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GEBZE

2018

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

İMMÜN TEST ÇALIŞMALARDA KULLANMAK İÇİN MUTANT İNSAN İMMÜNREAKTİF TRİPSİNOJEN (IRT) GELİŞTİRİLMESİ

MUSTAFA DİVYAPICIGİL
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
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DR. ESİN AKÇAEL

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GEBZE TEKNİK ÜNİVERSİTESİ	**YÜKSEK LİSANS JÜRİ ONAY FORMU**

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 27/06/2018 tarih ve 2018/33 sayılı kararıyla oluşturulan jüri tarafından 25/09/2018 tarihinde tez savunma sınavı yapılan Mustafa DİVYAPICIGİL'in tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

JÜRİ

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..…/…..../……… tarih ve ……./…..... sayılı kararı.

SUMMARY

In this thesis, it is aimed to develop recombinant IRT proteins that do not have a proteolytic activity to be used as a standard in Immunoreactive Trypsinogen (IRT) ELISA kits.

IRT is a protein used as a marker for the diagnosis of cystic fibrosis (CF) in newborns. In addition to CF; diabetes, pancreatitis, and pancreatic cancer are possible fields for using IRT as a marker. According to the literature, there are two important IRT isoforms called trypsinogen 1 (cationic trypsinogen, IRT1) and trypsinogen 2 (anionic trypsinogen, IRT2) in human pancreatic juice used in the diagnosis of CF. The active form of IRT is challenging to use in immunoassays because it causes the cleavage of antibodies, other protein-based components, and even itself due to its proteolytic activity.

In this study, recombinant human IRTs, that developed have K23D mutation, which changes lysine (K) residue at the 23rd position to aspartic acid (D), inhibits its activation and proteolytic activity by preventing the cleavage of inactive trypsinogen to active trypsin. Mutated genes were expressed in P. pastoris. The antigenic properties of mutant proteins were tested and confirmed by ELISA (Enzyme-Linked Immunosorbent Assay) and Western Blot. It has been shown that mutant IRTs have the same antigenic properties as wild-types, except the proteolytic activity.

As a result, recombinant human IRT protein with K23D mutation which gains ease of use in immune tests has been successfully developed.

Key Words: Immunoreactive Trypsinogen, Cystic fibrosis, Immunoassay, Proteolytic activity, ELISA, Western Blot.

ÖZET

Bu tez çalışmasında Immunreaktif Tripsinojen (IRT) ELISA kitlerinde standart olarak kullanmak üzere kesim aktivitesi olmayan rekombinant IRT proteinlerinin geliştirilmesi hedeflenmiştir.

IRT, yenidoğan bebeklerde kistik fibrozisin (KF) tanısı için marker olarak kullanılan bir proteindir. KF'ye ek olarak diyabet, pankreatit ve pankreas kanserinin teşhis ve takibinde, IRT biyolojik marker olarak kullanım potansyeline sahiptir. İnsan pankreas salgısı içerisinde; literatürde, tripsinojen 1 (katyonik tripsinojen, IRT1) ve tripsinojen 2 (anyonik tripsinogen, IRT2) olarak adlandırılan iki önemli IRT izoformu bulunur ve her ikisi de KF tanısında kullanılır. IRT'nin aktif formu antikorlar, diğer protein bazlı bileşenler ve hatta kendisini proteolitik aktivitesi nedeniyle kestiği için immün testlerde kullanılması zordur.

Bu çalışmada, inaktif form olan tripsinojenin aktif tripsine dönüşümünü engellemek amacıyla 23. amino asit olan lizini (K) aspartik aside (D) dönüştüren K23D mutasyonu yapılmış ve proteolitik aktivitesi inhibe edilmiş rekombinant insan IRT proteinleri geliştirilmiştir. Mutasyona uğramış genler daha sonra P. Pastoris'te ürettirilmiştir. Üretilen proteinler ELISA (Enzyme-Bağlantılı İmmünosorbent Assay) ve Western Blot immün testleri ile test ve tasdik edilmiştir. Rekombinant IRT'nin, yabanıl tiple aynı antijenik özelliklere sahip olduğu ve kesim aktivitesinin bulunmadığı gösterilmiştir.

Sonuç olarak, hedeflenen mutasyonla proteolitik aktivite engellenerek, immün testlerde kullanım kolaylığı getiren K23D mutasyonuna sahip rekombinant insan IRT proteini başarılı bir şekilde geliştirilmiştir.

Anahtar Kelimeler: İmmünreaktif Tripsinojen, Kistik fibrozis, İmmünoesey, Proteolitik aktivite, ELISA, Western Blot.

ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my supervisors, Prof. Tamer YAĞCI, who gave me a precious chance to prove myself in the academic area, and Dr. Esin AKÇAEL, who not only shared her profound scientific knowledge with me but also taught me great lessons of life. I also would like to thank Assoc. Prof. Fatıma YÜCEL, who always supported, encouraged me and gave opportunities to improve myself. Their support, suggestions, and encouragement gave me the drive and will to complete this work. I would also like to thank my jury members Prof. Ayşegül KARAHASAN and Assoc. Prof. Nuri ÖZTÜRK for spending their valuable times to evaluate this thesis.

I wish to express my warm and sincere thanks to Özlem ERTEKİN, Şerife Şeyda PİRİNÇCİ, Bengü ERGENOĞLU, İlham BAHHAR and Tuğçe Nur ERALP for their collaboration to this research.

I special thanks to my dear lab friends, Gamze BÜRTEK, Hatice ÇELİK, İsmail Selim YIDIRIM, Seda SEMERCİ, İlkay Göksu POLAT, Göknur Gizem DİNÇ, Göksu GÖK, Gizem AKTUĞ, Zülal BİLİCİ and Harun KOCAAĞA for their help.

Finally, I want to thank, İbrahim HOCAOĞLU, Sevecen TUĞLU GÜRPINAR, Aydan SARAÇ and Fatih KARAKAYA for their friendship.

This study was carried out in Scientific and Technical Research Council of Turkey (TÜBITAK) Marmara Research Center (MAM) Genetic Engineering and Biotechnology Institute (GMBE) under the project "Development of Immunoreactive Trypsinogen (IRT) and Pancreatic Related Protein (PAP) ELISA Kits for Early Diagnosis of Cystic Fibrosis in Newborn Screening" supported by the Scientific and Technical Research Council of Turkey (TÜBITAK) 1003-SBAG, under Grant No. 115S124.

TABLE of CONTENTS

LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		Explanations
µg	:	Micrograms
ng	:	Nanograms
pg	:	Picograms
µl	:	Microliters
kDa	:	Kilodaltons
kb	:	Kilobases
rpm	:	Revolutions per minute
A	:	Amper
V	:	Volt
3D	:	Three-dimensional
APS	:	Ammonium persulfate
BAEE	:	Nα-Benzoyl-L-arginine ethyl ester hydrochloride trypsin substrate
CDS	:	Coding DNA sequence
CF	:	Cystic Fibrosis
ELISA	:	Enzyme-Linked Immunosorbent Assay
Fig.	:	Figure
GMBE	:	Genetic Engineering and Biotechnology Institute
GTÜ	:	Gebze Technical University
His Tag	:	Polyhistidine-tag
Ig	:	Immunoglobulin
IRT	:	Immunoreactive Trypsinogen
LB	:	Luria Bertani
LS-LB	:	Low Salt Luria Bertani
MAM	:	Marmara Research Center
MCS	:	Multiple cloning sites
min	:	Minutes
OD	:	Optical density
PBS	:	Phosphate-buffered saline
PBS-T	:	Phosphate Buffered Saline with Tween 20

PVDF	:	Polyvinylidene fluoride or polyvinylidene difluoride
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	:	Tris-buffered saline
TBS-T	:	Tris-buffered saline with Tween 20
TTL	:	Diagnostic Technologies Laboratory
TÜBİTAK	:	The Scientific and Technological Research Council of Turkey
WT	:	Wild-type
YPD	:	Yeast Extract-Peptone-Dextrose
YPDS	:	Yeast Extract-Peptone-Dextrose with Sorbitol

LIST of FIGURES

LIST of TABLES

1. INTRODUCTION

Immunoreactive trypsinogen (IRT) is a pancreatic enzyme precursor which is not in the active form as first secreted from pancreas [Brodrick et al., 1979; Nshimyumukiza et al., 2014]. After it is transported to the small intestine, it is activated and converted to trypsin [Kukor et al., 2003]. The inactive form is called as trypsinogen, and the active type is called as trypsin [Paju et al., 2000]. IRT is normally found in the bloodstream, but in some cases, such as cystic fibrosis, the level in the blood can rise. Significant changes of IRT concentration in the bloodstream can be used as a diagnostic marker in Cystic Fibrosis (CF) diagnosis of newborns. [Ratjen et al., 2015]. IRT levels of adult CF patients and healthy people have not a significant difference, so IRT is not an option for adult diagnosis. [Therrell et al., 2012]. In addition to CF, recent articles point to the potential of IRT for use as a diagnostic marker in pancreatitis and several cancer types such as ovarian, gastric and colorectal cancers [Itkonen, 2008; Korvuo et al., 1996; Koivunen et al., 1990; Paju et al., 2006].

