Guerin (BCG) aşısı yaptıran hastalarda TDT sıklıkla yanlış pozitif sonuç vermektedir. Bu kullanılan protein karışımı yerine saflaştırılmış MPT64 proteini gibi alternative bir antijen kullanmak, BCG aşısından kaynaklanan yanlış pozitif sonuçları ortadan kaldırmak için büyük bit potansiyele sahiptir. Yani MPT64 proteini duyarlılığı arttırarak testin daha iyi sonuç vermesini sağlayabilir. Bu çalışmanın amacı MPT64 proteinini üretmek ve saflaştırmaktır. MPT64 protein elde etmek için tek bir kromatografik adım kullanımı amaçlanmıştır. Bu tez, *Escherichia coli* ER2566 bakterisinde intein etiketli pTXB1 ve pTYB21 plazmitlerine eklenen rekombinant MPT64 proteininin ekspresyonunun ayrı ayrı nasıl yapıldığını ve MPT64 protein saflaştırmasının tiyol reaktifleri sayesinde kendi kendine saflaştırma sağlayabilen bir kitin kolonu içeren IMPACT sistemini açıklar.

Anahtar Sözcükler: İntein Etiketi, MPT64 Proteini, *Mycobacterium tuberculosis*, Saflaştırılmış Protein Türevi, Tüberkülin Deri Testi

1. BACKGROUND AND AIM OF THE STUDY

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (1).This disease is one of the leading causes of death in the world according to World Health Organization (WHO). Disease is spread when healthy people are exposed to people with active tuberculosis disease (2). Usually, Mycobacterium tuberculosis infects lungs and affects host's immune system while creating granuloma which is detected by X-ray imaging (3). Percentage distribution of symptoms of TB is divided as 10% of the patients show active disease and 90% of the cases show no symptoms to detect tuberculosis disease (4). This dormant state of tuberculosis is called latent tuberculosis that is not risky for spreading disease but may develop in the future. Active and latent tuberculosis can be detected by Tuberculin Skin Test (TST) or blood test (5).

The year of 2020 has shown negative effect so far in so many things including tuberculosis as well due to COVID-19 pandemic. The estimated case number was 10 million in 2019. But 7.1 million people were diagnosed in 2019 and this proves people who has tuberculosis disease could not reach the healthcare they need. So, they cannot be diagnosed (2). Most of the cases of tuberculosis are detected in developing countries. This directly affects the burden of tuberculosis since the healthcare system cannot offer the proper diagnosis and treatment (6).

In order to overcome the undiagnosed patients and false positive or negative cases, more practical and inexpensive diagnosis techniques are in demand. TST is one of the practical and cheap techniques to detect active tuberculosis and latent tuberculosis. This technique is applied intradermally since 1912 as introduced by Charles Mantoux (7). Basically, TST detects the response against the injected reagent called purified protein derivative (PPD). This response is called delayed-type hypersensitivity (DTH) (8). It takes 48 to 72 hours to give result to the patients by measuring the size of the

inflammation on the forearm where injection is done (9). Size of the induration gives the information about patient. If measured induration is smaller than 5 mm, it is interpreted as negative result. Between 5 to 15 mm induration is accepted as positive result and may require additional diagnosis to make sure it is not false positive result (7).

Before PPD, Old Tuberculin (OT) was used by Robert Koch in early 1900s. OT was firstly obtained as protein mixture of tubercle bacillus. Later, a developed version of OT was produced and named as PPD. OT left its place to PPD for a better and standardized reagent. It is obtained by extracting and precipitating proteins from culture of *Mycobacterium tuberculosis* (9).

TST has shown changes throughout the time but the low specificity problem remained unsolved. While results are interpreted correctly with high sensitivity, the specificity is low due to Bacillus Calmette-Guérin (BCG) vaccinated people due to some antigens are in common both in PPD and BCG vaccine. Common antigens of both is resulted in giving positive result for vaccinated people (10).

BCG vaccine is an attenuated strain of *Mycobacterium bovis*. There are various vaccines with different qualities. The strain used for vaccine does not have region of difference 2 (RD-2) which makes it different from strain used for PPD production (11). This is the region that contains specific antigens such as MPT64 (24 kDa). This region also creates the difference by expressing these proteins in order to separate *Mycobacterium tuberculosis* from other mycobacteria (12).

MPT64 is one of the proteins that is found in PPD which is a mixture of proteins obtained from *Mycobacterium tuberculosis*. So, this protein has the potential to be

used alone in TST rather than using PPD, the protein mixture that can give false positive results due to BCG vaccination. Using an immunogenic protein MPT64 may give more sensitive results in TST (13).

In this study, cloning, expression and purification of MPT64 were aimed for MPT64 protein to be used in diagnosis of tuberculosis in the future. There were similar studies done for protein expressions of RD-2 protein. In those studies, they used blaF promoter to increase level of MPT64 expression (14) or using multiple chromatographic steps for purification (15) or preferring synthetic gene coding to overcome expression problem in *E. coli* (16). Unlike studies in the literature, this study offers a new way to produce MPT64. In order to do that, Intein Mediated Purification with and Affinity Chitin-binding Tag (IMPACT) system was used. IMPACT system has two vectors, pTXB1 that allows fusion of protein from C-terminus to intein tag and pTYB21 that allow fusion of protein from N-terminal to intein tag and also uses *E. coli* ER2566 as expression system. In this system, proteins can be purified in single chromatographic step by using intein tag.

2.INTRODUCTION

2.1.Tuberculosis Key Facts and WHO Strategy

Tuberculosis or shortly TB is a disease that easily spreads, and it is caused by *Mycobacterium tuberculosis.* Mostly, TB disease affects lungs rather than other parts of the body (4). Also, there is latent tuberculosis infection shortly LTBI. In the case where person has LTBI means that they already are infected by bacteria, but they show no symptoms specific to this disease and no clinical evidence. However, people with LTBI have potential to develop active tuberculosis later (17).

Every year, 10 million people get infected. According to WHO's 2019 report for tuberculosis, tuberculosis caused death of between 1,1 and 1,3 million HIV-negative people and 251000 HIV-positive people. Additional to HIV cases, drug resistance is a bigger problem for treatment of people with tuberculosis. Drug resistance results in inefficient usage of rifampicin which is one of the most effective drugs against tuberculosis (18).

WHO planned a new strategy for tuberculosis and named this project as The End TB Strategy. Strategy includes twenty years following 2015. There are two main targets for 2025 and 2035. Rate of reduction in death caused by tuberculosis is 75% and 95% respectively in 2025 and 2035. To achieve these, WHO follows key concepts such as patient-centered care, better and systematic disease screening in different high-risk groups. Also, working with government, increasing engagements with civil society organization for awareness and taking more action in research part for better and more rapid diagnosis and treatment techniques, making more investments for new technologies to adopt to use in research are included in their strategy statement (19).

2.2.Challenges in Tuberculosis Diagnosis

To minimize the risk of spreading tuberculosis, diagnostics has an important role in it. There is always need for better and easier diagnosis techniques. It is known that TB is a disease more frequently seen in poor countries. So, this brings more challenges with it since poor countries have less resources to provide better care for patients. There are many challenges that affect the fight against tuberculosis. These challenges can be explained in categories such as technical inadequacies, financial difficulties, multi-drug resistance and patients' life standards (20).

If these categories are examined, there are several parameters that make these conditions challenge. The number of places that serve as point-of-care or shortly POC. In addition to POC, the number of qualified personnel with enough equipment in health facilities are important and dependent on financial resources that governments and organizations can offer. Also, the techniques which can be performed in these health facilities is important because all diagnostic systems have different advantageous and limitations. So, this is a critical parameter that results in statistics obtained showing infected and healthy people at the end. Apart from technical and financial parameters, the opportunities that people have are crucial such as providing basic needs. The wealth plays an important role in this disease because it directly affects the life standards of people. Especially, poverty, bad habits like smoking and coinfection with HIV are important. Also, education is critical because infected people spread this disease without realizing it and people with no knowledge end up in using wrong drugs for treatment which makes the situation worse for patient themselves (21). Another problem is multidrug-resistance (MDR). Multidrug-resistance condition is detected by molecular diagnostic systems which some of them are easy to perform but all are expensive techniques. There still are undefined mechanisms trigger the multidrug resistance which sometimes cannot be detected. Unfortunately, not all resistance can be detected by current applications so far (22).

2.3.Diagnosis of Tuberculosis

Required strategy to fight against tuberculosis is to find more efficient, cheaper and rapid methods. Efficiency is crucial in order to reduce the morbidity rate caused by false negative results and unidentified tuberculosis patients. Cheaper methods are required because these kinds of methods can be used in places where TB burden is high due to poverty and low life standards leading people to have poor hygiene habits. Also, rapid methods are in demand because these methods are more applicable in POC (23).

There are several techniques to detect tuberculosis disease. Each technique has different advantageous and disadvantageous such as rapid but false positive results, time consuming but repeatable and accurate (24). They can be grouped as radiological, microbiological, molecular and immunological methods as shown in the Table 2.1:

Table 2.1. List of methods used for diagnosis of tuberculosis.

Radiological	Microbiological	Molecular	Immunological
Chest radiography	Sputum smear microscopy Culture	PCR Loop-mediated isothermal amplification Gene Xpert MTB/RIF Assay	Lipoarabinomannan urine strip test Interferon-γ release assay Tuberculin Skin Test

2.3.1. Radiological methods

X-rays are used to obtain an image of lungs inside in **chest radiography**. X-rays allow to visualize cavities in lungs that disease causes. The film from chest x-ray shows the overlapping images of tissue in order to identify the abnormalities (25).

2.3.2. Microbiological methods

Sputum smear microscopy is one of the widely used methods for the detection of tuberculosis disease. It is fast and simple method done by using unconcentrated sputum sample from patient (26). Common Gram stain is not applicable to mycobacteria which are called as acid-fast bacili due to its high lipid content. Instead of Gram staining, Ziehl-Neelsen staining is used (27).

Culture is the gold standard among many methods for diagnosis of tuberculosis. There are solid and liquid media available for culture. Generally, bronchoscopy, gastric aspiration, coughing sample or sputum samples are used (28) . In case of extra pulmonary tuberculosis, different specimens such as urine or biopsy samples are preferred (29).

2.3.3. Molecular methods

Nucleic Acid Amplification Tests (NAATs) have better results compared to other techniques due to some advantageous such as rapid results. **Polymerase Chain Reaction (PCR)** is one of the most common techniques among molecular methods (30). For PCR, it is possible to use extracted DNA from sputum sample. (31).

Loop-mediated isothermal amplification which is known as LAMP is a new fast test for diagnosis of tuberculosis. There is a commercial product using LAMP for tuberculosis developed by Eiken Chemical Company. This test is done by using sputum sample from patient. LAMP requires 4 primers to detect 6 regions of a target DNA. This process is completed under two hours and at a single temperature 65°C. Then the results are obtained and can be seen under UV light (32).

Gene Xpert MTB/RIF assay is another rapid molecular assay which is a nucleic acid amplification test that allows to detect even if there is a small quantity of genetic material in sputum sample (33). This assay offers rapid and automated system with its cartridge and simple usage which is its greatest advantage. But it is an expensive system which is a challenge for poor countries (34). Assay does not only detect tuberculosis disease but also detects rifampicin resistance. Drug resistance detection helps to identify multi-drug resistant TB. The sensitivity and specificity rates are known as 88% and 95% respectively for MTB/RIF (30).

2.3.4. Immunological methods

2.3.4.1. Lipoarobinomannan urine strip test

Common accepted tests are good enough to detect tuberculosis but not practical enough to perform in POC (35). So, more practical applications are on demand and actually WHO called for new methods to diagnose active TB patients faster and decrease the burden of tuberculosis (36).

Lipoglycan component called Lipoarabinomannan (LAM) is found in the cell wall of *Mycobacterium tuberculosis*. It can be detected in urine (37). People with

active TB might have LAM in their blood stream and this can be used to detect LAM in urine later (35).

There is a commercial product to detect LAM. It is a simple strip test called Determine TB LAM Antigen assay (firstly manufactured by Alere, currently manufactured by Abbott). This cheap test only required 60 µL of urine sample from patient and the result is obtained in 30 minutes (38). Easy-to-perform and results obtained in short time features are advantageous this test offers but the test is useful for heavily ill patients who are HIV-positive. The reported sensitivity and specificity of LAM test are 42% and 91%, respectively (39).

2.3.4.2.Interferon-gamma release assay

In addition to diagnosis of active TB, people with LTBI is also important to reduce the TB burden. Different approaches are much needed for better diagnosis techniques. Interferon-gamma release assay (IGRA) is one of them and it is an alternative to Tuberculin Skin Test (TST) (40). There are two types of IGRA which are T-SPOT.TB (manufactured by Oxford Immunotec) and QuantiFERON-TB Gold In-Tube (QFT-G IT) (manufactured by Cellestis Limited) (41).

In T-SPOT.TB which is an in vitro test, response from T cells is detected. The peptide mixture containing culture filtrate protein-10 (CFP-10) and early secretory antigenic target-6 (ESAT-6) was used. Peripheral blood mononuclear cells obtained from whole blood are used and it is the difference from other IGRA technique (42).

QuantiFERON-TB was approved by FDA in 2001. The test is again based on measuring patient’s immune response to *Mycobacterium tuberculosis* (43). Whole

blood sample is taken from patient and if patient is infected, white blood cells will release interferon-gamma due to being mixed with specific antigens. Purified protein derivative (PPD) was used for TST but later more specific antigens were chosen to rule out false positive results. The antigens were CFP-10 and ESAT-6. These antigens are encoded by *Mycobacterium tuberculosis.* Developed version of the test was also approved by FDA in 2004 and it is called QFT-TB Gold (QFT-G) (27). The latest version containing an additional antigen called TB7.7 and the test is applied by adding blood to the tube. Test is named as QFT Gold In-Tube (Approved in 2007 by FDA) (44).

2.3.4.3.Tuberculin skin test

Tuberculin Skin Test (TST) is one of the oldest methods that has been used to diagnose tuberculosis disease. The method was developed by Robert Koch in 1890. The research for finding a cure for TB was resulted in Koch's discovery on tuberculin as a diagnostic tool (45).

Tubercle bacilli was the key to obtain a mixture of substances called old tuberculin, OT which was later used by Charles Mantoux and the application of intradermal technique was adopted for diagnosis by using this mixture in 1912 (7). Proteins were extracted from culture of *M. tuberculosis* and eventually purified protein derivative (PPD) was developed. The test is done by applying PPD intradermally to the patient and delayed-type hypersensitivity was measured for interpretation of results (46). The reaction usually takes 48 to 72 hours to complete. At the end, a visible red, swollen mark forms on the forearm of patient and the diameter of induration is measured (47). The size of the induration is interpreted as strong positive if it is bigger than 10 mm and as intermediate if size varies between 5-9 mm and as negative if it is smaller than 5 mm (48). Results are highly affected by patients' current condition. Test gives positive results if patient has active or latent TB. Also, the case of positive result

happens when there are other diseases included such as infection by MOTT or cancer or HIV or the case of previous organ transplant operation etc. Another parameter is age of patient for reading the test result correctly. Addition to this list, Bacille Calmette-Guerin (BCG) vaccinated people get positive results. So, there are some cases that patients might get false positive results, and this affects the treatment process and TB burden overall since TST is widely used by many health facilities (49).

Sensitivity percentage for TST was reported as 77% and non-BCG vaccinated populations have a better chance to show high specificity such as 97% unlike BCG vaccinated populations (50). The specificity of TST varies between 46-73% since there are populations vaccinated but still has high TB burden (51).

2.4.MPT64 Proteins as New Antigens for TST

BCG vaccination has effects on TST results since it is reason some of the false positive results. The vaccination is one of the methods to prevent tuberculosis disease. In 1921, Bacillus Calmette-Guerin (BCG) vaccine was created in Pasteur Institute (52). BCG vaccine still is the only vaccine used against TB. The vaccination usually applied to infants after birth and children (53).

BCG vaccine is known with its protection percentage as 60-80%. Even though this offered protection, LTBI could be the case for the patient. TST has low level of true positive results because people who vaccinated before also give positive results. Unfortunately, test fails to differentiate true positivity caused by TB disease itself (54).

An isolated strain of *Mycobacterium bovis* from milk was the source to this vaccine developed in early 1900s. After vaccination got popular, substrains of BCG were started to emerge. Transferring this strain to different countries and passaging it continuously has led to obtain new substrains as a natural outcome (52).

The variation of substrains were characterized over the years. Further characterization of *M. tuberculosis* helped to identify many proteins that can be used for better and improved diagnostic methods. The BCG vaccine obtained from *M. bovis* and PPD that obtained from *M. tuberculosis* share many common proteins. These proteins cause false positive results. In order to minimize the risk of false positive results, PPD could be developed to be more specific to detect TB patients by TST. There are many proteins secreted by *M. tuberculosis* and some of these are not encoded by BCG vaccine substrains (55).

Identifying proteins of *M. tuberculosis* is important since some of these proteins are secreted by the organism and are the first things that cells interact in the early stage of infection as an immune response (56). Predominantly secreted proteins characterized from culture filtrate of *M. tuberculosis* are potential candidates for new treatments and diagnostics (57).

Some of the antigens found in TB cells are defined as protective antigens. MPT64 is one of these secreted antigens (58). During tuberculosis infection, MPT64 can weaken the immune response coming from macrophages by damaging unfolded proteins in host cell. So, these protective antigens are the weapons of TB to alter the pathways of host organism to defend itself (59).

The activity of MPT64 protein is dependent in type VII secretion system which regulates the secretion of virulence factors of *Mycobacterium tuberculosis* (60). In a

research done for screening proteins secreted by *Mycobacterium tuberculosis,* it was reported that full-length MPT64 and MPT64_24-113 successfully inhibited human growth hormones in HeLa cells. It was confirmed that full length or N-terminus protein of MPT64 hold potential to be used in various diagnostics and treatments (61).

Mycobacterium Protein Tuberculosis 64 (MPT64), (Rv1980c), a 24 kDa protein is encoded in *M. tuberculosis, M. africanum* and *M. bovis* (62). One of the most popular candidates against current PPD is MPT64 which is encoded by Region of Difference 2 (RD2). Many BCG substrains lost this region that encodes MPT64 over the years due to continuous passaging. So, this minor difference can eliminate the effect of BCG vaccine for false positive results of TST (12). T helper type 1 (Th1) cells in humans can recognize MPT64 antigens since MPT64 is secreted predominantly (62). Even though *M. bovis* has the gene for MPB64 (has the identical sequence and amino acids of MPT64), some BCG vaccine substrains do not have either gene or protein expression such as *M. bovis* BCG substrains Danish, Tice, Montreal, Glaxo and Pasteur (52).