CF is an autosomal recessive genetic disease affecting multiple systems and organs simultaneously, including lungs, pancreas, sweat glands and external glands in the respiratory and digestive system [Ratjen et al., 2015]. Early detection of the disease is vital to start an effective treatment strategy to increase the life quality of the patients. Therefore, in recent years, CF was included in the neonatal screening programs in many countries such as USA, New Zealand, Australia, most European countries and Turkey [Kleven et al., 2008; Castellani et al., 2009; İçke et al., 2017]. IRT levels are relatively high in newborns with CF and therefore, it is used as a neonatal screening marker for the disease. For diagnosis of cystic fibrosis, IRT levels are tested in those screening programs; using the membrane absorbed heel prick samples of newborns [Barbara et al., 2013; Crossle et al., 1979; Therrell et al., 2012]. It is essential to develop a sensitive, cost-effective and easy method for efficient monitoring and early diagnosis of these diseases. ELISA tests are commonly used in the screening programs and the development of efficient diagnostic tests is the key for reliable diagnosis of the disease. For the detection of Cystic fibrosis, protein-based ELISA kits are widely used [Therrell et al., 2012]. In every ELISA test, a calibration curve by using standard protein needs to be constructed to measure the concentration of the unknown sample [Voller et al., 1976]. However, usage of active trypsin limits the efficiency of the test since it cleaves the antibodies used as recognition material [Smith, M.A., Kalaria, R.N., Perry, 1993[Siriwardena et al., 2012; Oiva et al., 2011]. In order to prevent protease activity of IRTs, protease inhibitors or high pH buffers may be used whereas both of these strategies limit the interaction between antibody and IRTs, thus interfering with the measurement of IRT levels [Haverback et al., 1960 Spelbrink et al., 2011; Farady et al., 2014 Porstmann et al., 1992].

ELISA tests depend on antigen-antibody interaction, and hence, the reactive components used in the analysis are mostly proteins [Gan et al., 2013]. In the developed kits, IRT is used for the establishment of the standard curve for quantification. IRT is supplied as absorbed to a membrane similar to that used in the collection of heel bleedings and extracted in a buffer together with the samples. As IRT is a self-activating protease enzyme, the stability of membrane absorbed enzyme is a challenge in long-term storage [Kukor et al., 2002; Kukor et al., 2003].

IRTs developed in this study do not possess protease activity thus do not cleave proteins used in immunoassay studies. This is expected to increase the stability and shelf life of the IRT standards used in ELISA kits.

1.1. The Purpose, Contribution, and Content of Thesis

It was aimed to deactivate human IRT by mutating the amino acid residue at the cleavage site of trypsinogen into active trypsin and block its protease activity to enable its usage in immunoassay studies. Mutant IRT was recombinantly produced in *Pichia pastoris* X-33 strain. To prevent the protease activity of IRT, the lysine residue at the 23rd position was changed to aspartic acid. To produce IRT, coding DNA sequence (CDS) was newly designed, synthesized and cloned in multiple cloning sites of the yeast expression vector. The product was produced as fused to 6xHis Tag at N-terminus. The yeast expression plasmids containing alpha factor secretion signal, IRT CDS and 6xHis Tag was transformed to *P. pastoris* X-33 strain, and the desired protein was produced and purified. After purification, enzymatic activities of the proteins were measured with Nα-BenzoylL-arginine ethyl ester hydrochloride (BAEE) which is a trypsin substrate, and we found that the enzymatic activity of IRT is diminished. In parallel, the antigenic activity of two IRT isoforms was assessed by ELISA and Western Blot techniques.

2. LITERATURE REVIEW

2.1. Enzyme & Proenzyme

Enzymes are proteins that catalyze a biochemical reaction. Enzymes consist of a protein part (apoenzyme) that gives specificity and a non-protein part (coenzyme) that is necessary for the activity. Enzymes bind to their substrates, allowing them to undergo metabolic exchange or to combine with another molecule from the reaction chain. Most of the chemical reactions catalyzed by an enzyme are in one of six main groups [Sigman et al., 1975].

• Hydrolases

Catlysis hydro (water) based reactions (e.g., proteases).

• Transferases

Catalyze the transfer of functional groups from the donor molecule to the receptor.

• Oxireductases

Catalyze transferring one or more electrons or hydrogen atoms from a molecule to be oxidized to another molecule to be reduced (oxidation-reduction reactions).

• Isomerases

Catalyzes the modification of the position of an atom or a functional group in a molecule.

• Ligases

Catalyzes the assembly of two substrate molecules to form another molecule.

• Lyases

Catalyzes the opening of a molecule by a single non-hygroscopic reaction by forming double bonds in one or both of the molecules to be formed.

Their target molecule is called as a substrate, and the result is the product. Enzymes are one of the mandatory elements of living organisms. Without enzymes, most of the chemical reactions cannot be actualized [Khan et al., 1998].

Proenzymes, also called zymogens, are inactive precursor forms of enzymes. Without a structural alteration, proenzymes cannot be reformed into enzyme structure [Khan et al., 1998; Rinderknecht et al., 1984].