There were many studies to clone and express MPT64 protein in order to characterize this protein or to utilize MPT64 for diagnostic purposes. A study for producing MPT64 to use it as an alternative to false positive results in TST was done by insertion of MPT64 into pRR3 plasmid and then transferred into *Mycobacterium smegmatis.* Since MPT64 expression level was low, blaF promoter were used to increase expression level and it was increased several times more (14). Another study to obtain strong immune response for TB mouse model was done by cloning MPT64 adjacent to ESAT6 by PGEM-T-easy cloning kit and expression was done and final product was obtained as insoluble protein (63). A study in 2018 was done to produce MPT64 as extracellular protein using pD861-SR plasmid in *E.coli* BL21 (DE3) offered a way to use rhaBAD promoter to increase expression level and pelB signal peptide to express the protein as extracellular protein (16). rhaBAD promoter that controls T7

promoter was also used to express MPT64 protein in *E.coli* BL21 (DE3) in order to obtain MPT64 as an antigen to differentiate TB infection from MOTT (64).

All these studies were about increasing expression levels of MPT64 proteins. In this thesis, we aimed to clone this protein into a vector including intein tag and chitin binding domain and to look for their expression levels in different conditions. For this this purpose Intein Mediated Purification with an Affinity Chitin-binding Tag (IMPACT) system (NEB, #E6901S) was used. This system has two plasmids, pTXB1 and pTYB21 which allow protein to be fused either from C or N terminus to the tag intein. Intein tag provides self-purifying protein to be obtain in a single affinity chromatography step. Chitin Binding Domain (CBD) which helps fusion protein to bind onto chitin resin and to remove other proteins from the target protein. during purification. Using two plasmids gives opportunity to clone MPT64 at its C- terminus in pTXB1 which creates MPT64+intein complex and N-terminus in pTYB21 which creates intein+MPT64 complex. According to this system, the self-cleavage of intein tag from the target protein can be done by introducing 1,4-dithiothreitol (DTT), a reducing reagent. Therefore, DTT is added into the cleavage buffer and let it break the disulfide bonds and prevent formation of new disulfide bonds between cysteine residues in proteins. As a result, DTT induces the peptide bond cleavage at its N-terminus for pTXB1 and C-terminus for pTYB21 (65).

3.MATERIALS AND METHODS

3.1.DNA Isolation from *M. tuberculosis* H37Ra

DNA of *Mycobacterium tuberculosis* grown in Löwenstein-Jensen media was used. Template DNA was obtained by using the boiling method. Briefly, a loopful bacteria was taken and put into 1 mL of buffer containing 10 mM Tris (Sigma Aldrich, #T1503-1KG), pH 8,0 and 1 mM EDTA (Bioshop, #EDT002). The tube was boiled at 100°C for 5 minutes then centrifuged at 15,000 *g* for 3 minutes. The supernatant was taken into a clean eppendorf tube and stored at -20°C (66).

3.2. Amplification of *mpt64* Gene

3.2.1. Primer design for mpt64 gene and pTXB1

Forward and reverse primers were designed by using software called SnapGene. By using SnapGene program, *mpt64* gene (NCBI Reference Sequence: NC_000962.3) from *Mycobacterium tuberculosis* H37Ra sequence was selected. Length of *Mycobacterium tuberculosis* H37Ra is 4419977 bp and length of *mpt64* gene is 687 bp.

On SnapGene software, required restriction enzyme site was added to the selected binding site. Also, a random sequence (GGTGGT) was added to create an efficient DNA cleavage. Nde1 restriction site was added to N-terminus of *mpt64* in order to create 34-mer forward primer with 50% GC content (Figure 3.1) and Sap1 restriction

site was added to C-terminus of *mpt64* (Figure 3.2) in order to create 36-mer reverse primer with 61% GC content.

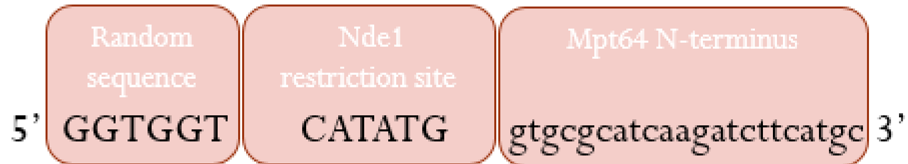

Figure 3.1. Forward primer designed for *mpt64* gene amplification and cloning into pTXB1.

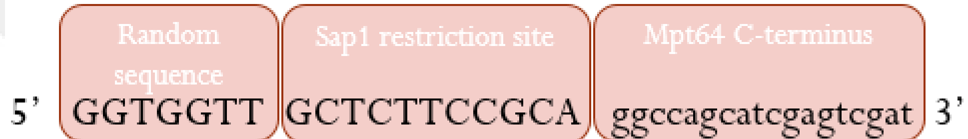

Figure 3.2. Reverse primer designed for *mpt64* gene amplification and cloning into pTXB1.

While N-terminus of target sequence was remained identical to the original as seen in the Figure 3.3, stop codon from C-terminus of the target sequence was excluded for the intein tag of pTXB1 (Figure 3.4).

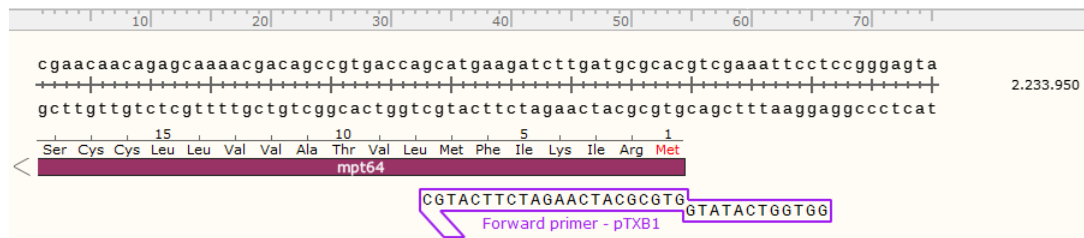

Figure 3.3. View of forward primer binding to N-terminus of mpt64 gene in SnapGene software.

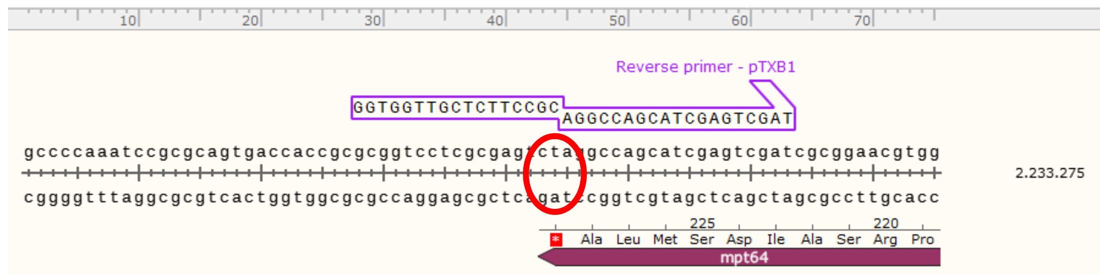

Figure 3.4. View of reverse primer binding to C-terminus of mpt64 gene with stop codon excluded in SnapGene software

3.2.2. Primer design for mpt64 gene and pTYB21

On SnapGene software, required restriction enzyme site was added to the selected binding site. Sap1 restriction site was added to N-terminus of *mpt64* in order to create 37-mer forward primer with 51% GC content (Figure 3.5) and Pst1 restriction site was added to C-terminus of *mpt64* (Figure 3.6) in order to create 36-mer reverse primer with 58% GC content.

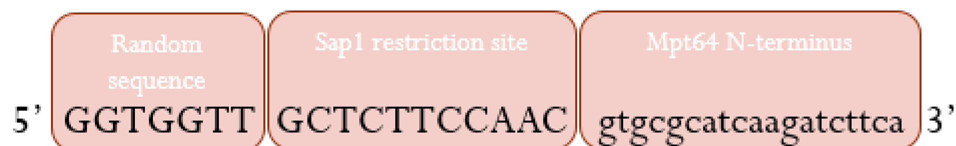

Figure 3.5. Forward primer designed for mpt64 gene amplification and cloning into pTYB21.

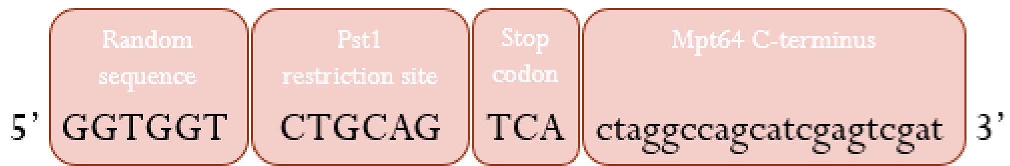

Figure 3.6. Reverse primer designed for mpt64 gene amplification and cloning into pTYB21.

While N-terminus of target sequence was remained identical to original as seen in the Figure 3.7, additional stop codon was included for the sake of expression of intein tag from pTYB21 (Figure 3.8).

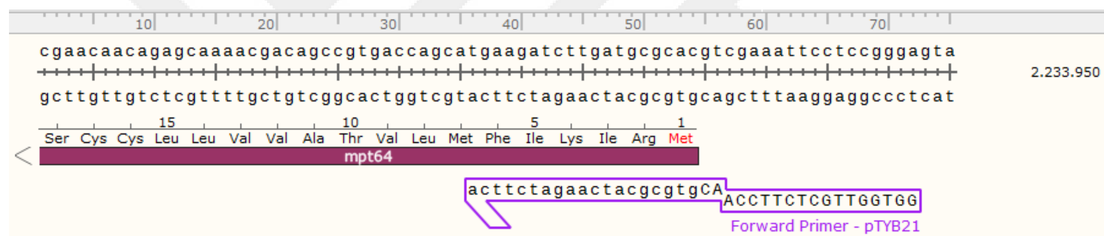

Figure 3.7. View of forward primer binding to N-terminus of mpt64 gene in SnapGene software.

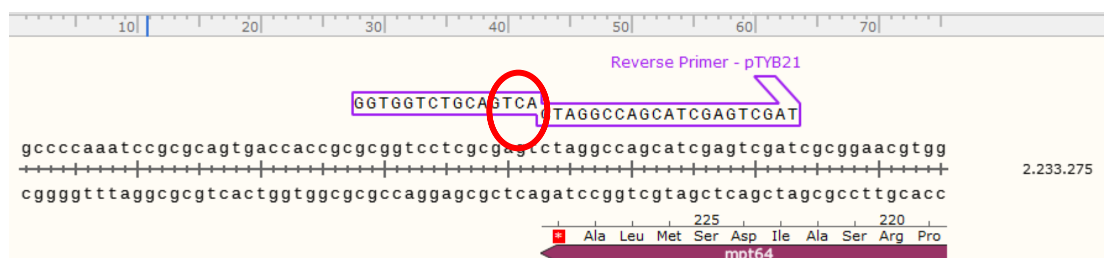

Figure 3.8. View of reverse primer binding to C-terminus of mpt64 gene with additional stop codon in SnapGene software.

3.2.3. Amplification of mpt64 gene

Amplification of *mpt64* gene was performed with two sets of primers at different temperatures by polymerase chain reaction (PCR). PCR was done by using GeneMark Master Mix II (5X) (#RP02-II-400). For negative control sample, 1 μL of dH_2O was added to PCR tube instead of the DNA template. Annealing temperatures of primers designed for pTXB1 were 60°C and 72°C while annealing temperatures of primers designed for pTYB21 were 62°C and 75°C. List of ingredients and volumes and PCR conditions were shown in the Table 3.1, 3.2 and Table 3.3:

Table 3.1. The list of reagents used in amplification of MPT64 coding DNA sequences

Reagent	Volume (μL)
dH_2O	17
Forward primer	1
Reverse primer	1
5X Master Mix	5
Template DNA of H37Ra	1
Total volume	25 μL/reaction

Table 3.2. The table of PCR conditions for amplification of *mpt64* in pTXB1.

Condition		Temperature (°C)	Time (min)	Cycle
Initial Denaturation		94	5	1
Denaturation		94	1	
Annealing	w/primers designed for pTXB1	60 & 72	1	30
Extension		72	1	
Final Extension		72	5	1

Table 3.3. The table of PCR conditions for amplification of *mpt64* in pTYB21.

Condition		Temperature (°C)	Time (min)	Cycle
Initial Denaturation		94	5	1
Denaturation		94	1	
Annealing	w/primers designed for pTYB21	62 & 75	1	30
Extension		72	1	
Final Extension		72	5	1

3.3. Cloning of Target Gene in *E. coli* ER2566

3.3.1. Digestion of vector and insert with restriction enzymes

3.3.1.1. Digestion of pTXB1 and *mpt64*

pTXB1 plasmid was purified by using Genemark Plasmid Miniprep Purification Kit, (#DP01) and the concentration of DNA was measured by Nanodrop 2000c Spectrophometer (Thermo Scientific).

Amplicons were purified by using Genemark Plus PCR Clean Up Kit, (#DP04P) and the concentration of DNA was measured by Nanodrop 2000c Spectrophometer.

The restriction enzymes, Nde1 (NEB, #R0111S) and Sap1 (NEB, #0169S) were used. The condition of reaction was set as 37°C for 1 hour then 65°C for 20 minutes for heat inactivation. Table 3.4 was followed for double digestion:

Table 3.4. The table of digestion reaction protocol for pTXB1 and *mpt64*.

Ingredient	Cut pTXB1	Uncut pTXB1	Cut mpt64	Uncut mpt64
dH_2O	8 µL	12,5 µL	8 µL	12,5 µL
DNA	35 µL	10 µL	35 µL	10 µL
10X Buffer	5 µL	2,5 µL	5 µL	2,5 µL
Nde1	1 µL	-	1 µL	-
Sap1	1 µL	-	1 µL	-
Total reaction volume	50 µL	25 µL	50 µL	25 µL

3.3.1.2.Digestion of pTYB21 and *mpt64*

pTXB1 plasmid was purified by using Genemark Plasmid Miniprep Purification Kit, (#DP01) and the concentration of DNA was measured by nanodrop. pTYB21 was digested by Pst1-HF (NEB, #R3140S).

The condition of reaction was set as 37°C for 1 hour then 65°C for 20 minutes for heat inactivation. Table 3.5 was followed for double digestion:

Table 3.5. The table of digestion reaction protocol for pTYB21 by Pst1.

Ingredient	Cut pTYB21	Uncut pTYB21
dH_2O	19 µL	20 µL
DNA	25 µL	25 µL
10X Buffer	5 µL	5 µL
Pst1	1 µL	-
Total reaction volume	50 µL	50 µL

After digestion with Pst1-HF, another reaction was prepared for digestion with Sap1. The concentration of DNA was measured by Nanodrop 2000C.

The condition of reaction was set as 37°C for 1 hour then 65°C for 20 minutes for heat inactivation. Table 3.6 was followed for digestion:

Table 3.6. The table of digestion reaction protocol for pTYB21 by Sap1.

Ingredient	Cut pTYB21	Uncut pTYB21
dH_2O	-	1 µL
DNA	21,5 µL	21,5 µL
10X Buffer	2,5 µL	2,5 µL
Sap1	1 µL	-
Total reaction volume	25 µL	25 µL

Amplicons were purified by using Genemark Plus PCR Clean Up Kit, (#DP04P) and the concentration of DNA was measured by nanodrop.

The condition of reaction was set as 37°C for 1 hour then 65°C for 20 minutes for heat inactivation. Table 3.7 was followed for double digestion:

Table 3.7. The table of digestion reaction protocol for *mpt64.*

Ingredient	Cut mpt64	Uncut mpt64
dH_2O	8 µL	12,5 µL
DNA	35 µL	10 µL
10X Buffer	5 µL	2,5 µL
Pst1	1 µL	-
Sap1	1 µL	-
Total reaction volume	50 µL	25 µL

3.3.2. Ligation with pTXB1 and pTYB21

For ligation of digested mpt64 and digested pTXB1, T4 DNA ligase (Takara, #2011A) was used. The condition of reaction was set as 16°C for one hour and 65°C for 10 minutes for heat inactivation. The ratio of the vector and insert was adjusted as 1:3, 1:5 and 1:10. Table 3.8 was followed for ligation:

Table 3.8. The table of ligation reaction protocol for pTXB1 and *mpt64*.

	Vector Insert Ratio (V:I)			Control Reactions		
Ingredient	**1:3**	**1:5**	**1:10**	**Cut vector**	**Cut vector + ligase**	**Insert + ligase**
dH_2O	33 µL	32,1 µL	30,8 µL	35 µL	34 µL	42 µL
pTXB1	10 µL	10 µL	10 µL	10 µL	10 µL	-
mpt64	1 µL	1,9 µL	3,2 µL	-	-	2 µL
5X Buffer	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL
Ligase	1 µL	1 µL	1 µL	-	1 µL	1 µL
Total rxn vol	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

For ligation of digested mpt64 and digested pTYB21, T4 DNA ligase (Genemark, #GDL001LS(1000U)) was used. The condition of reaction was set at 16°C for overnight and 65 °C for 10 minutes for heat inactivation. The vector:insert ratio was adjusted as 1:3, 1:5 and 1:10. Table 3.9 was followed for ligation:

Table 3.9. The table of ligation reaction protocol for pTYB21 and *mpt64*.

	Vector Insert Ratio (V:I)			Control Reactions		
Ingredient	**1:3**	**1:5**	**1:10**	**Cut vector**	**Cut vector + ligase**	**Insert + ligase**
dH_2O	4 µL	3,5 µL	1,9 µL	6 µL	5 µL	10 µL
pTXB1	10 µL	10 µL	10 µL	10 µL	10 µL	-
mpt64	1 µL	1,5 µL	3,1 µL	-	-	5 µL
4X Buffer	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL
Ligase	1 µL	1 µL	1 µL	-	1 µL	1 µL
Total rxn vol	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL

3.3.3. Preparation of competent *E. coli* ER2566

3 mL of *E. coli* ER2566 was grown at 37°C overnight. Then, 500 µL from culture was transferred into 50 mL of LB Broth in 250 mL Erlenmeyer flask. 50 mL culture was mixed in shaker at 37°C, 200 rpm. When OD value reached between 0,30-0,45 at 600 nm, the culture was transferred into 50 mL falcon tube and centrifuged at 10,000 *g* for 10 minutes. Supernatant was discarded. 10 mL of ice cold $CaCl_2$ solution (0,06 M $CaCl_2$ (MP Biomedicals, #195088) 0,01 M PIPES (Sigma Aldrich, #P6757), pH 7, 15% Glycerol (Sigma Aldrich, #15524-1L-R)) was added and pellet was washed. Tube was kept on ice for 30 minutes. Washed pellets were centrifuged at 5,000 *g*, +4°C for10 minutes. The supernatant was discarded. 2 mL of $CaCl_2$ solution was added and pellet was resuspended. Competent cells were obtained and portioned as 100 µL/tube then tubes were stored at -80°C.

3.3.4. Transformation into competent *E. Coli* ER2566

Competent *E.coli* ER2566 bacteria and products from ligation process put on ice. 100 µL of competent bacteria and 5 µL of each ligation product that had V:I ratio of 1:3, 1:5 and 1:10 were mixed separately. Tubes were incubated on ice for 30 minutes. Then, tubes were incubated at 42°C for 90 seconds on heat block. After that tubes were immediately put on ice for heat shock. 900 µL of LB media (Sigma Aldrich, #L7658-1KG) was added and tubes were incubated at 37°C for 45 minutes. At the end of incubation, tubes were centrifuged at 13,000 RPM for 30 seconds. 900 µL of supernatant was discarded and leftover supernatant was used to resuspend the pellet and then spread onto LB agar (Sigma Aldrich, #L7533) plates containing 100 µg/mL ampicillin (Sigma Aldrich, #A9518). The plates incubated at 37°C overnight. Colony numbers were counted the following day.

3.3.5. Colony PCR

The colonies obtained on LB agar plates were used for colony PCR. Carefully selected colonies were used as template in PCR and also, they were cultured in liquid media simultaneously. The amplification protocol was used in this step. The PCR conditions for *E.coli* ER2566 containing pTXB1 with mpt64 was given in Table 3.10 and The PCR conditions for *E.coli* ER2566 containing pTYB21 with mpt64 was given in Table 3.11:

Table 3.10. The table of colony PCR conditions.

Condition		Temperature (°C)	Time (min)	Cycle
Initial Denaturation		94	5	1
Denaturation		94	1	
Annealing	w/primers designed for pTXB1	60	1	30
Extension		72	1	
Final Extension		72	5	1

Table 3.11. The table of colony PCR conditions.

Condition		Temperature (°C)	Time (min)	Cycle
Initial Denaturation		94	5	1
Denaturation		94	1	
Annealing	w/primers designed for pTYB21	62	1	30
Extension		72	1	
Final Extension		72	5	1

3.3.6. Confirmation by sequencing

In order to confirm the cloning, Sanger sequencing was done. Randomly selected Colony 5 that contain mpt64 insert with pTXB1 in *E. coli* ER2566 were sequenced by Eurofins Genomics Company. T7 universal primer (5' TAATACGACTCACTATAGGG 3') was used.

For sequencing the Colony 13 that contain mpt64 insert with pTYB21 in *E. coli* ER2566, new primer was designed which were (5' AGGAAGACGATTATTATGGG 3') as forward primer.

3.4.Expression of MPT64 Protein

3.4.1. Induction of protein expression

Bacteria were inoculated into 15 mL LB broth and incubated at 37°C 200 rpm and left for overnight. After incubation, 5 mL of starter culture was added to 500 mL LB broth media containing 100 µg/mL ampicillin. The culture was incubated at 37°C, 200 rpm and OD600 value was checked until reaching 0,5. When OD600 value reached 0,5, 200 µL IPTG was added to 500 mL culture in order to express protein in 0.4 mM IPTG presence. 500 mL culture was incubated at 37°C, 200 rpm for 4 hours. At the end of incubation, the culture was spun down at 5,000 *g* for 15 minutes at 4°C. Supernatant was discarded. Pellet was weighed then stored at -80°C.

3.4.2. Media optimization for protein expression by pTYB21

Media optimization for expression was done with different media such as Mueller-Hinton Broth (Merck, #70192-500G), Tryptic Soy Broth (Merck, #22092), Brucella Broth (Merck, #B3051-500G), Brain-Heart Infusion Broth (Sigma Aldrich, #53286-500G), LB Broth (Sigma Aldrich, #L7658-1KG) and LB Broth (Merck, #110285) ((all containing 100 µg/mL ampicillin). When OD600 value of culture was reached 0.5, 0.4 mM IPTG was added and culture was incubated at 200 rpm and 37°C for 4 hours. After the induction of protein expression, cell pellet obtained by centrifugation at 5000 G for 15 minutes at 4°C and then was resuspended in column buffer (20 mM Tris-HCl pH 8.5, 500 mM NaCl (Sigma Aldrich, #13423), 0.5% Triton X-100 (Sigma Aldrich, #X100-500ML)). Resuspended pellet homogenized for 5 minutes with 30 seconds on and 30 seconds off on ice by sonication. Homogenized cell pellet was spun down for 45 minutes at 10,000 *g* and 4°C. Supernatant and pellet were used in further analysis.

3.4.3. Handling inclusion body

In this step, the protocol from IMPACT kit was followed. According to the protocol, the buffers for this process were prepared (65). Pellet obtained from culture was resuspended with 20 mL of column buffer containing (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). Resuspended cell pellet was homogenized on ice for 5 minutes (30 sec on and 30 sec off) at 20% power with 40% pulse. Homogenized cell lysate was centrifuged at 10,000 *g* for 45 minutes at 4°C. Supernatant and pellet were separated and taken into clean tube to work with later.

Pellet was used after this step. 20 mL of lysis buffer containing (20 mM Tris-HCl, 500 mM NaCl, 7 M Guanidine-HCl (Gold Biotechnology, #G-211)) as recipe given Table 3.12 was used to dissolve pellet. After dissolving pellet by pipetting, the mixture was left to mix at 4°C for 1 hour. At the end of 1 hour, mixture was centrifuged at 10,000 *g* for 45 minutes at 4°C and 19 mL of supernatant was taken into a clean tube.

Dialysis for supernatant containing guanidine-HCl was done in presence of urea (Merck, #57-13-6). Buffer A, B, C, D and E were used, and the buffer ingredients were shown in Table 3.13, 3.14, 3.15, 3.16, 3.17 below. Supernatant was dialyzed against 200 mL of buffer A, B, C, D once and E twice. The product obtained after dialysis was ready to use in purification step.

Table 3.12. Recipe of lysis buffer.

Lysis Buffer (100 mL for resuspending pellet obtained from 1 L culture)			
Chemical	Stock	Final	Volume Required
Tris HCl pH 8,5	1 M	20 mM	2 mL
NaCl	5 M	0,5 M	10 mL
Guanidine HCl	8 M	7 M	87,5 mL
		Total volume:	99,5 mL + 0,5 mL dH_2O

Table 3.13. Recipe of renaturation buffer A.

Renaturation Buffer A (1 L for dialysis of pellet resuspended in 100 mL lysis buffer)			
Chemical	Stock	Final	Volume Required
Tris HCl pH 8,5	1 M	20 mM	20 mL
NaCl	5 M	0,5 M	100 mL
Urea	10 M	8 M	800 mL
		Total volume:	920 mL + 80 mL dH_2O

Table 3.14. Recipe of renaturation buffer B.

Renaturation Buffer B (1 L for dialysis of pellet resuspended in 100 mL lysis buffer)			
Chemical	Stock	Final	Volume Required
Tris HCl pH 8,5	1 M	20 mM	20 mL
NaCl	5 M	0,5 M	100 mL
Urea	10 M	6 M	600 mL
		Total volume:	720 mL + 280 mL dH_2O

Table 3.15. Recipe of renaturation buffer C.

Renaturation Buffer C (1 L for dialysis of pellet resuspended in 100 mL lysis buffer)			
Chemical	Stock	Final	Volume Required
Tris HCl pH 8,5	1 M	20 mM	20 mL
NaCl	5 M	0,5 M	100 mL
Urea	10 M	4 M	400 mL
		Total volume:	520 mL + 480 mL dH_2O

Table 3.16. Recipe of renaturation buffer D.

Renaturation Buffer D (1 L for dialysis of pellet resuspended in 100 mL lysis buffer)			
Chemical	Stock	Final	Volume Required
Tris HCl pH 8,5	1 M	20 mM	20 mL
NaCl	5 M	0,5 M	100 mL
Urea	10 M	2M	200 mL
Oxidized glutathione (Gold Biotechnology, #G-060-1)	1 mM	0,1 mM	100 mL
Reduced glutathione (Gold Biotechnology, #G-275-25)	100 mM	1 mM	10 mL
		Total volume:	430 mL + 570 mL dH_2O

Table 3.17. Recipe of renaturation buffer E.

Renaturation Buffer E* (1 L for dialysis of pellet resuspended in 100 mL lysis buffer)			
Chemical	Stock	Final	Volume Required
Tris HCl pH 8,5	1 M	20 mM	20 mL
NaCl	5 M	0,5 M	100 mL
Oxidized glutathione	1 mM	0,1 mM	100 mL
Reduced glutathione	100 mM	1 mM	10 mL
		Total volume:	230 mL + 770 mL dH_2O

*:Renaturation buffer E is used two times in the last step of dialysis. So, the volume should be doubled.

3.5. Purification by Affinity Chromatography

Firstly, 4 mL of chitin resin was poured into column to sink in. Then, column was washed with 40 mL column buffer. 27 mL of dialyzed protein mixture was poured into column at rate of 1 mL/min. Sample from flow through was taken. After this, column was washed with 80 mL of column buffer at rate of 2 mL/min. Sample from washed column was collected. 12 mL of cleavage buffer containing DTT (20 mM Tris-HCl, 500 mM NaCl, 50 mM DTT) was prepared just before cleavage step. Cleavage buffer was quickly flushed and then column was incubated at room temperature for 40 hours. At the end of 40 hours incubation, elution was done. Purified protein was collected in 1 mL fractions. Column was again washed with 13 mL of column buffer and elution was completed. Chitin column was regenerated by washing with 12 mL of stripping solution (0.3 M NaOH). Sample was taken labelled as stripped w/NaOH.

3.6. Analysis of Protein Expression and Purification

3.6.1. SDS-PAGE analysis

Resolving gel (12%) with stacking gel (4%) was prepared. Gel ingredients were mixed as given recipe in Table 3.18 and poured between 0,75 mm PAGE glasses.

Table 3.18. Ingredients and volumes for two SDS gels.

	Ingredient	Volume for two gels (µL)
Stacking Gel	30% Acrylamide/bisacrylamide	650
	1 M Tris-HCl, pH 6,8	1250
	20% SDS	25
	Distilled water	3075
	Ammonnium persulfate	50
	TEMED	10
Resolving Gel	30% Acrylamide/bisacrylamide	4000
	1,5 M Tris-HCl, pH 8,8	2500
	50% Glycerol	750
	Distilled water	2750
	Ammonnium persulfate	100
	TEMED	10

Samples were mixed with 3X loading buffer (NEB, #B7703) then loaded into gel. Gel was run at 110V for 90 minutes. After that gel was stained with Coomassie blue staining solution overnight. Next day, gel was washed with destaining buffer (5:4:1 dH_2O:Methanol:Acetic acid) for 30 minutes. Washing step was repeated 3 times. The gel image was obtained by ChemiDoc.

In addition to Coomassie staining, silver staining was done for only MPT64 expression in *E. coli* ER2566 containing pTXB1 with *mpt64* by using Silver Stain Plus kit (Bio-Rad, #161-0449).

3.6.2. Lyophilization

All fractions from elution step of purification were collected into clean 50 mL tube. Tube was placed in FreeZone -84C benchtop freeze dryer (Labconco) and left overnight for drying. Powder form was weighed to assess efficiency of end product.

3.6.3. Capilla TB-Neo strip test

100 µL of selected fraction obtained after elution of MPT64 purification process was placed onto Capilla TB-Neo strip test (Tauns Laboratories, #CATB0870). Test was read after 15 minutes for positivity.

3.6.4. Western blotting

For Western blotting, as mentioned in SDS-PAGE analysis, same gel percentage was used as given in Table. Each sample was loaded into gel at same volume after mixing with 3X loading buffer.

Gel was run at 110 V for 90 minutes. At the end of running, gel was put into 1X transfer buffer (Biorad, #1610734). PVDF membrane (Biorad, #170-4156) was kept in methanol for 2 minutes. Then, membrane was put into 1 X transfer buffer for 10 minutes as wet transfer sandwich was prepared as shown in Figure.

The cassette holding sandwich was placed into tank which was filled with ice-cold 1X transfer buffer. Magnetic stirrer and cooling unit were also placed into tank. The transfer was done in cold room +4°C for 2 hours at 50V.

At the end of transferring process. The membrane was checked if bands were transferred successfully or not. Then, membrane was incubated in 25 mL of 5% non-fat milk (Biorad, #170-6404) at room temperature for 1 hour on shaker.

During incubation, primary antibody was diluted in 5% non-fat milk, anti-CBD monoclonal antibody (NEB, #E8034) ratio was adjusted as 1:1000 and kept at +4°C (For long term -20°C). At the end of incubation, blocking milk was discarded and primary antibody solution was poured onto membrane. Membrane was incubated at +4°C overnight on shaker.

Next day, primary antibody was collected, and membrane was washed with 1X Tris-buffered saline tween 20 (1X TBS-T), (1M Tris-HCl pH 7,4, 5 M NaCl and 1% Tween-20 (Sigma Aldrich, #5727) for 5 minutes. This step repeated 3 times. Then, anti-mouse secondary antibody (Cell Signaling, #7076S) diluted beforehand with 5% non-fat milk (1:5000). Secondary antibody solution was poured onto membrane and membrane incubated at room temperature for 1 hour. At the end of incubation, membrane was washed with 1X TBST for 30 minutes. This step was repeated 3 times.

For visualization, ECL (Thermo Scientific, #32132) reagent was used. Reagent was carefully placed onto membrane and kept in dark 5 minutes then visualized by ChemiDoc.

4.RESULTS

4.1.Cloning and Expression of MPT64 Protein inserted into pTXB1 in *E. coli* ER2566

4.1.1. DNA Isolation from *M. tuberculosis* H37Ra

Concentration of DNA extracted from *Mycobacterium tuberculosis* H37Ra was measured as 17,1 ng/µL by NanoDrop 2000C.

4.1.2. Transformation of *mpt64* Gene within pTXB1 into *E. coli* ER2566

4.1.2.1.Amplification of *mpt64* gene for pTXB1 vector

Concentration of *mpt64* PCR product used for pTXB1 was measured as 20 ng/ ng/µL by NanoDrop 2000C. *mpt64* PCR product was obtained as 714 bp at 60°C annealing temperature and optimization of PCR for *mpt64* with primers for cloning into pTXB1 was shown in Figure 4.1. In the figure, desired PCR product shown in lane 1.

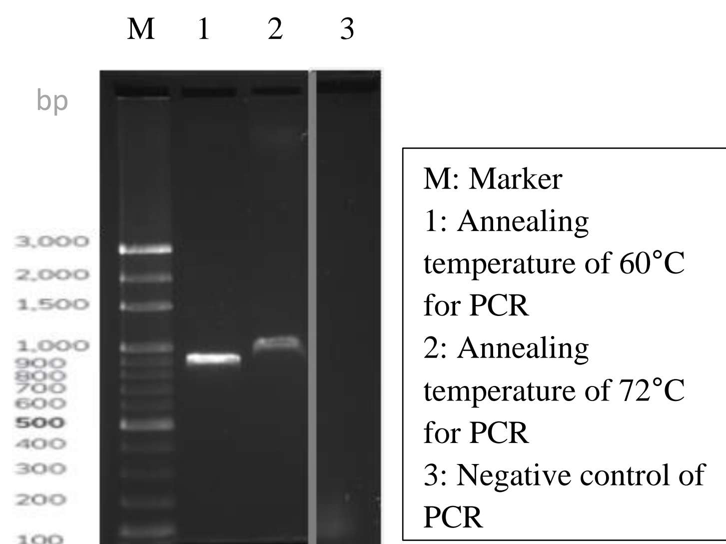

Figure 4.1. 1,2% agarose gel image of PCR product of *mpt64* for pTXB1.

4.1.2.2.Digestion of vector and insert with restriction enzymes

In order to clone *mpt64* into pTXB1, amplified *mpt64* and pTXB1 were digested by Sap1 and Nde1. Digested fragments of *mpt64* (689 bp) shown as 1 and pTXB1 (6664 bp) shown as 3 were run in gel electrophoresis to confirm digestion as shown in Figure 4.2.

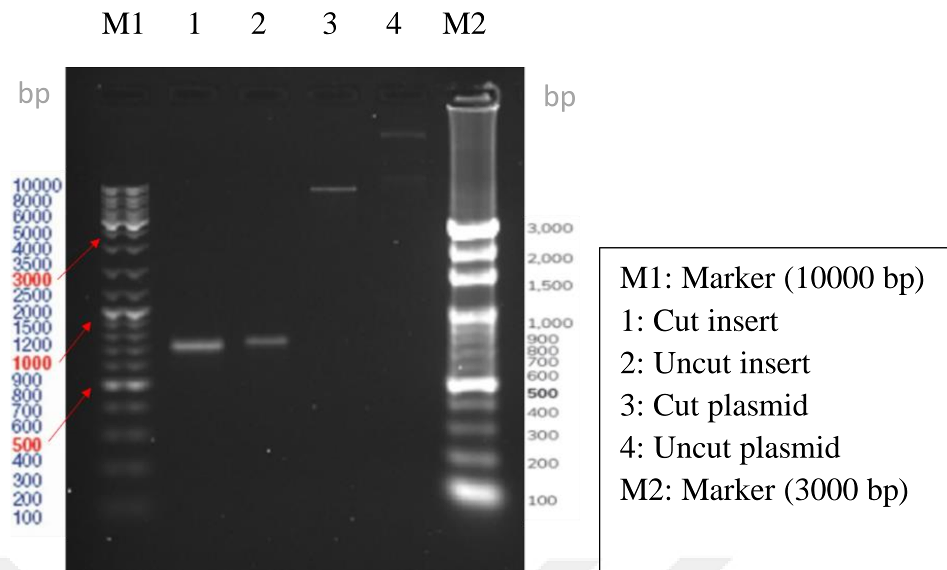

Figure 4.2. 1,2% agarose gel image of digested *mpt64* and pTXB1.

After digestion process, digested *mpt64* as insert were used in ligation with pTXB1 vector. The concentration of inserts and vectors were measured by NanoDrop 2000C and the concentrations accepted for ligation step were given in Table 4.1 below

Table 4.1 DNA concentrations of digested *mpt64* and pTXB1 plasmid for ligation.

Ligation of *mpt64* and pTXB1	
Digested *mpt64*	Digested pTXB1
6 ng/µL	3,4 ng/µL

4.1.2.3.Transformation into competent *E. coli* ER2566

At the end of ligation step, transformation was done in order to obtain colonies on LB agar plates. Then, the plasmids containing the desired target gene was used for transformation into *E.coli* ER2566.

Also, different V:I ratio proved that using higher concentrations of insert increased number of colonies obtained. With 1 hour of ligation process for *mpt64* and pTXB1, increasing V:I ratio resulted in a few more colonies than previous ratio as seen in Figure 4.3.