Enzymes and proenzymes are generally protein-based molecules. However, a few are catalytic RNA molecules [Guerrier-Takada et al., 1983].

2.2. Protease

Protease is an enzyme that catabolizes different kinds of proteins. It is also called proteinase or peptidase. Protein is catabolized via hydrolyzation of their peptide bonds by proteases. Thus, proteins are converted into smaller chains like peptides or even smaller units as amino acids [Farady et al., 2014; Rinderknecht et al., 1984]. According to their specialization proteases are categorized into seven main groups:

- Asparagine peptide lyases

  Proteases that use asparagine for digestion.

- Aspartic proteases

  Proteases that use aspartate carboxylic acid for digestion.

- Cysteine proteases

  Proteases that use cysteine thiol for digestion.

- Glutamic proteases

  Proteases that use glutamate carboxylic acid thiol for digestion.

- Metalloproteases

  Proteases that use metal ions for digestion.

- Serine proteases

    Proteases that use serine alcohol for digestion.

- Threonine proteases

    Proteases that use secondary threonine alcohol for digestion.

Proteases can be found in plants, bacteria, animals, archaea and even in viruses. They are essential molecules that have an essential role in the human digestion process.

2.3. Immunoreactive Trypsinogen (IRT)

Immunoreactive Trypsinogen (IRT) is the proenzyme form of the serine protease trypsin that is secreted from human pancreas for protein digestion [Therrell et al., 2012]. It has three major isoforms, IRT1 (UniProt P07477, GenBank NG_008307), IRT2 (UniProt P07478, GenBank NG_008322) and IRT3 (UniProt P35030, GenBank NG_001337). However, IRT1 and IRT2 are studied because they are widely used as diagnostic markers for CF. [Therrell et al., 2012]. The main difference between them is ionic, IRT1 is cationic while IRT2 is anionic [Kimland et al., 1989]. Other features are similar for both. IRT cleaves peptide chains of a substrate from lysine or arginine mostly [Kimland et al., 1989]. IRT is in trypsinogen form when it is first produced in the pancreas. IRT is approximately 24 kDa and 247 amino acids in length. IRT has a signal peptide between 1st and 15th amino acids, and an activation peptide resides between 16th and 23rd amino acids [Frossard, 2001; Gudgeon et al., 1990] (Fig. 2.1).

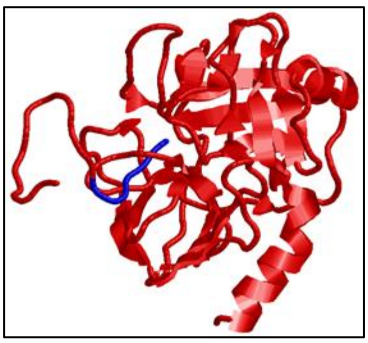

Figure 2.1: 3D Structure of IRT1. Activation peptide is shown as blue.

Because of high pH conditions of pancreatic juice, IRT can conserve its inactive form and migrates into the small intestine via duodenal papilla [Sahin-Toth, 2005; Kimland et al., 1989; Haverback et al., 1960]. Afterward, IRT is activated by enterokinases, autoactivation and low pH of the stomach [Haverback et al., 1960]. In some circumstances like Cystic Fibrosis, which is a genetic disease, IRT secretion into the small intestine is blocked. Hence IRT starts to become active and digests pancreas [Kimland et al., 1989; Haverback et al., 1960; Therrell et al., 2012]. So IRT can be found more than 70 ng/ml in the bloodstream of CF patients. [Barbara et al., 2013; Therrell et al., 2012; Crossle et al., 1979]. ELISA tests that detect CF use dried blood spots as the sample source, Table 2.1 points out the correlation between nanograms of IRT in 100 µl ELISA well and nanograms of a sample that dehydrated blood spot incubated with [Crossle et al., 1979; Li et al., 2006].

Table 2.1. Correlation of IRT amount between ELISA wells and blood sample.

IRT ng/ml blood	IRT ng/well
3000	9
2000	6
1000	3
500	1,5
250	0,75
150	0,45
50	0,15

2.4. Antibodies

Antibodies are one of the main components of the adaptive immune system, which is centered on lymphocytes bearing antigen receptors that are generated by somatic recombination, arose approximately 500 million years ago in jawed fish. The basic structure of an antibody molecule (Immunoglobulin, Ig) is a Y-shaped protein produced by different polypeptide chains. Plasma cells such as B-Cell produces these Y-shaped proteins. The general structure of immunoglobulin contains two heavy chains (H chains) and two light chains (L chains). Both consist of variable and constant amino acid sequence regions. Chains are held together with disulfide bonds in specific regions [Mariuzza et al., 2010].