Figure 4.3. Pictures of agar plates after transformation of pTXB1 with insert into *E.coli* ER2566 at different V:I ratios.

4.1.2.4.Colony PCR

Carefully selected single colonies from agar plates were used for colony PCR as template DNA and then, PCR products were run in gel electrophoresis to confirm the products that contain the target gene fragments. Colonies of that contain *mpt64* and pTXB1 (7342 bp) showed in Figure 4.4.

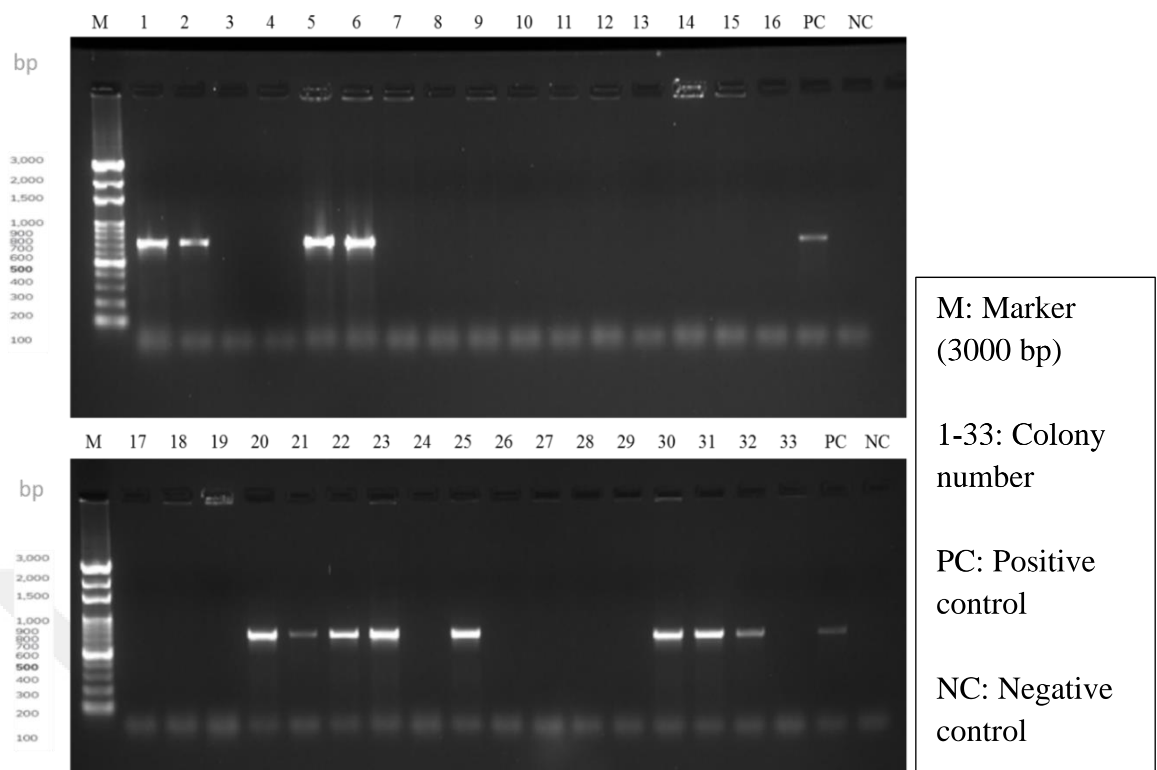

Figure 4.4. Image of 1,2% agarose gel electrophoresis after colony PCR for insert with pTXB1.

Successfully cloned colonies are 1, 2, 5, 6, 20, 21, 22, 23, 25, 30, 31 and 32nd colonies for *mpt64* within pTXB1 (12 out of 33 colonies). The calculated percentage for this is 36,36%.

4.1.2.5.Confirmation by sequencing

The confirmation of successful cloning was done. Randomly selected colonies were sent to Sanger sequencing. Raw data of Sanger sequencing for colony given in Appendix 1.

The ending and beginning of *mpt64* sequence for pTXB1 were confirmed in sequencing analysis. In Figure 4.5, 61st base is the beginning of *mpt64* was with a red arrow and 714th base is the last base of *mpt64*. The next base (715th base) where intein tag starts in the sequence was shown with a blue arrow.

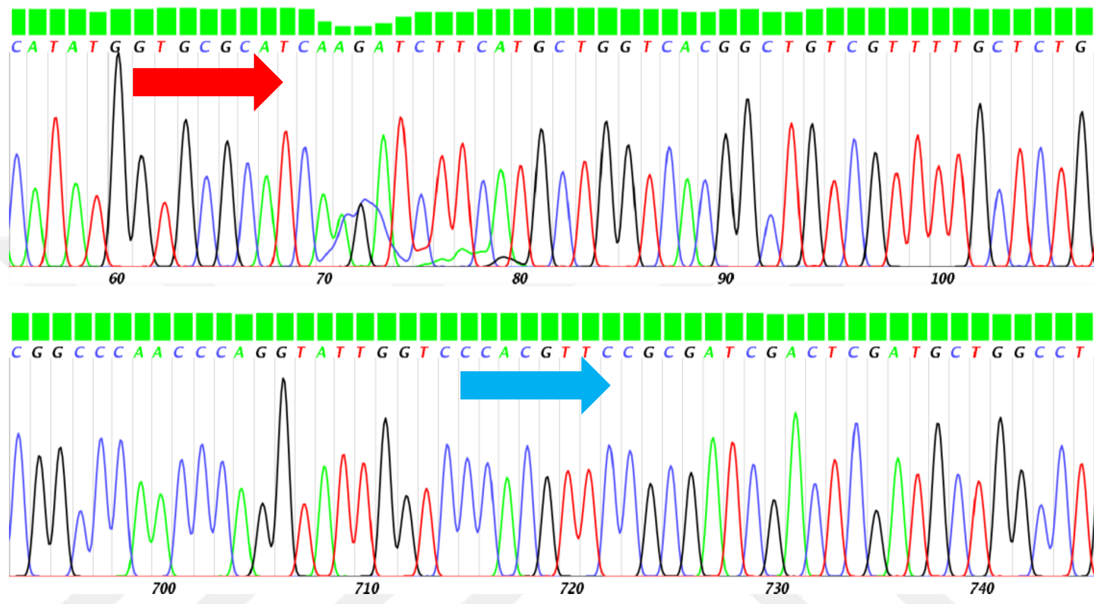

Figure 4.5. Image of sequence analysis by T7 universal primer shows beginning and end of *mpt64* inserted into pTXB1.

4.1.3. Analysis of Protein Expression

4.1.3.1. Induction of MPT64 protein expression

E.coli ER2566, *E.coli* ER2566 containing pTXB1 which has only intein tag and *E.coli* ER2566 containing pTXB1 with *mpt64* were grown in LB Broth and protein induction was done as mentioned in section 3.4.1. After resuspension of cell pellet, mixture was centrifuged. Supernatant which is the soluble protein fraction shown in gels with S and pellet which is the inclusion body fraction shown in gels with P were

separated. In order to compare addition of IPTG to the expression, uninduced culture was grown simultaneously. Uninduced pellet and supernatant were shown under -IPTG and induced culture samples were shown under 0.8 mM in Figure 4.6. SDS-PAGE gel was run and then stained with Coomassie blue as shown in Figure 4.6.

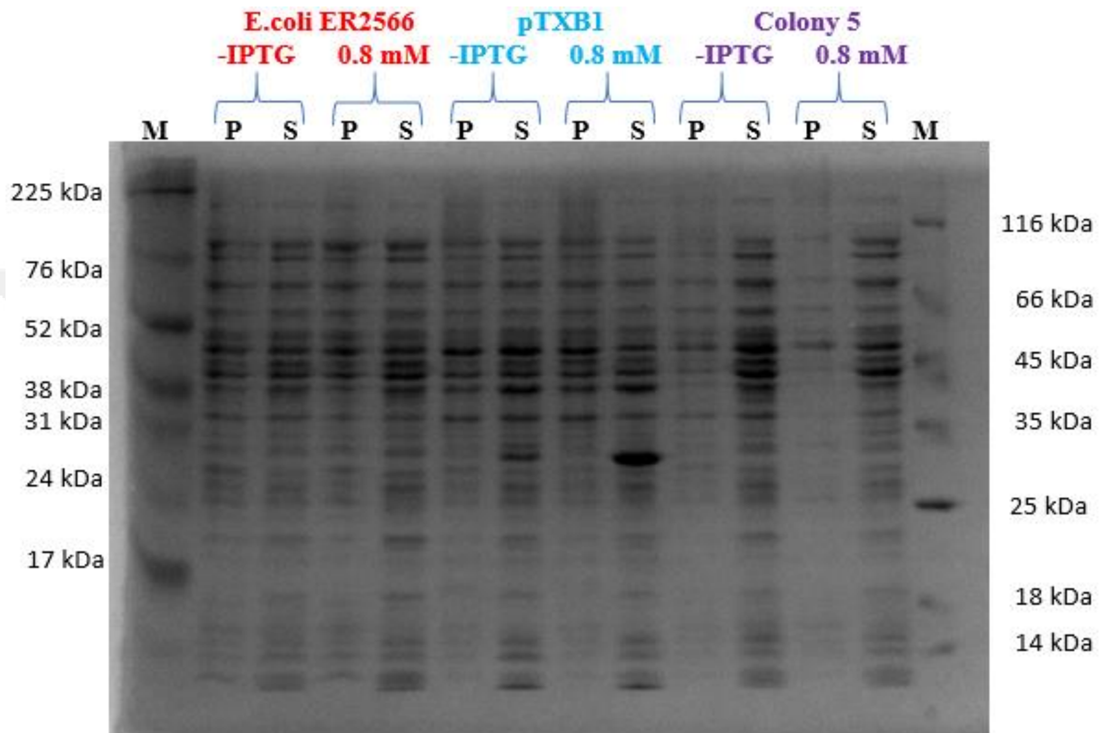

Figure 4.6. Image of SDS-PAGE gel stained with Coomassie blue.

Desired band for MPT64 fused with intein tag was not obtained even though intein was visible when expressed by *E.coli* ER2566 containing only pTXB1 as seen in lane for supernatant of pTXB1 in Figure 6. This gel image shows that the expression level was low and could not be detected by SDS-PAGE. The intein tag (28 kDa) became easily visible in soluble part when expressed by *E. coli* ER2566 and stained with Coomassie blue stain. But expression of MPT64 and intein (52 kDa) could not be obtained with or without IPTG addition

In order to elaborate expression better, after series of expression trials, Silver staining was applied for protein induction done at 23°C for 16 hours. Silver stained gel image was shown below as Figure 4.7 and bands of expressed MPT64 became visible as expected at 52 kDa band.

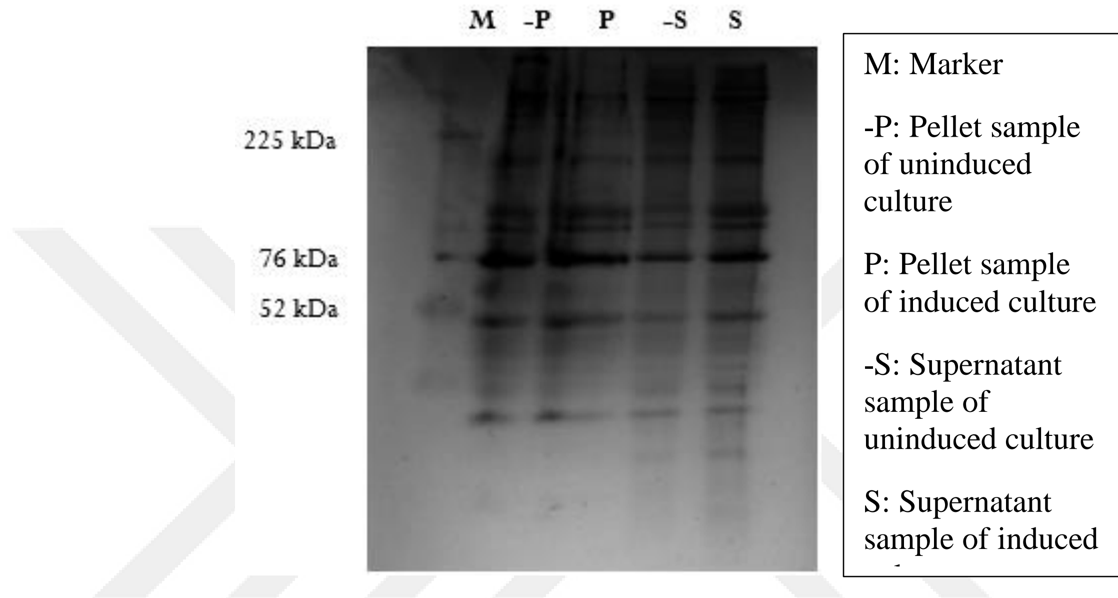

Figure 4.7. Gel image of MPT64 fused with intein tag being visible after silver staining.

4.1.3.2.Western blotting

Western blotting was done for MPT64 expression. Randomly selected colony was used for protein induction at two different conditions, 18 hours at 15°C as cold condition and 16 hours at 23°C as room temperature condition in order to analyze protein expression better. The samples from these cultures were used for Western blotting. Supernatant which is the soluble fraction was shown as S and pellet which is the insoluble fraction was shown as P in membrane images.

The primary antibody is specific to intein tag. So, calculated protein size for protein+tag is 52 kDa for colony. In cold condition, expression was observed even without using IPTG (which was shown with "-") in both supernatant (S) and pellet (P) as shown in 4.8. In room temperature condition, amount of tag was slightly increased in pellet and decreased in supernatant when protein expression was induced by addition of IPTG. But uninduced cultures of room temperature showed no sign of tag presence, this also proved that colony fails to express MPT64 without IPTG addition in room temperature.

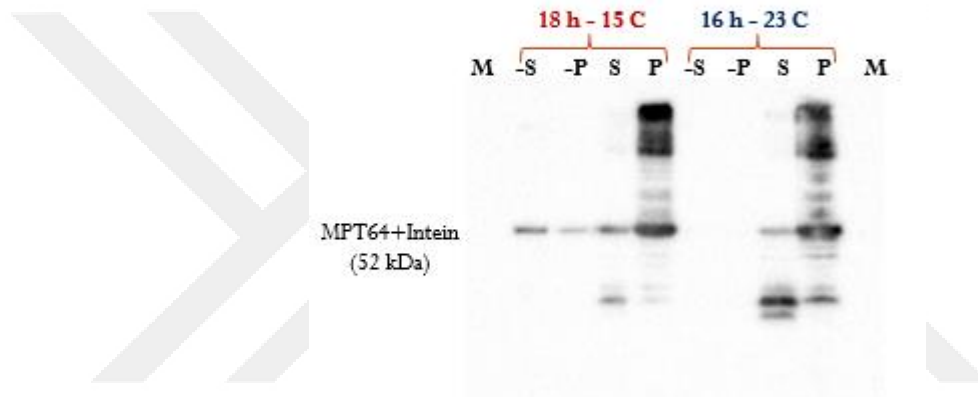

Figure 4.8. Image of Western blot analysis for MPT64 expression at cold condition and room temperature condition.

Series of expression trials were done for MPT64+intein tag complex in *E. coli* ER2566 but the level of expression was not sufficient to go further. So, second plasmid pTYB21 was used for cloning of *mpt64* and intein tag was put before MPT64 protein sequence.

4.2. Cloning and Expression of MPT64 Protein inserted into pTYB21 in *E. coli* ER2566

4.2.1. DNA Isolation from *M. tuberculosis* H37Ra

Concentration of DNA extracted from Mycobacterium tuberculosis H37Ra was measured as 17,1 ng/µL by NanoDrop 2000C.

4.2.2. Transformation of *mpt64* Gene within pTYB21 into *E. coli* ER2566

4.2.2.1. Amplification of *mpt64* gene for pTYB21 vector

Concentration of *mpt64* PCR product used for pTYB21 was measured as 33,5 ng/ ng/µL by NanoDrop 2000C. *mpt64* PCR product was obtained as 720 bp at 62°C annealing temperature and optimization of PCR for *mpt64* with primers for cloning into pTYB21 was shown in Figure 4.9. In the figure, desired PCR product shown in lane 1.

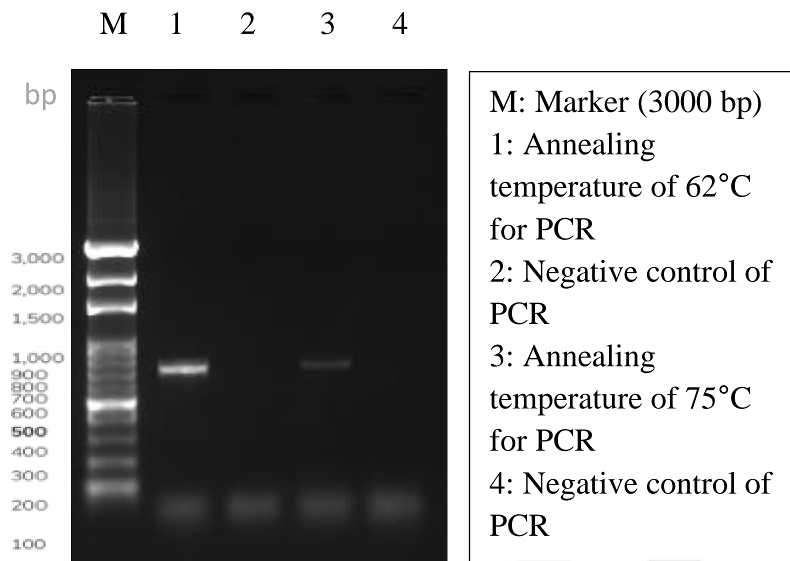

Figure 4.9. 1,2% agarose gel image of PCR product of *mpt64* pTYB21.

4.2.2.2.Digestion of vector and insert with restriction enzymes

In order to clone *mpt64* into pTYB21, amplified *mpt64* and pTYB21 were digested by Sap1 and Pst1-HF. Digested fragments of *mpt64* (691 bp) shown as 1 and pTYB21 (7451 bp) shown as 4 were run in gel electrophoresis to confirm digestion as shown in Figure 4.10.

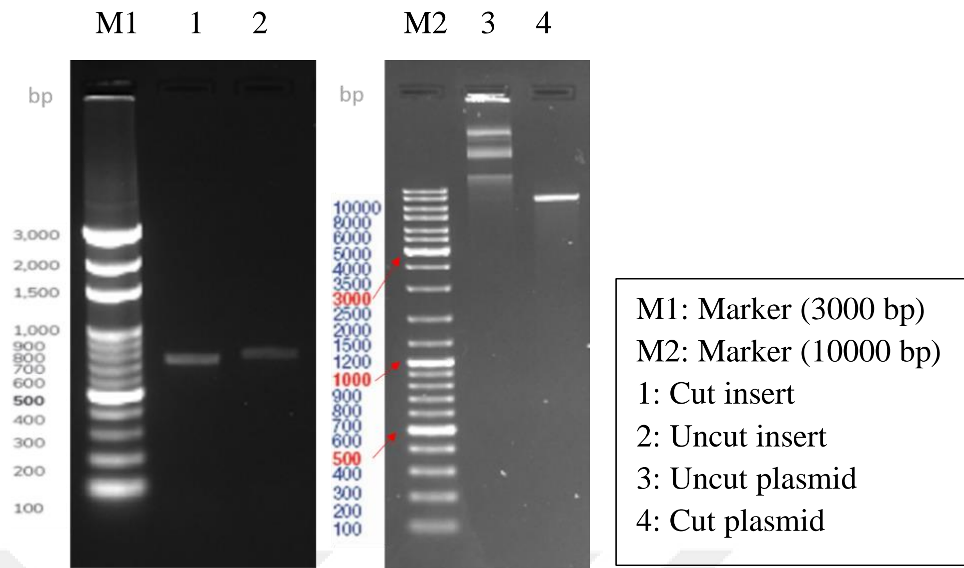

Figure 4.10. 1,2% agarose gel image of digested *mpt64* and pTYB21.

After digestion process, digested *mpt64* as insert were used in ligation with pTYB21 as vector. The concentration of insert and vector were measured by NanoDrop 2000C and the concentrations accepted for ligation step were given in Table 4.2 below.

Table 4.2. DNA concentrations of digested *mpt64* and pTYB21 plasmid for ligation.

Ligation of *mpt64* and pTYB21	
Digested *mpt64*	Digested pTYB21
6,1 ng/µL	2,3 ng/µL

4.2.2.3.Transformation into competent *E. coli* ER2566

At the end of ligation step, transformation was done in order to obtain colonies on LB agar plates. The ligation was done by using the same V:I ratio for both plasmids.

Then, the plasmids containing the desired target gene was used for transformation into *E.coli* ER2566. Ligation time with overnight incubation for *mpt64* and pTYB21 resulted in more colonies than ligation for one hour for *mpt64* and pTXB1 overall.

Also, different V:I ratio proved that using higher concentrations of insert increased number of colonies obtained but increasing concentration of insert too much showed decrease in number of colonies obtained. With 1 hour of ligation process for *mpt64* and pTXB1, increasing V:I ratio resulted in a few more colonies than previous ratio. In addition to this, longer ligation period for *mpt64* and pTYB21 showed drastic increase in number of colonies obtained which proves the effect of ligation time. V:I ratio resulted in different amounts of colonies as shown in Figure 4.11.

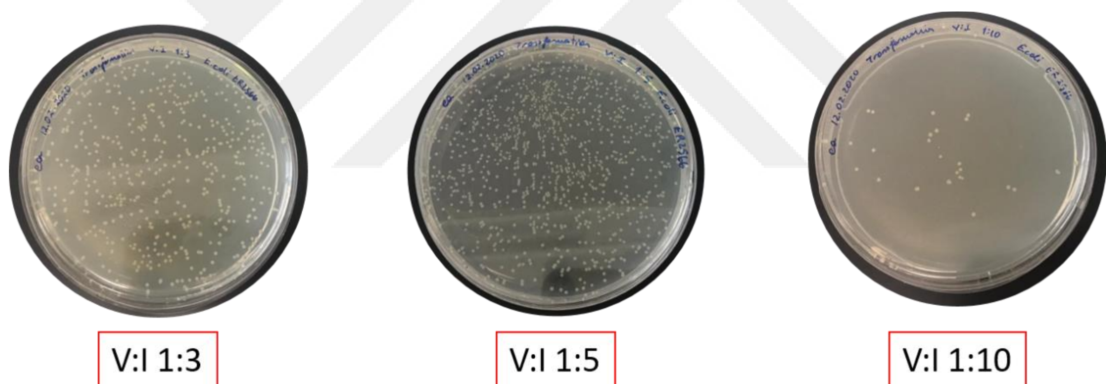

Figure 4.11. Pictures of agar plates after transformation of pTYB21 with insert into *E.coli* ER2566 at different V:I ratios.

4.2.2.4.Colony PCR

Carefully selected single colonies from agar plates were used for colony PCR as template DNA and then, PCR products were run in gel electrophoresis to confirm the products that contain the target gene fragments. Colonies containing *mpt64* and pTYB21 (8149 bp) showed in Figure 4.12.

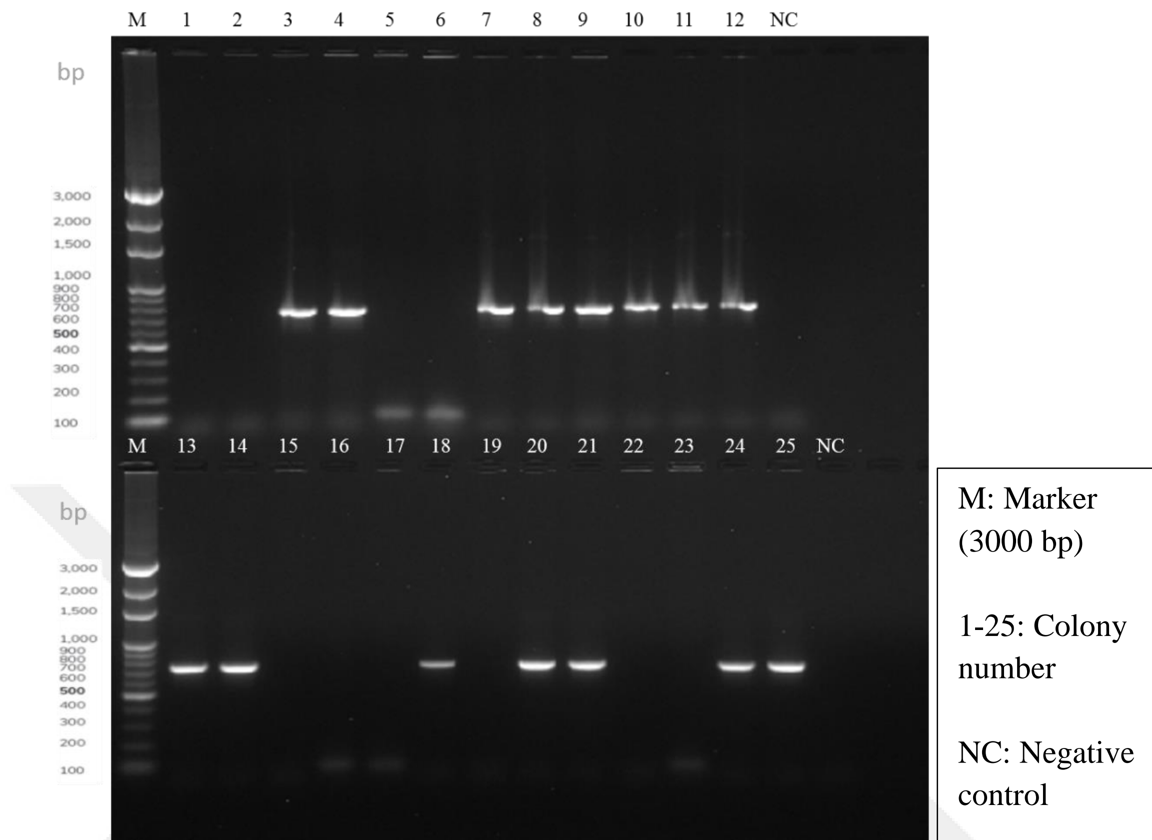

Figure 4.12. Image of 1,2% agarose gel electrophoresis after colony PCR for insert with pTYB21.

Successfully cloned colonies: 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 18, 20, 21, 24 and 25th colonies for *mpt64* and pTYB21 (15 out of 25 colonies). The calculated percentage for this is 60%. The amount of successfully cloned colonies showed the efficacy of ligation process was much better for *mpt64* and pTYB21.

4.2.2.5.Confirmation by sequencing

The ending and beginning of *mpt64* sequence for pTYB21 were confirmed in sequencing analysis. In Figure 4.13, 56th base is the end of the intein tag and, 57st base is the beginning of *mpt64* was shown with a red arrow and 743th base is the last base of *mpt64* was shown with blue arrow.

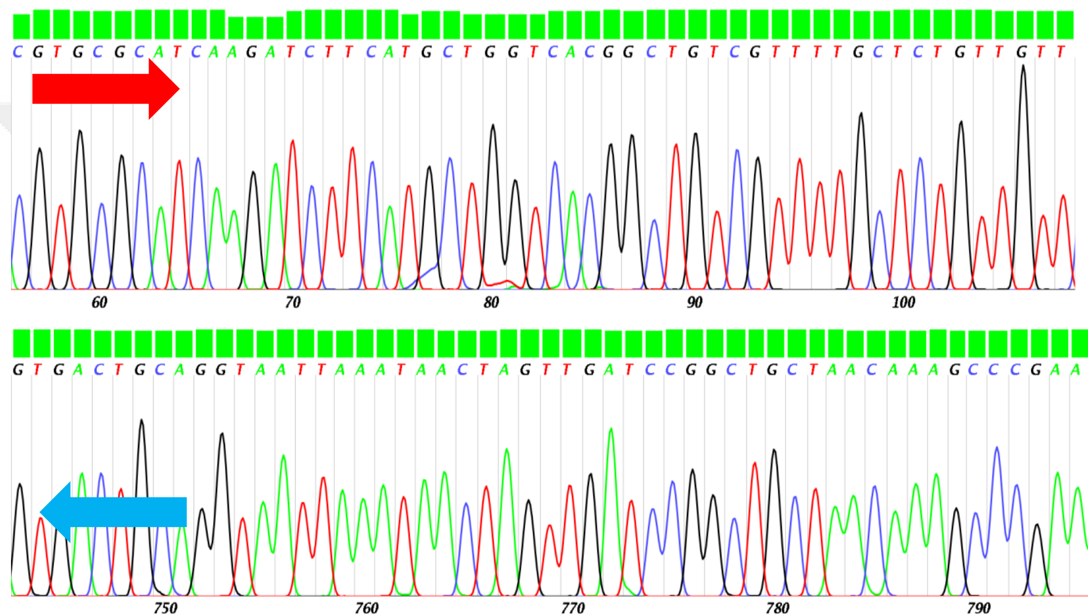

Figure 4.13. Image of sequence analysis by forward primer shows beginning and end of *mpt64* inserted into pTYB21.

4.2.3. Analysis of Protein Expression and Purification

4.2.3.1. Induction of protein expression

After low expression of MPT64 with pTXB1, plasmid pTYB21 plasmid was used in order to put intein tag before MPT64 protein sequence. *mpt64* inserted into pTYB21 was expressed in *E.coli* ER2566 successfully. To compare the intein tag itself and

expression level of MPT64, *E.coli* ER2566 containing only pTYB21 vector and *E.coli* ER2566 containing pTYB21 with *mpt64* were grown in LB Broth and protein induction was done. Also, uninduced cultures were grown simultaneously. The expression of protein+intein tag (80 kDa) and intein tag of pTYB21 (56 kDa) were compared in SDS-PAGE image as shown in Figure 4.14. Samples from uninduced cultures were shown with – and samples of induced cultures were shown with +. Only tag was expressed easily by *E.coli* ER2566 and after handling resuspended cell pellet the tag found in insoluble fraction both shown in induced culture sample of pTYB21 of resuspended pellet and pellet part while expression of MPT64 is lower compared to expression of only tag. Also, MPT64 protein fused with intein tag was expressed and after handling of resuspended cell pellet, MPT64 obtained as insoluble protein in pellet as shown in induced culture sample of MPT64 of resuspended pellet and pellet.

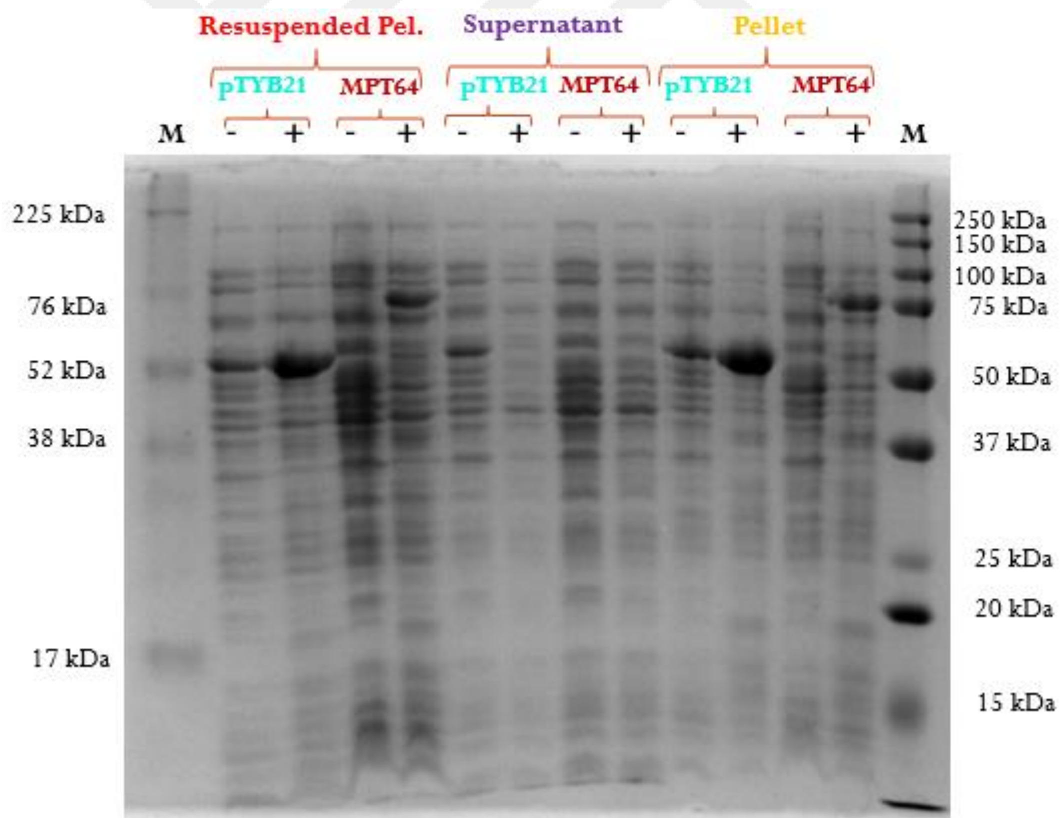

Figure 4.14. Image of SDS-PAGE gel stained with Coomassie blue.

4.2.3.2.Media optimization for protein expression by pTYB21

After successfully expression MPT64 fused with intein tag by inducing protein expression in *E. coli* ER2566, media optimization was done to improve expression. As given in Figure below, 6 different media were used. As brain-heart infusion broth showed improvement on expression of MPT64 as seen in BH lane of Figure 4.15, other media trials were not effective as this. Previously used LB broth media was from Sigma Aldrich, the product obtained by using this media was shown in L1 and changing brand resulted in less or no product which could not seen in the gel image either in soluble or insoluble part (L2).

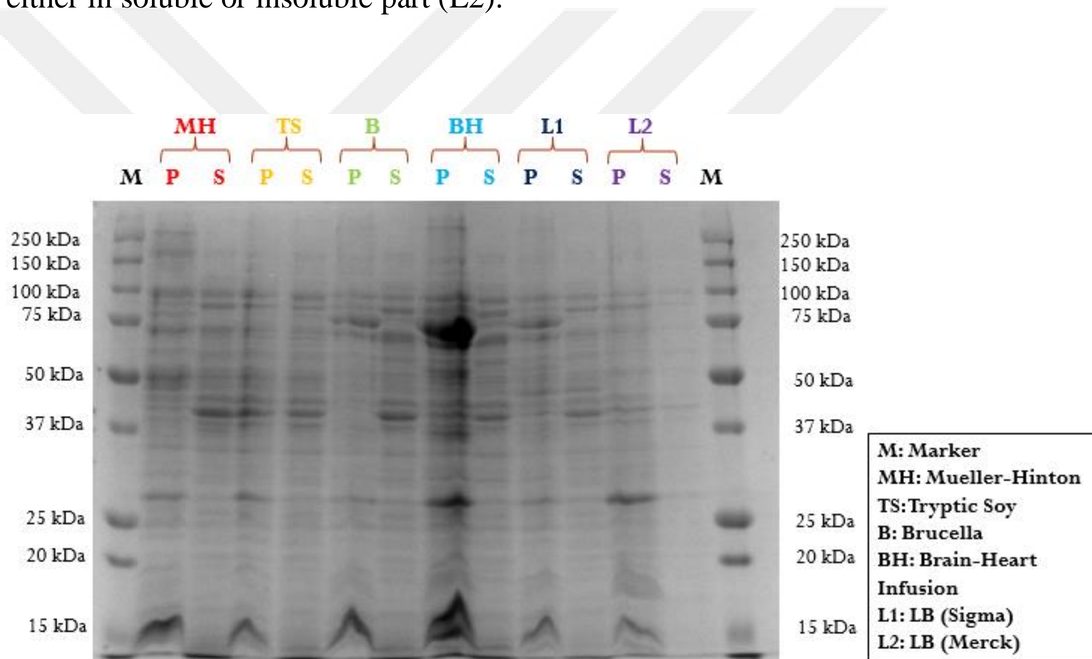

Figure 4.15. Image of SDS-PAGE gel stained with Coomassie blue.

4.2.3.3.Purification of proteins

After confirming the expression of MPT64 and optimum media selection, 500 mL culture of colony was induced in presence of 0,4 mM IPTG. The cell pellet obtained from the culture was weighed as 2,2 g and cell pellet was resuspended in 20 mL of column buffer then pellet as inclusion body was obtained. 20 mL of lysis buffer was

used to dissolve pellet and 19 mL of this fraction was dialyzed against 200 mL of dialysis buffers. At the end of dialysis, 28 mL of protein mixture was obtained. 27 mL of this protein mixture was used in purification and approximately 25 mL in small fractions was eluted at the end of purification. The samples from culture and purification were run on SDS-PAGE. In the Figure 4.16, the intein+MPT64 complex easily visible in CP sample. After treating pellet to solubilize the protein, the amount of intein+MPT64 complex was lost and the bands in dialyzed pellet (D) and the flow through sample (FT) taken after dialyzed pellet loaded onto chitin column were slightly less visible compared to CP. Most of the protein complex was captured by the chitin column itself and chitin column sample (C) taken before addition of cleavage buffer was shown in Figure 4.16. After immediate addition of cleavage buffer containing DTT, the band intensity did not change as seen in DW lane. At the end of 40 hours of incubation, eluted protein was collected in fractions and some of the fractions were run on gel as shown as F1, F3, F5, F7 and F9. The IMPACT system dictates that desired product is obtained in first fractions. As the system offers, the intensity of purified MPT64 decreases as fractions collected as shown between F1-F9. Lastly, regeneration of chitin column by washing with 0,3 M NaOH solution resulted in showing the low efficiency of purification.