The most important part of the antibody is the antigen binding site. The structure of the antibody recognizes a specific target on the foreign molecule (antigen), named epitope. The binding region of each antibody is unique and specific to a particular epitope on the foreign molecule (antigen). An antigen is defined as a foreign molecule, and it interacts with the antibody via six hypervariable regions, three of which are light and three of which are in the heavy chain. Amino acid sequences in hypervariable regions differ from one molecule to another. Although the function differs in details, they share relatively simple basic features. Regions outside of the hypervariable regions are not highly variable and are generally species-specific [Nössner et al., 1995; Tjandra et al., 1990].

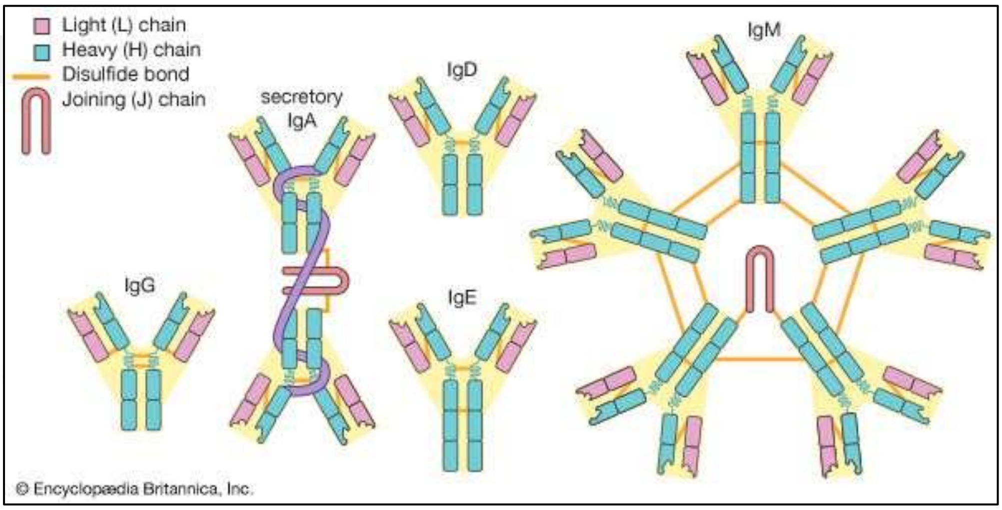

Figure 2.2: Immunoglobulin types.

As a result, antibodies can bind to the broad spectrum of different antigenic molecules because of their hypervariable regions, and another antibody can detect them because of their conserved regions. These features make them suitable for detection of a specific target and being recognized at the same time [Tjandra et al., 1990].

Antibodies are divided into five classes according to their conserved regions and function as shown in Fig. 2.2 [Web 1, 2018]. Each class name is formed by a letter appended to the letters "Ig", the abbreviation for the immunoglobulin term: IgG, IgM, IgA, IgD and IgE [Martin Flajnik and Masanori Kasahara, 2010; Nössner et al., 1995]. IgG is the most valuable immunoglobulin type for immunoassay researches because B cells secrete it into the media, that gives an easy opportunity for gathering. It also has a favorable structure that increases binding affinity, which is an essential factor for immunoassays.

2.5. Immunoassays

Immunoassays are biochemical detection test methods based on antibody-antigen interaction, in order to use the inherent ability of antibodies to bind to specific structures of target molecules. Because antibodies are developed and selected by use of one particular structure of antigens, or analytes, they are highly specific and can bind only to their target structure. Monoclonal and polyclonal antibodies are favorable components to detect and quantify particular target molecules. Two main commonly used immunoassay techniques are Western blot also called immunoblotting, and Enzyme-Linked Immunosorbent Assay (ELISA) [Porsch-Özcürümez et al., 2004; Cabrini et al., 1990].

Western blotting is a molecular detection technique, which can be applied after gel electrophoresis. Once the gel electrophoresis has been carried out, samples are transferred to the membrane, which has unique ability to bind any protein. After that, the membrane is blocked via a well-known non-reactive protein such as bovine serum albumin (BSA) or casein. After prevention of nonspecific binding of a protein to nitrocellulose or PVDF membrane, firstly primary antibody specific to the target is applied to the membrane. After that, the enzyme-labeled secondary antibody is used for tagging of the interaction. Monitoring of specific antibody-antigen interaction is achieved by application of substrate [Porsch-Özcürümez et al., 2004].

The other widespread immunoassay is enzyme-linked immunosorbent assay (ELISA) technique. Enzyme-linked immunosorbent assay (ELISA) is an enzyme-linked immunoassay method that is primarily used in immunology to detect the presence of an antibody or antigen in a sample. ELISA is used as a useful diagnostic method in many areas such as the food industry, medicine, and pathology. Basically, in an ELISA application, an antigen is bound to the surface of a well on a 96 well plate, and then a specific antibody is applied on the surface to attach the antigen. This antibody is bound to an enzyme, and in the last step, a substrate is added that can generate a detectable signal. The detectable signal means a change in the color. A spectrophotometer measures the resulting color change. The resulting absorbance value allows us to quantitate the amount of the product. ELISA technique is not such a different method from western blotting, but it offers us quantitative results also, while western blotting can only give us qualitative results [Porsch-Özcürümez et al., 2004; Pradelles et al., 1985; Cabrini et al., 1990].

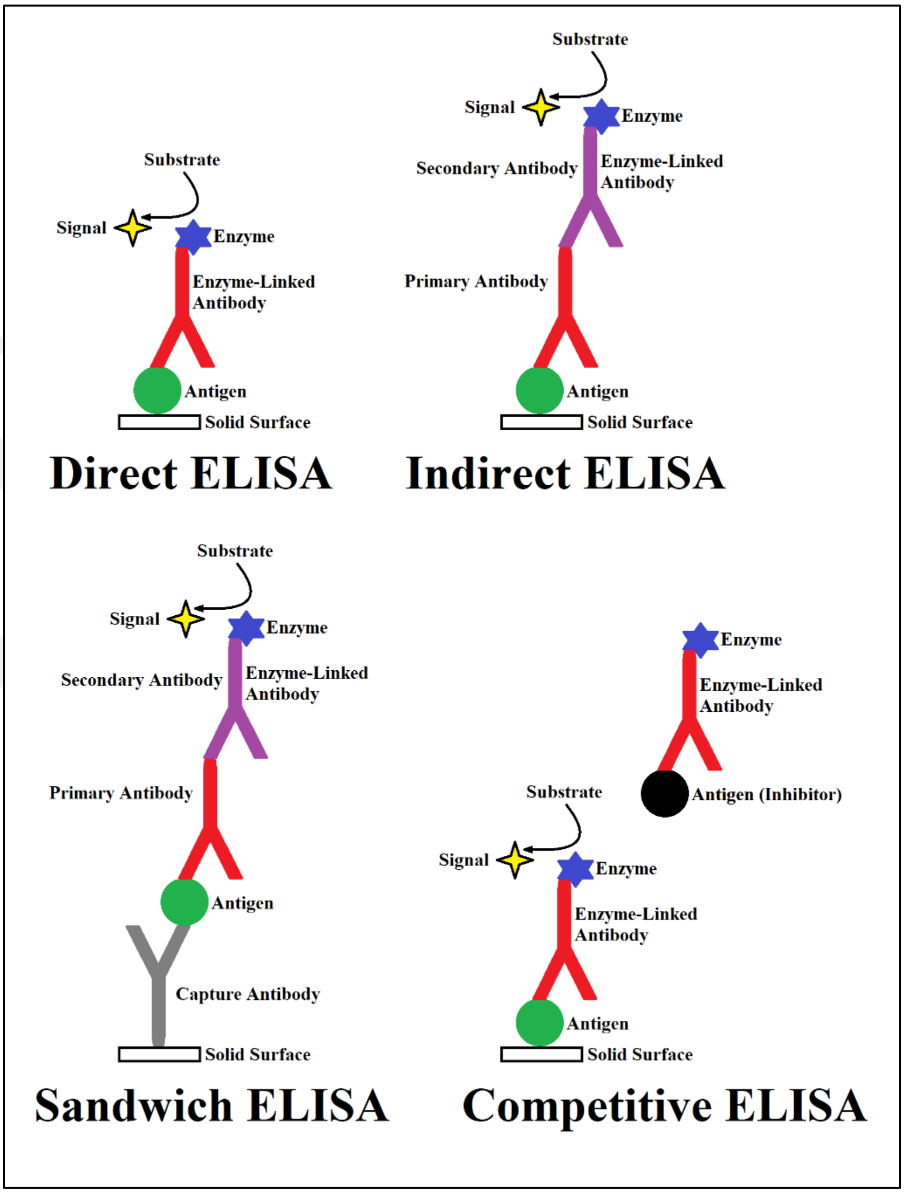

Figure 2.3: ELISA Types.

Four typical ELISA formats are sandwich, direct, indirect and competitive ELISA [Porstmann et al., 1992]. They can be defined as follows:

- Direct ELISA

In the direct ELISA method, the antigens in the sample are bound nonspecifically to the surface of a 96 well plate, then the labeled antibody is added to the wells. It is incubated for certain periods. With the washing procedure performed, unbound labeled antibodies are removed from the medium. The amount of bound enzyme-labeled antibody in the wells is determined by the color change caused by the addition of the substrate of the enzyme.