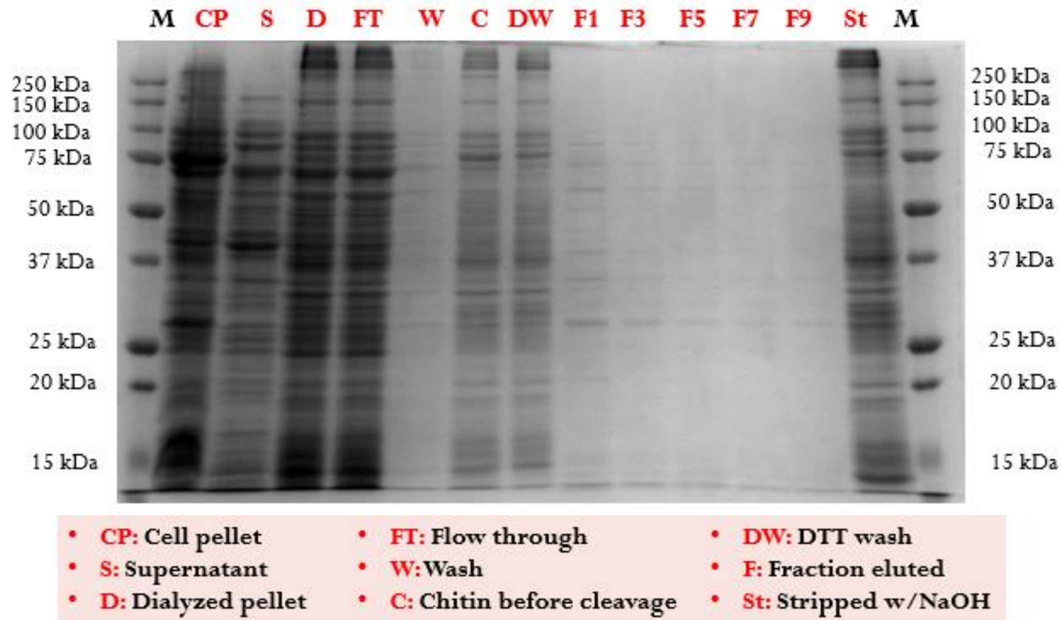

Figure 4.16. Image of SDS-PAGE gel for expression and purification samples stained with Coomassie blue.

Fractions obtained from purification process were collected into one tube. Total amount of protein was obtained by freeze drying the tube. Powder form of purified protein was weighed as 0,88 g. Dried MPT64 was resuspended in 4 mL of distilled water to obtain concentrated protein solution.

In addition to Western blotting image of samples from colony for MPT64 within pTXB1, samples from protein expression of colony for MPT64 within pTYB21 culture and sample of purification step were visualized in Western blotting. So, in Western blot analysis, the primary antibody has shown the intein tag only which helped to elaborate the expression and purification level. The end product of expression for this case was 80 kDa. The Figure 4.17 below showed that not only protein and tag fusion was expressed but also tag was expressed separately. In cell pellet (CP), the intein+MPT64 fusion was visible in addition to the only tag expression which was slightly more visible than intein+MPT64. Cell pellet was manipulated to handle

inclusion bodies which was MPT64 in this case and dialysis was done. Dialysis sample (D) and the flow through sample (FT) which was obtained when dialyzed protein was loaded onto chitin column have almost the same amount of expression. This shows the low level of purification for MPT64. Column was washed with column buffer after loading dialyzed protein and the sample was taken as wash (W). Wash sample showed no tag or protein presence which showed the inefficacy of washing column. Eluted protein which lacks the intein tag was expected to show no band as given in Figure 4.17 below. For regeneration of chitin column, 0,3 M NaOH solution was used to wash column and sample was collected (S). Stripping the intein tags and non-specific bound proteins from column showed there were still intein+MPT64 complex left intact since there is an 80 kDa band visible. Additionally, intein tag band became more pronounced since some of the intein+MPT64 complex were successfully purified and tag obtained alone.

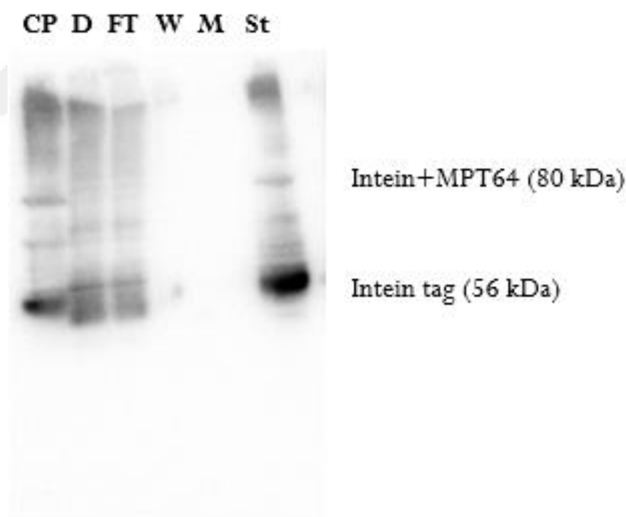

Figure 4.17. Image of Western blot analysis for expression and purification MPT64.

Capilla TB-Neo strip test was used to confirm MPT64 presence. The negative control was tested by using only column buffer. Some of the eluted fractions obtained purification step were tested on strip tests to confirm MPT64 antigen presence as seen in Figure 4.18. It shows that only column buffer trial gave negative result since it does

not contain any MPT64 protein. Also, first fractions have more of the purified protein while last fractions have less to no MPT64 protein to show a positive band in strip test.

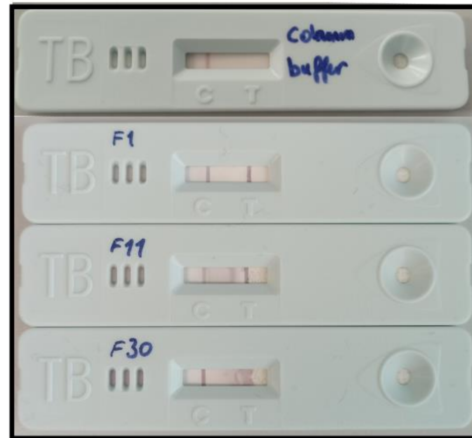

Figure 4.18. Image of Capilla TB-Neo strip tests done for column buffer and different fractions.

After lyophilization and resuspension of dried protein, the concentrated MPT64 was tested on strip to confirm its presence. The positive band as shown in Figure 4.19 was much more intense in compared to single fraction eluted.

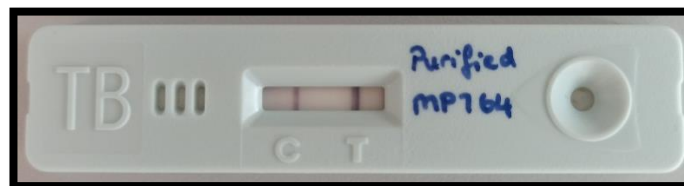

Figure 4.19. Image of Capilla TB-Neo strip tests done for purified MPT64 protein.

5.DISCUSSION AND CONCLUSION

In this study, cloning, expression and purification of MPT64 proteins were aimed. For this purpose, two different plasmid vectors such as pTXB1 and pTYB21 were used. These plasmids are including an intein tag and a chitin binding domain. Chitin binding domains help proteins to bind to the chitin column and other proteins can be washed away. Then, these purified proteins can be removed by adding a reducing agent like DTT on intein tag sites which has the ability for self-excision. As a result, no additional enzymes such as proteases are required for cleaving the target protein and the chitin binding protein. Therefore, no impurities are found in the final product including target proteins.

pTXB1 and pTYB21 plasmids chosen in this thesis are used for expression of protein with addition of intein tag. So, they are similar, but not the same entirely. pTXB1 vector is used to fuse the target protein at its C-terminus whereas pTYB21 vector is used for N-terminus. So, system can be used for both ends separately. The mini intein found in pTXB1 is from *Mycobacterium xenopi gryA* gene. Desired protein (MPT64, 24 kDa in this case) can be purified at its C-terminus from 28 kDa intein tag in the presence of redox reagent, DTT. The situation is reversed for pTYN21, vector has another intein tag and it is from *Saccharomyces cerevisiae VMA1* gene. Again, by introducing DTT, purification at protein’s N-terminus from intein tag (56 kDa). The IMPACT system acquires affinity chromatography and using self-cleavable intein tag adjacent to chitin binding domain helps to finish purification in single step.

In this study, the expression level of MPT64 proteins in pTXB1 vector was very low. There were no significant change of recombinant protein expression under different conditions of temperature, the amount of IPTG, incubation time etc. The intein tag+CBD alone was successfully expressed and found as soluble protein in supernatant. But the recombinant protein expression was not detected in gel after

staining with Coomassie blue. In order to confirm protein expression, silver nitrate staining was chosen instead of Coomassie blue staining, which is more sensitive. After silver nitrate staining, the protein band of MPT64+intein tag+CBD could be detected as very low. These results are compatible with the literature published in 2008 which clarifies the issue with low expression level of MPT64 (59). Since expression starts from MPT64 gene sequences, rare codons found in desired gene fragment which causes expression to remain low by meaning tRNAs in *E.coli* that do not prefer required codons for MPT64 (62). Low abundance of some codons may result in low expression which makes it inefficient for protein production and purification becomes impossible at this point.

As an alternative, pTYB21 was used to express MPT64 proteins with a high yield. In this vector, MPT64 gene sequences are followed by the chitin binding domain and the intein tag. The CBD + intein tag in pTYB21 alone expressed successfully, however the expression level slightly decreased when MPT64 gene was cloned into the vector (CBD+intein+*mpt64* gene) .

In order to increase the expression level, media optimization was also performed. Six different broth media were used. This showed not only the effect of media type but also the effect of brand preferred for same media type. The highest level of expression was obtained with Brain-Heart Infusion Broth media.

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Proteins were obtained as inclusion bodies, therefore refolding and dialysis steps were required. Since the expression levels of MPT64 proteins was already very low, most of proteins were lost during all these additional steps and purification step. Western blot results showed that the amount of the intein tag+CBD was increased after cleavage reaction with DTT by meaning MPT64 proteins were eluted from the chitin columns. Because of the very low concentrations of MPT64 proteins after column purification, Capilla TB-Neo strip test which includes specific antibodies to MPT64

proteins were used to detect proteins in the elute. Results showed that MPT64 proteins were obtained succesfully.

As a conclusion, MPT64 proteins are very important antigens to detect tuberculosis in clinical samples such as urine, blood samples, and sputum. At the same time, the MPT64 protein is a very good candidate for the TB vaccination instead of BCG. In this thesis study, MPT64 proteins were cloned into plasmid vectors having an intein tag and a chitin binding domain. These proteins were successfully expressed; however the yield was quite low. In future, codon optimization and the use of synthetic gene blocks instead of the natural gene from *M. tuberculosis* will be performed to increase the expression level of MPT64 proteins in the same vector and bacteria. As second option for better expression, different host cells like *M. smegmatis* instead of *E.coli* will be used. Different type of expression vectors will be tried as another option.

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7.APPENDICES

7.1. Appendix 1. Data of Sanger Sequencing of *mpt64* Gene with pTXB1 in *E. coli* ER2566 by T7 Universal Primer

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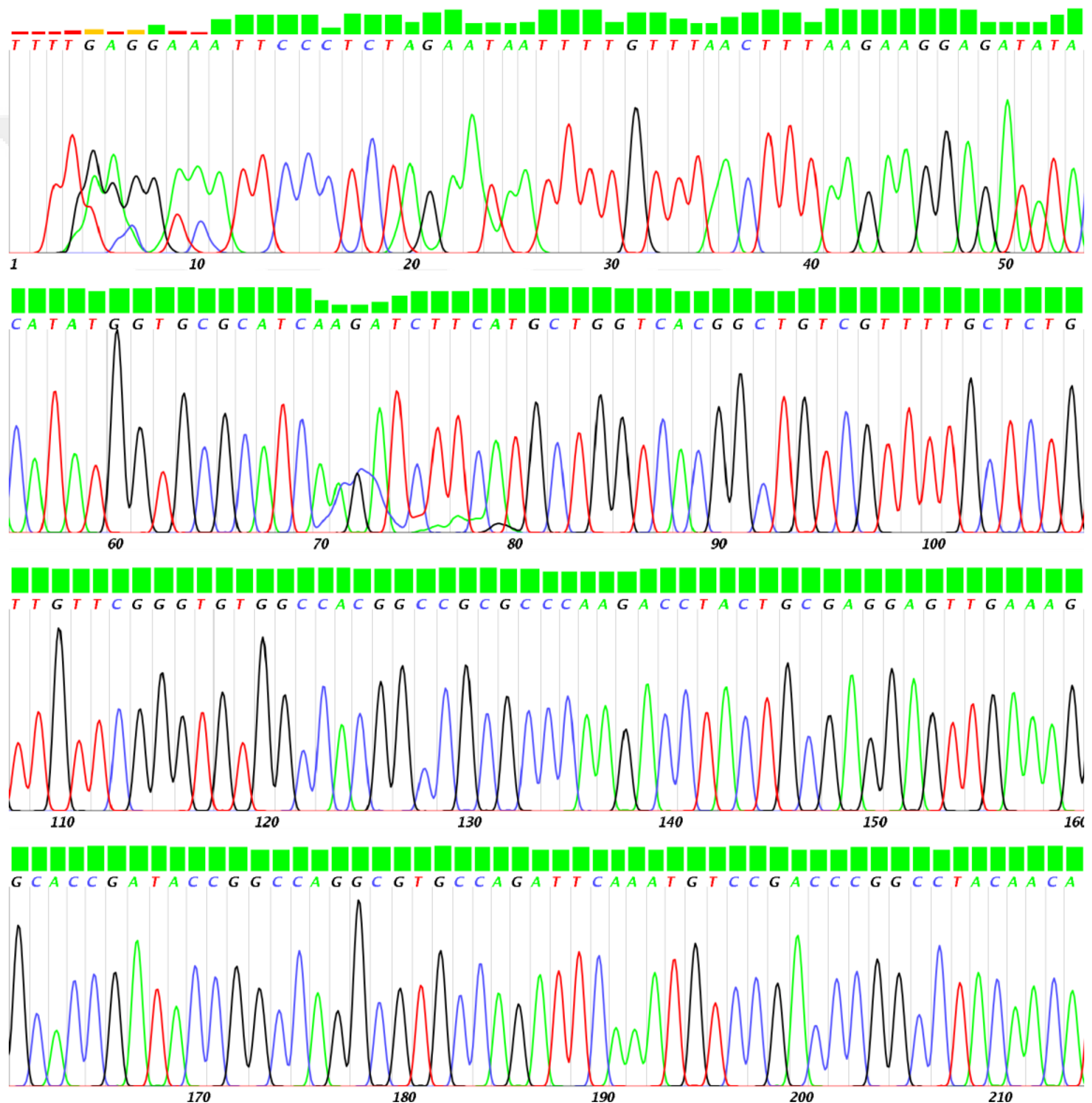