- Indirect ELISA

Indirect ELISA requires two antibodies, unlike direct ELISA. The first antibody is the unlabelled antibody that binds to the antigen, and the second antibody is the labeled antigen which attaches to the first antibody. The antigens in the sample bind to the well surface as same as direct ELISA. First, unlabeled primary antibodies are added to wells. Antigen and primer antibody is incubated for a certain period. The primary antibodies which are not bound by the washing procedure are removed from the medium. Subsequently, an enzyme-labeled secondary antibody specific for the primary antibodies are added to the wells. It is incubated for certain periods. The labeled secondary antibodies that are not bound by the repetitive washing procedure are removed from the medium. Finally, the enzyme is added to the substrate wells, and the resulting color change identifies antibodies. The organism from which the two antibodies used are derived is different, and this system is obtained by targeting this difference. For example, if the primary antibody is the mouse antibody, the secondary antibody is the anti-mouse antibody.

- Sandwich ELISA

This test determines the amount of antigen in samples. The color change caused by direct or indirect ELISA indicates the presence of the antibody, while the color change occurring in the sandwich ELISA suggests the presence of the antigen. In the sandwich, ELISA, antibodies (capture antibody) are immobilized to the solid phase of the wells of the 96 well plate. The antigen-containing sample is then added, and the antigen-antibody complex is expected to form. Washing steps remove unbound proteins. A second antibody (detection antibody) labeled with an enzyme is linked to a different epitope of the antigen bound to the capture antibody. After removal of the unbound detection antibody by washing, the substrate of the enzyme is added. The substrate reacts with the enzyme bound to the detection antibody and causes a color change. This color change is measured by a spectrophotometer. Color product formation is directly proportional to the concentration of the unmarked ligand (antigen or antibody in the serum). The main advantages of this technique are that the antigen does not need to be purified before use and the sandwich ELISA is very specific. The disadvantage of this technique is that each antibody cannot be used. Combinations of selected monoclonal antibodies should identify different epitopes on the same antigen. Structure of the antigen is also crucial for sandwich ELISA because if the surface of the antigen is not suitable for multiple antibody-antigen interactions, sandwich ELISA system will not work.

• Competitive ELISA

The primary purpose of this technique is to compare antibody-antigen interaction. The yield of antigen-antibody interaction can be measured by adding an antigen-coated antibody in the well that is coated with the antibody, which is specific to the same antigen. Alternatively, the pre-incubated antibody-antigen can be added to the antigen coated well, in order to detect the binding affinity. What is important is that the amount of signal generated in this technique is inversely proportional to the antigen-antibody interaction yield. Its steps are as follows: The antibody is bound to the solid phase of the wells of the 96 well plate. Sample containing labeled and the unlabeled ligand are added to the wells. During the incubation, the enzyme-labeled ligand competes with the unlabeled ligand to bind the immobilized antibody. In the competitive ELISA format which includes the immobilization of the antigen, labeled antibody and unlabelled antigen is added in the second step. Hence immobilized antigen and the free antigen compete for binding to a labeled antibody. After incubation and washing, the substrate is added. The color change resulting from the reaction is inversely proportional to the amount of unlabeled ligand in the sample. Measured signal decreases with increasing antigen amount in the sample.

3. MATERIALS

3.1. General Kits and Reagents

Table 3.1: List of kits and reagents.

EcoRI, Restriction Enzyme	R0101S, New England Biolabs (NEB), USA
NotI, Restriction Enzyme	R0189S, New England Biolabs (NEB), USA
T4 DNA Ligase	M0202S, New England Biolabs (NEB), USA
T4 DNA Ligase Reaction Buffer	B0202, New England Biolabs (NEB), USA
Thermo Scientific GeneJet Miniprep	K0502, Thermo Scientific™, USA
Zeocin™, Antibiotic	11006-33-0, Invitrogen, USA
Ampicillin, Antibiotic	A5354, Sigma-Aldrich, USA
Aprotinin, Protease inhibitor	000000010236624001, Roche, USA
pGAPZα A, *Pichia pastoris* Expression Vector	V20520, Invitrogen, USA
QIAquick Gel Extraction Kit	28704, QIAGEN, USA
PureLink™ PCR Purification Kit	K310001, Invitrogen, USA
Gel Loading Dye, Blue (6X)	B7021S, New England Biolabs (NEB), USA
1 Kb Plus DNA Ladder	10787018, Invitrogen, USA
Nα-Benzoyl-L-arginine ethyl ester hydrochloride, Trypsin substrate	B4500-10G, Sigma-Aldrich, USA
HisTrap HP histidine-tagged protein purification columns	17524701, GE Healthcare, UK
NBT/BCIP Ready-to-Use Tablets	11697471001, Roche, USA
3,3′,5,5′-Tetramethylbenzidine dihydrochloride hydrate powder, ≥98%	T8768, Sigma-Aldrich, USA
4-Nitrophenyl phosphate disodium salt hexahydrate	N4645, Sigma-Aldrich, USA
Pierce™ BCA Protein Assay Kit	23225, Thermo Scientific™, USA
Streptavidin-POD conjugate	11089153001, Roche, Switzerland
BLUeye Prestained Protein Ladder, 10 to 245 kDa	PM007-0500, GeneDireX, Taiwan
PageRuler™ Prestained Protein Ladder, 10 to 180 kDa	26616, Thermo Scientific™, USA
GangNam-STAIN™ Prestained Protein Ladder, 10 to 245 kDa	24052-3, iNtRON Biotechnology, Inc., South Korea
4x Laemmli Sample Buffer	1610747, Bio-Rad, USA