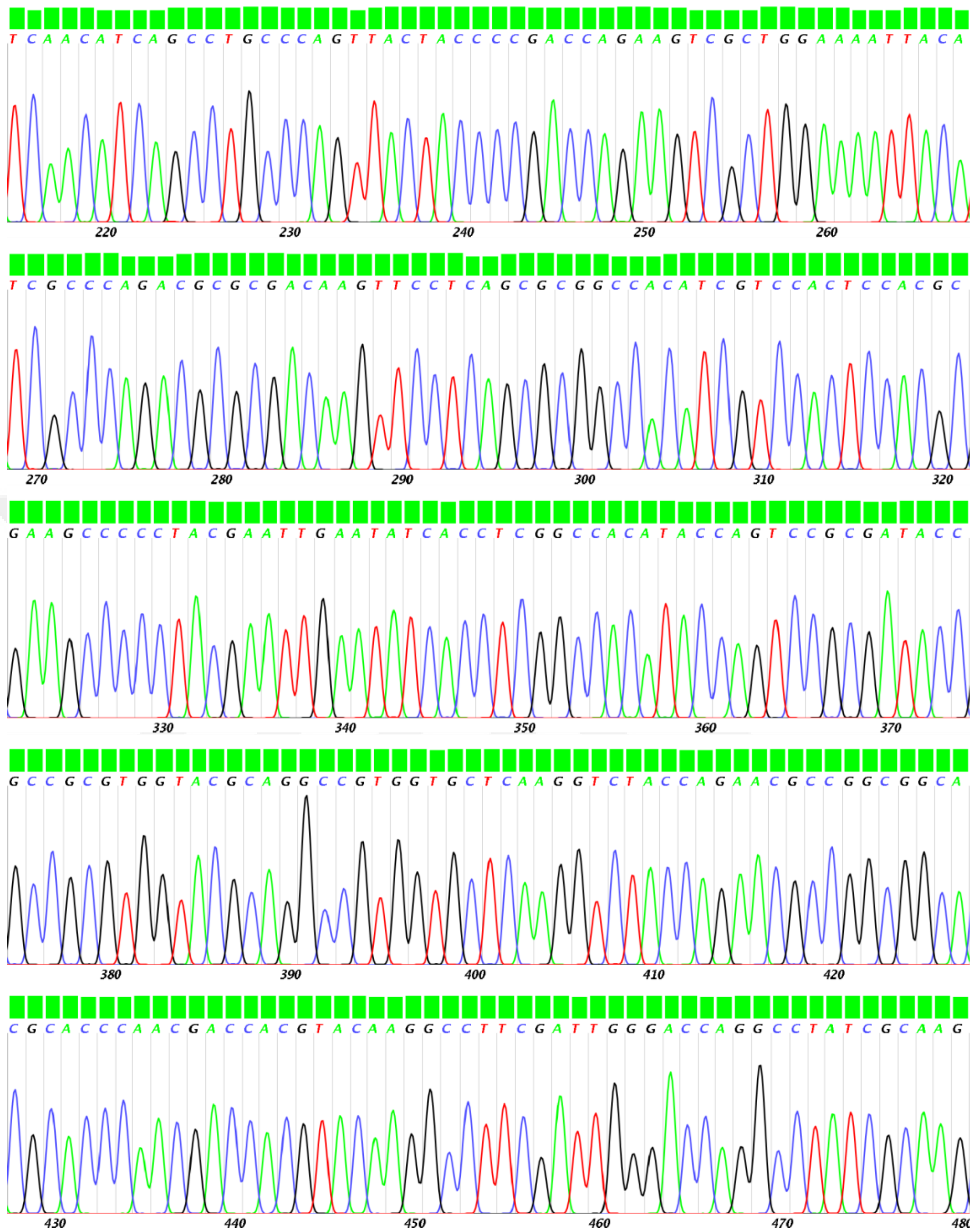
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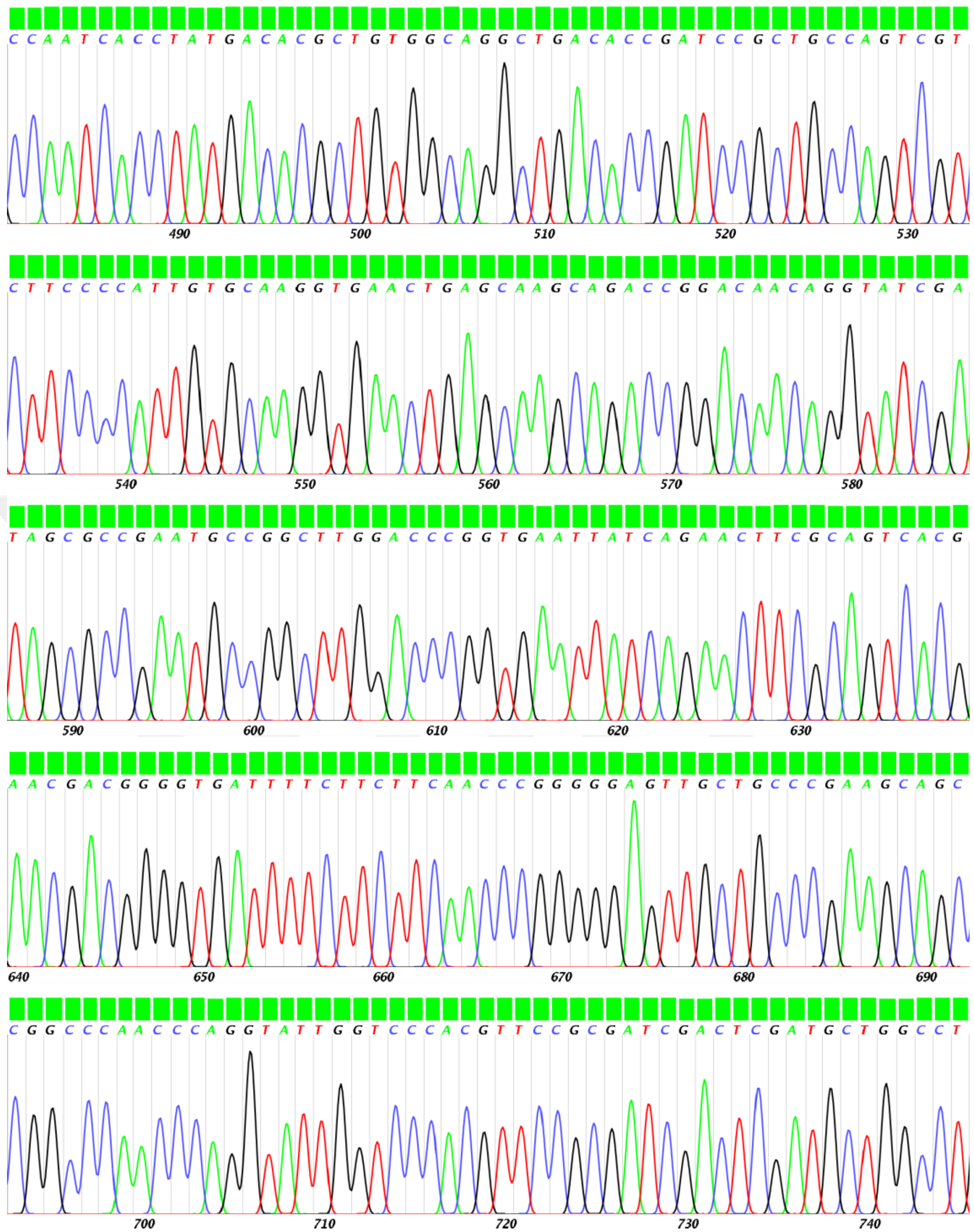
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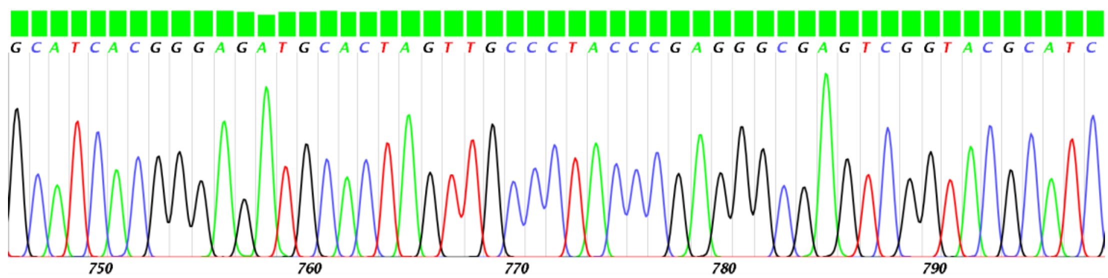
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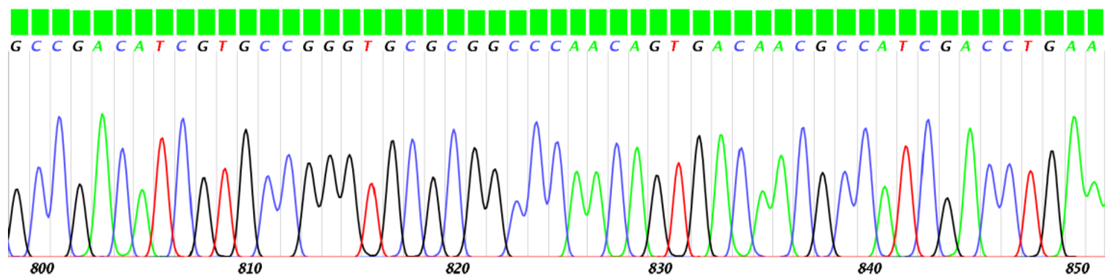
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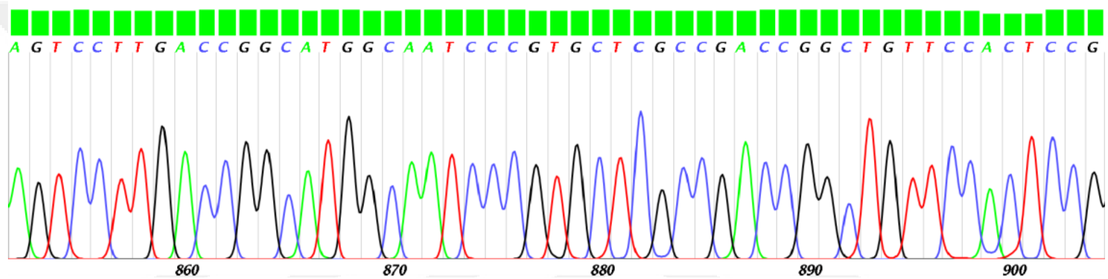
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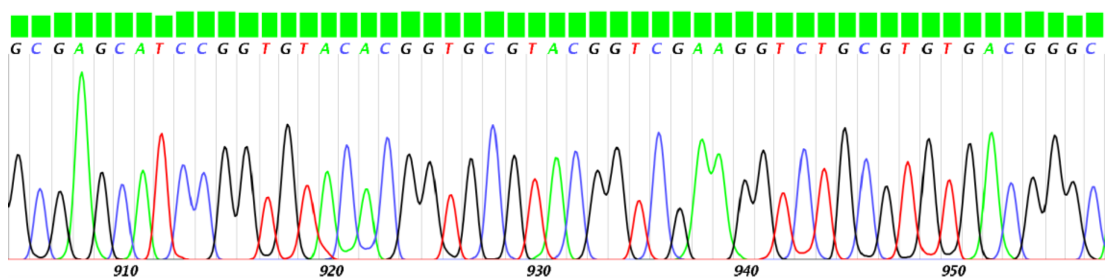
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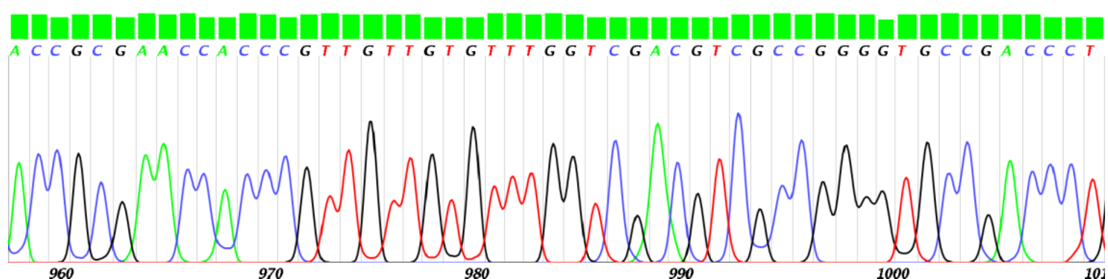
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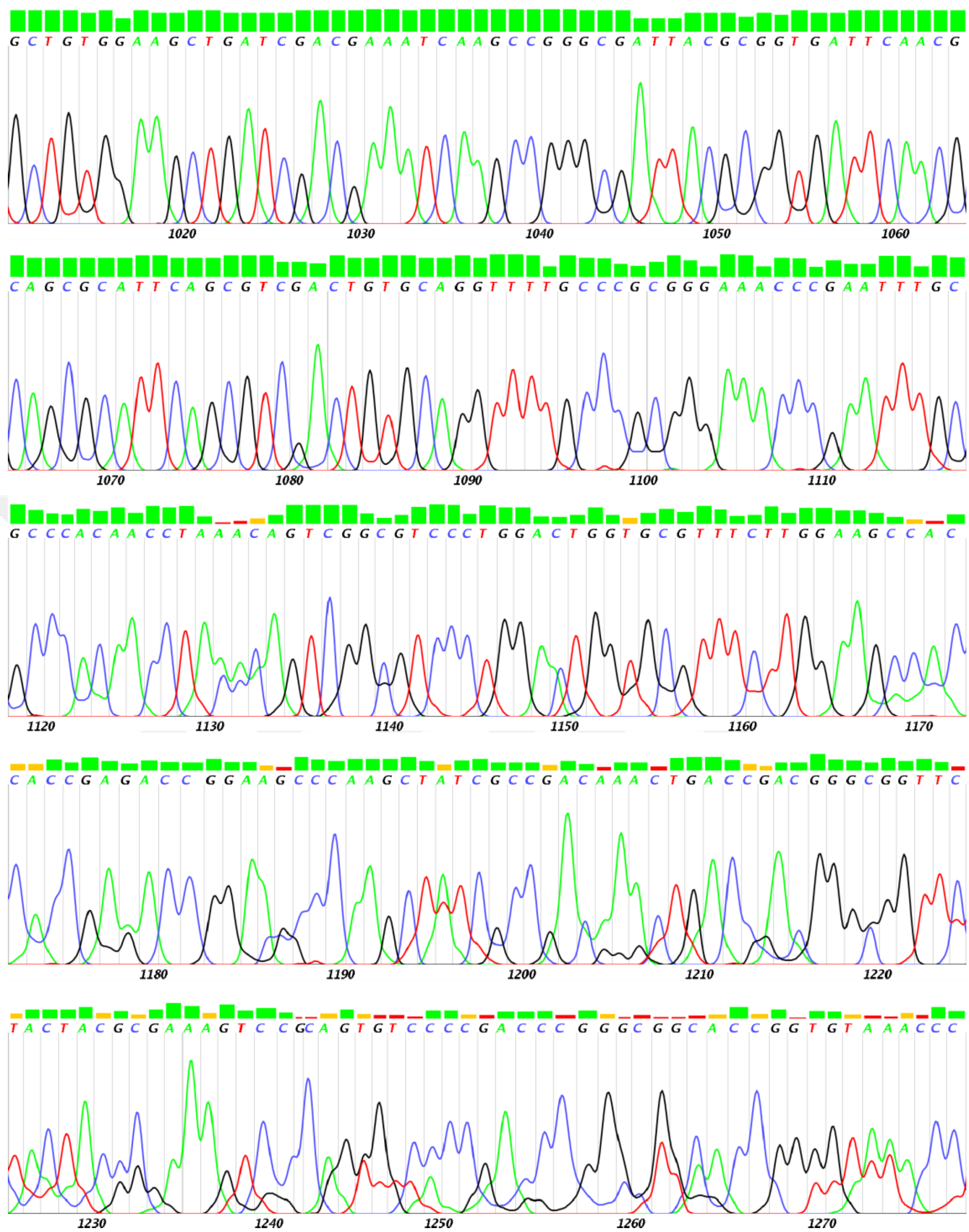
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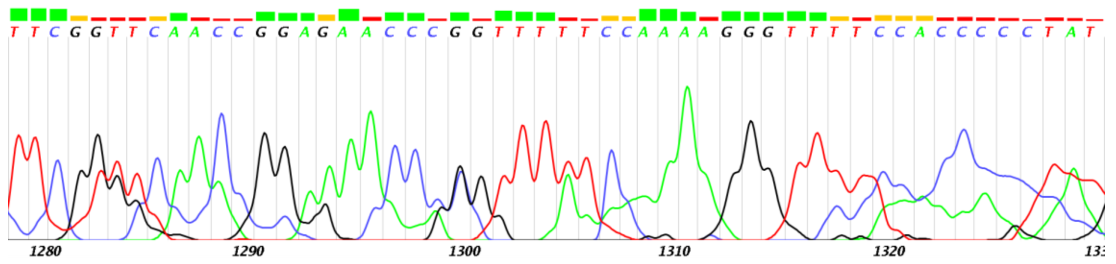
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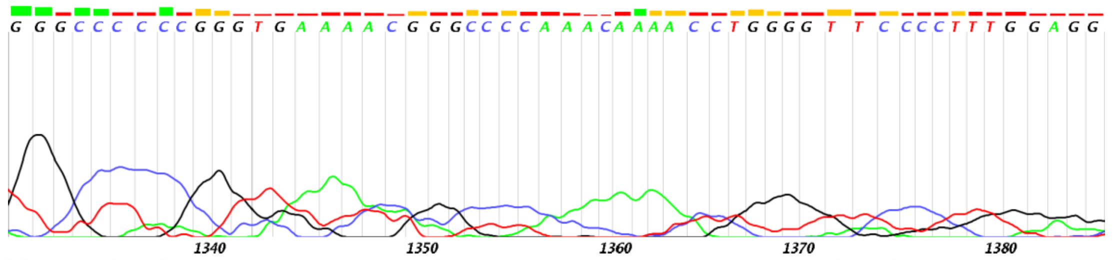
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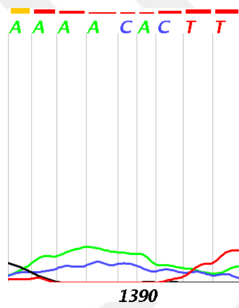
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7.2. Appendix 2. Data of Sanger Sequencing of *mpt64* Gene with pTYB21 in *E. coli* ER2566by Forward Primer

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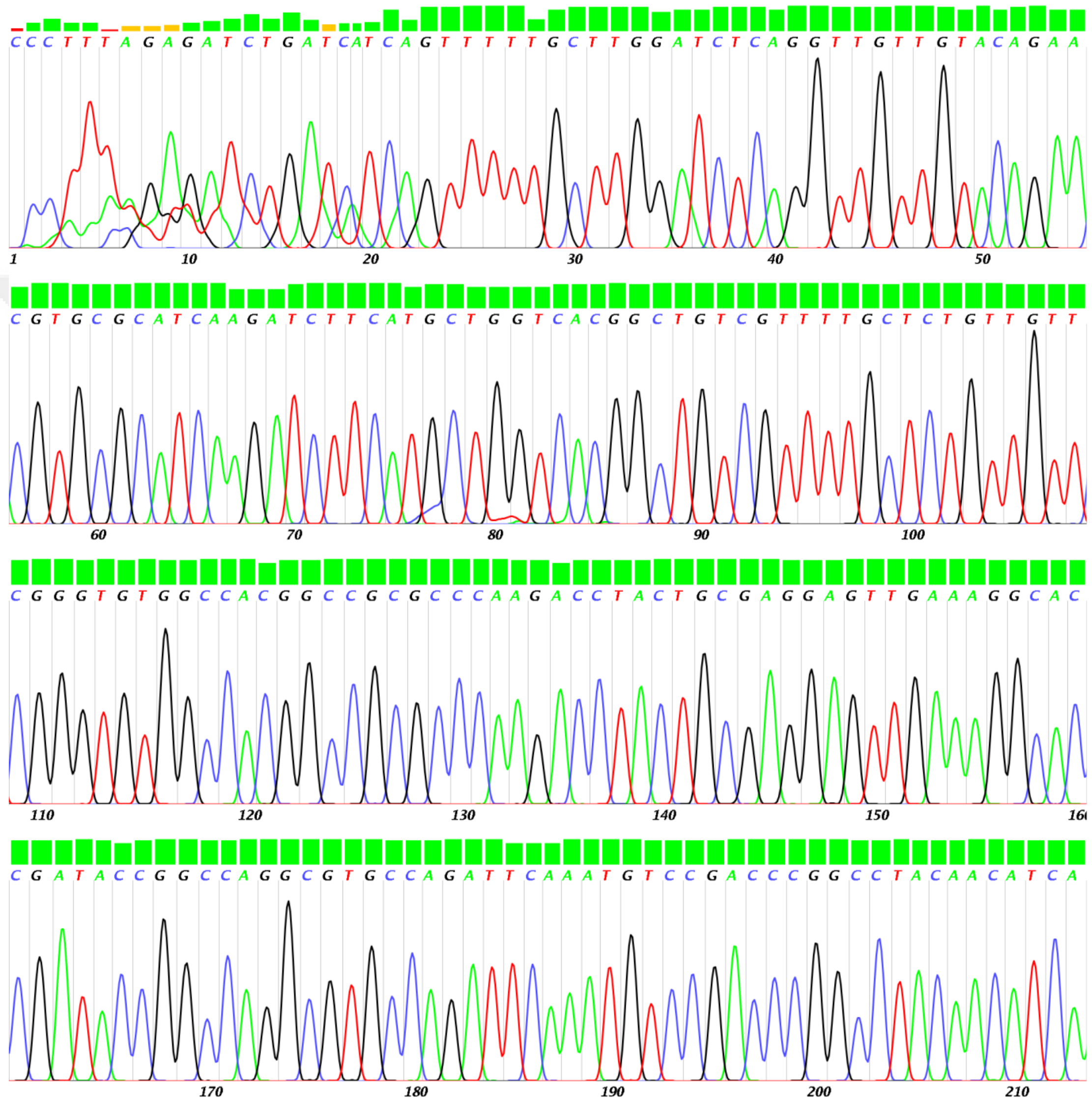

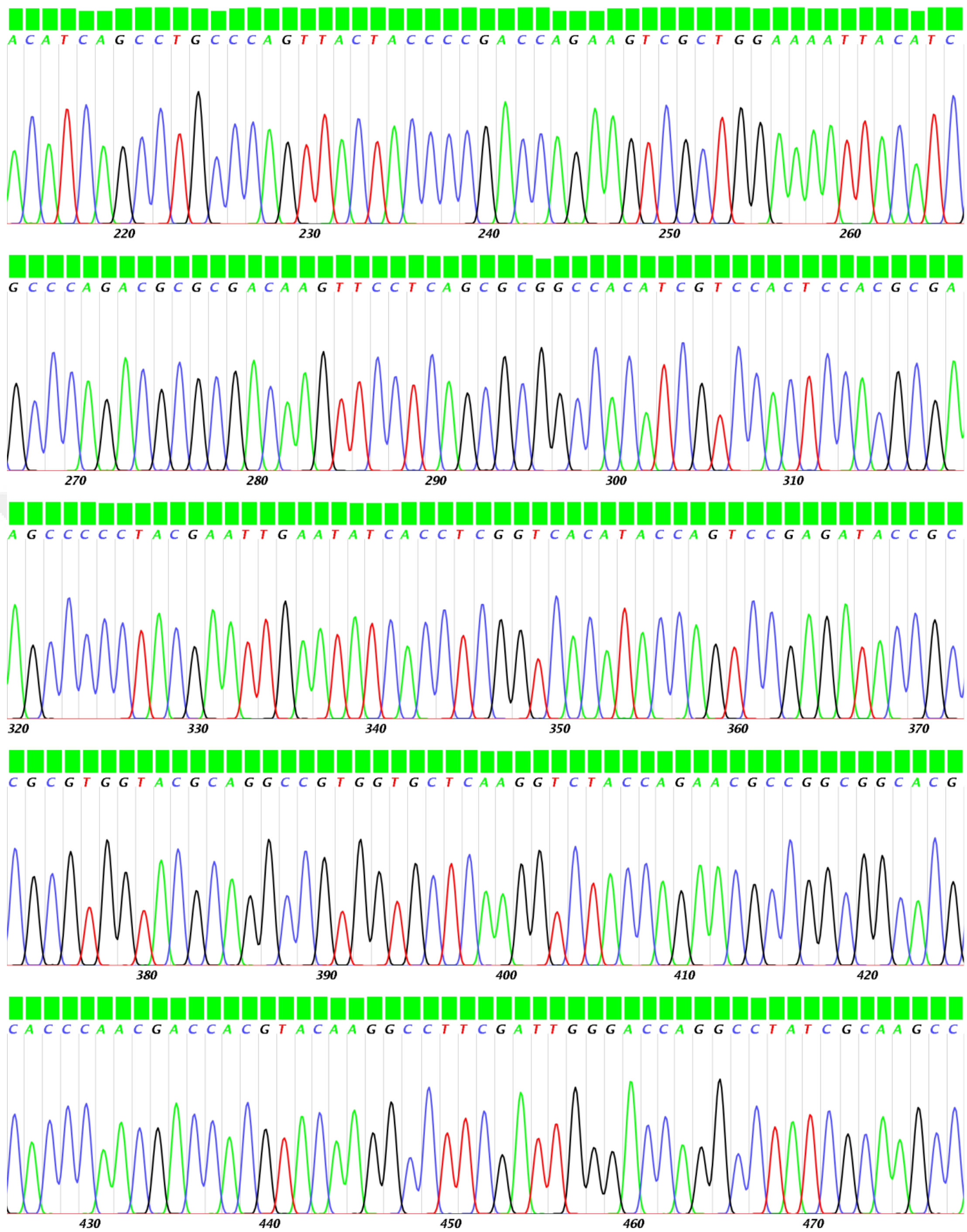
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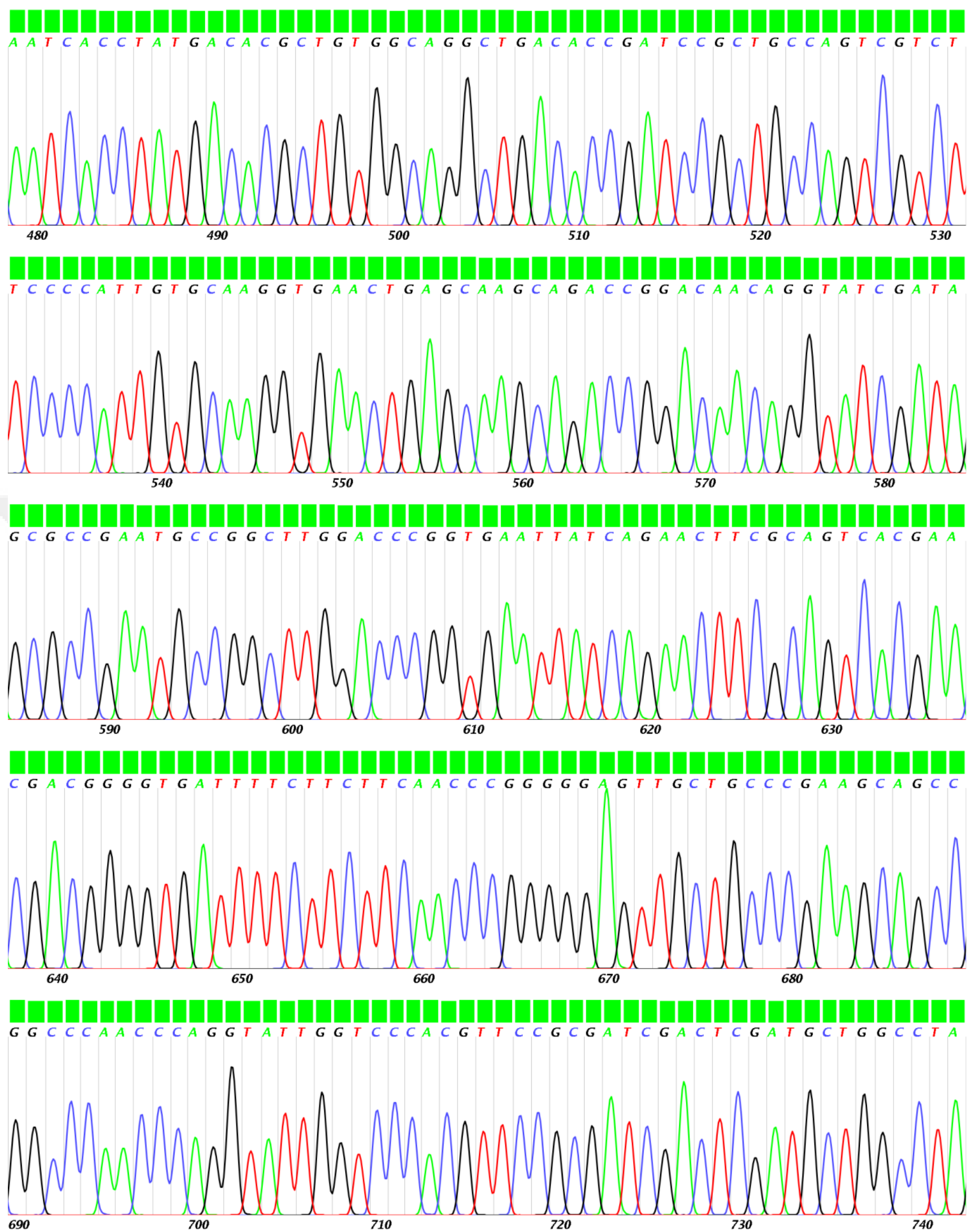
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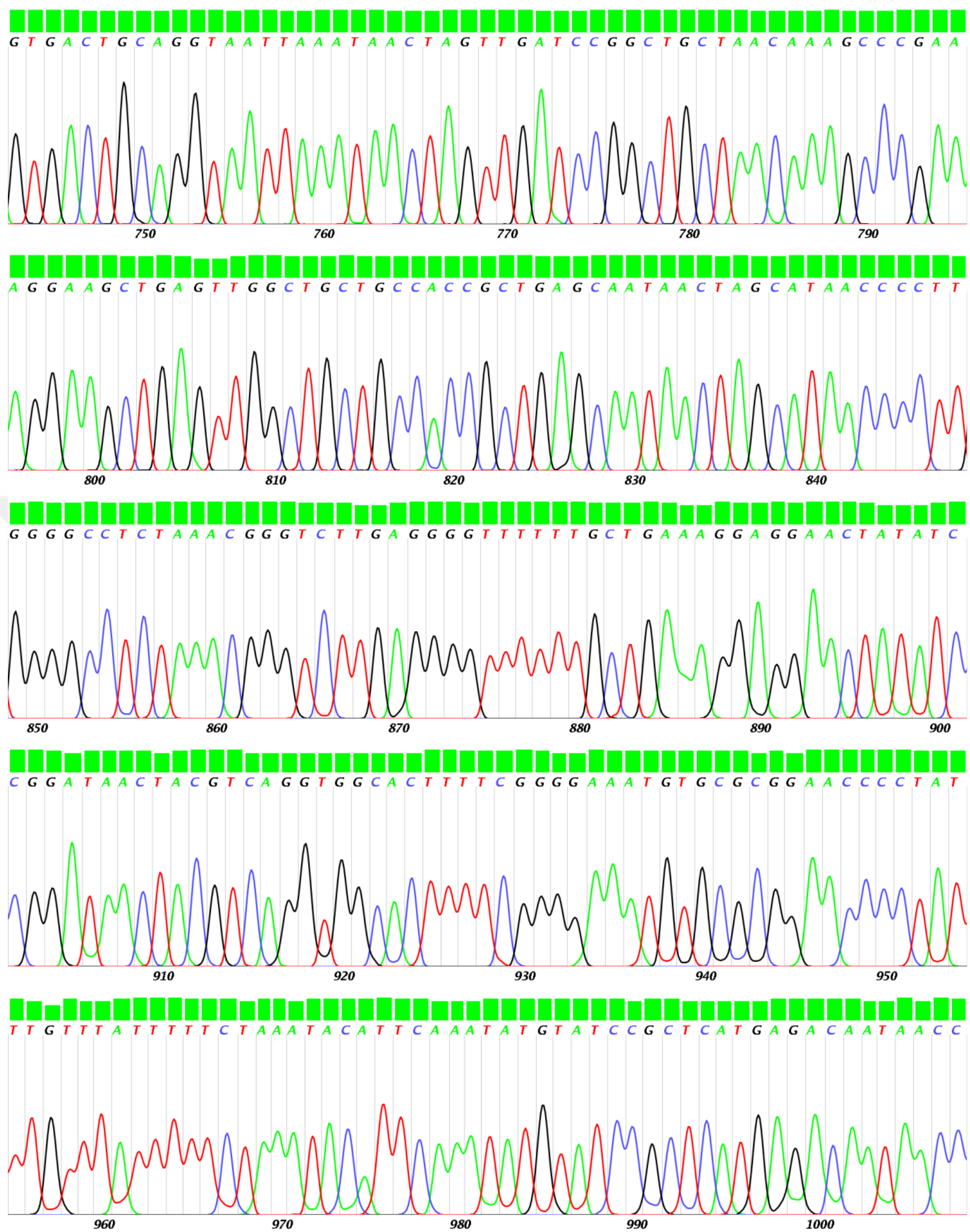
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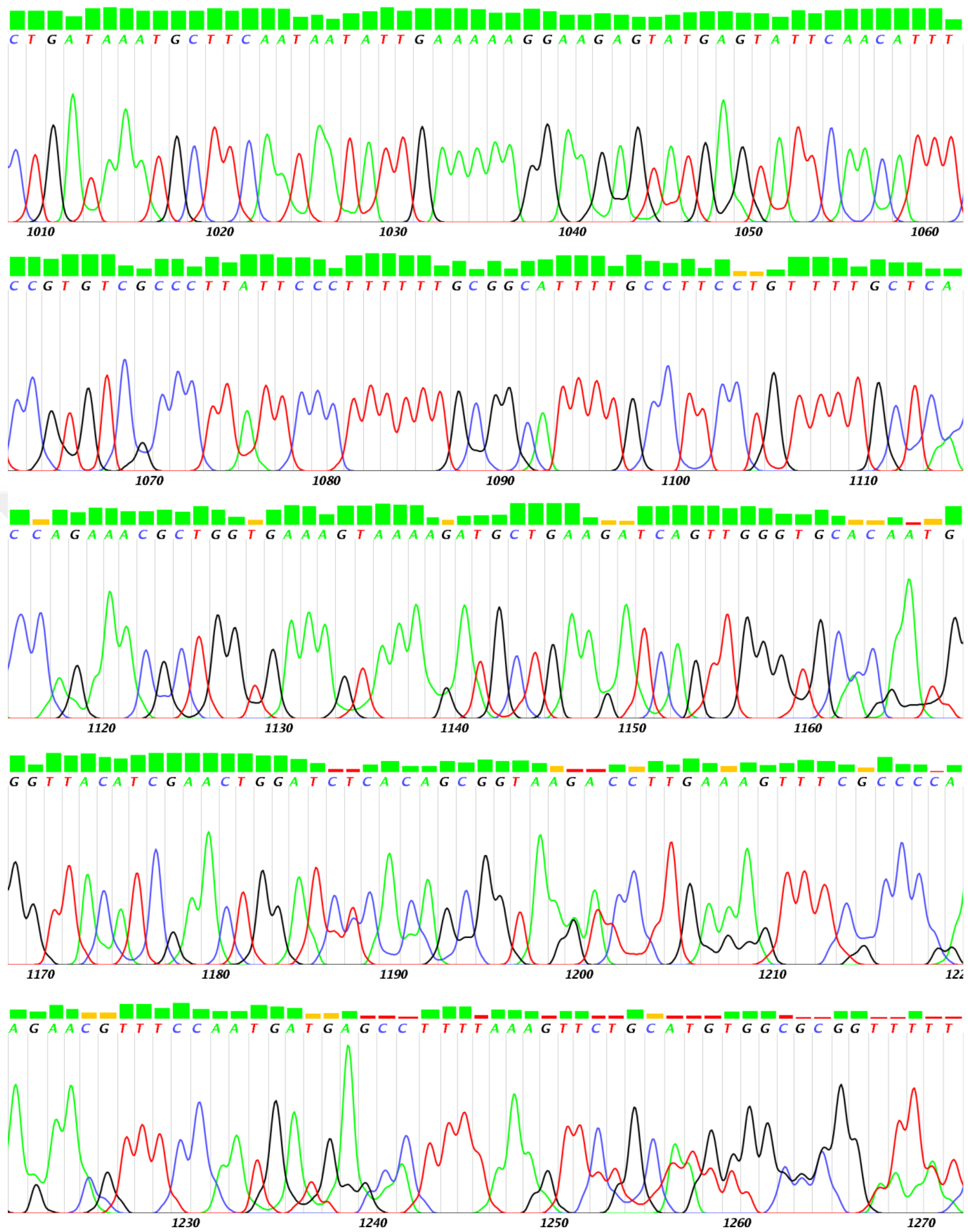

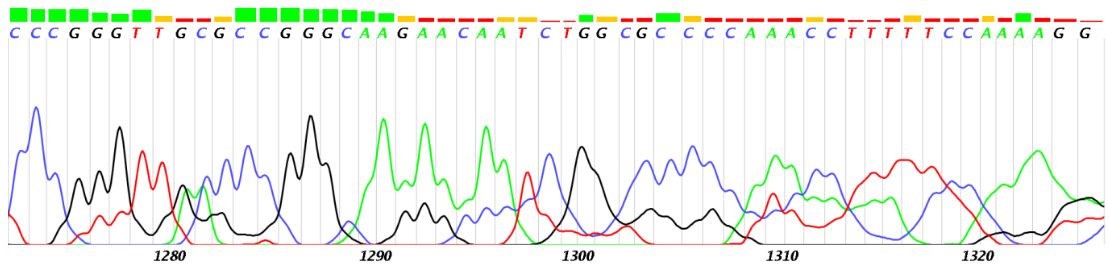

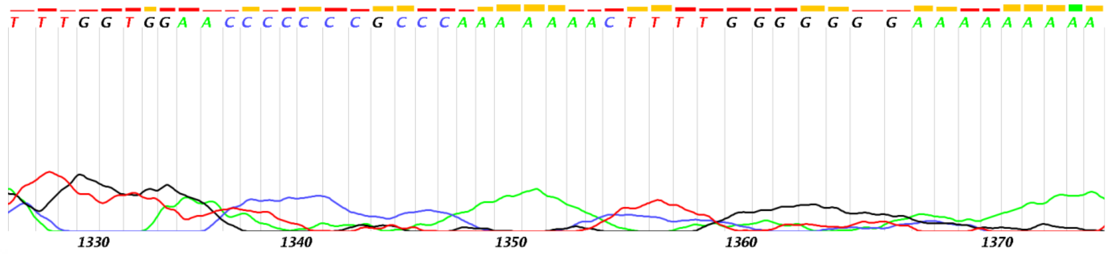

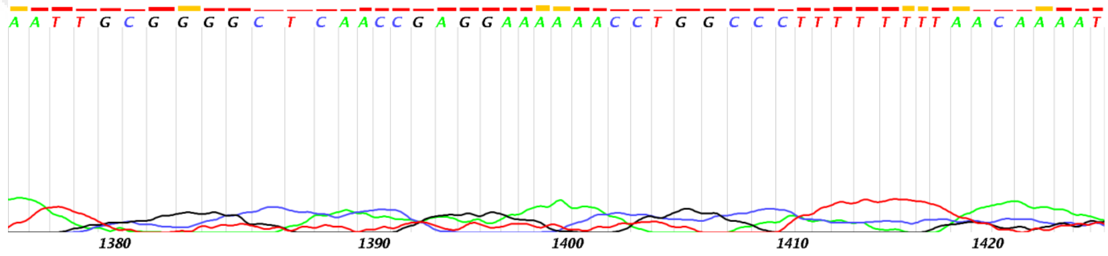

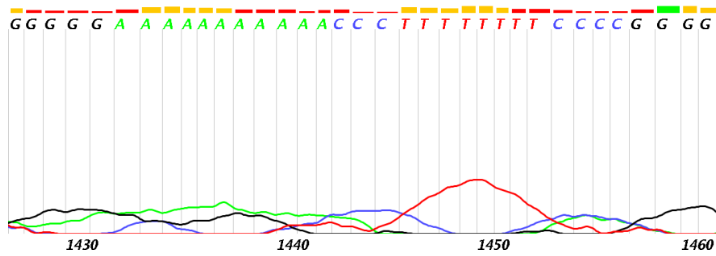

8. CURRICULUM VITAE