3.2. Buffers and Solutions

Table 3.2: List of buffers and solutions.

10X SDS Running Buffer	250 mM Tris-Base (pH:8.3) 1.90 M Glycine 1% SDS
Silver Staining Fix Buffer (1L)	50% Methanol 12% Acetic acid 0,5 ml Formalin (37% Formaldehyde)
Silver Staining Washing Buffer	50% Methanol
Silver Staining Sensitizing Buffer (1L)	0.2 g Na_2S2O_3
Silver Staining Band-Fix Buffer (1L)	2 g $AgNO_3$ 0,75 ml Formalin (37% Formaldehyde)
Silver Staining Developer Buffer (1L)	60 g Na_2CO_3 0,5 ml Formalin (37% Formaldehyde) 4 mg $Na_2S_2O_3.5H_2O$
Silver Staining Stop Buffer	50% Methanol 12% Acetic acid
10X TBS	200 mM Tris-Cl (pH: 7.6) 1.5 M NaCl
1X Transfer Buffer	10% 10X SDS Running Buffer 20% Methanol
1X TBST	1X TBS 0.1% Tween-20
5% Blocking Solution	5% non-fat dry milk in 1X TBS-T
1.5% Casein Blocking Solution	1,5% Hammerstein casein 1% Polyvinylpyrrolidone In 1xPBS
10X TBE (1L)	108g Tris base 55g Boric acid 40 ml 0.5M EDTA (pH 8.0)
1x PBS	137 mM NaCl 27 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4
1x PBS-T	137 mM NaCl 27 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 0.05% (v/v) Tween 20
Aprotinin buffer	50mM tris-HCl pH7.2 150mM NaCl 1 mM EDTA
LB Broth Medium (1L)	5 g Yeast Extract 10 g Trypton 10 g NaCl

Table 3.2: Continuation of the table.

LB Agar (1L)	5 g Yeast Extract 10 g Trypton 5 g NaCl 15 g Agar
Low Salt LB Broth Medium (1L)	5 g Yeast Extract 10 g Trypton 5 g NaCl
Low Salt LB Agar (1L)	5 g Yeast Extract 10 g Trypton 5 g NaCl 15 g Agar
YPD Broth Medium	1% Yeast Extract 2% Peptone 2% Dextrose
YPD Agar	1% Yeast Extract 2% Peptone 2% Dextrose 2% Agar
YPDS Broth Medium	1% Yeast Extract 2% Peptone 2% Dextrose 1 M D-Sorbitol
YPDS Agar	1% Yeast Extract 2% Peptone 2% Dextrose 1 M D-Sorbitol 2% Agar
His-Tag Binding Buffer	20 mM sodium phosphate 0.5 M NaCl 10 mM imidazole pH 7.4
His-Tag Elution Buffer	20 mM sodium phosphate 0.5 M NaCl 0.5 M imidazole pH 7.4

Table 3.3: List of SDS-PAGE separating gel recipe.

12% separating gel	5 ml	10 ml	15 ml
ddH_2O	2.15	4.3	6.45
40% Acryl:Bis	1.5	3	4.5
1.5 M Tris pH8.8	1.25	2.5	3.75
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.002	0.004	0.006

Table 3.4: List of SDS-PAGE separating gel recipe.

5% Stacking Gel	2 ml	4 ml	6 ml	8 ml
ddH_2O	1.46	2.92	4.38	5.84
40% Acryl:Bis	0.25	0.5	0.75	1
1 M Tris pH 6.8	0.25	0.5	0.75	1
10% SDS	0.02	0.04	0.06	0.08
10% APS	0.02	0.04	0.06	0.08
TEMED	0.002	0.004	0.006	0.008

3.3. Antibodies

Table 3.5: List of Antibodies used in this study.

Name	Target	Supplier
13C9	IRT	MAM-13C9, TÜBİTAK
4A7	IRT	4IT3, HyTest Ltd
6G7	IRT	MAM-6G7, TÜBİTAK
11F9	IRT	MAM-11F9, TÜBİTAK
2C4	IRT	4IT3, HyTest Ltd
5B1	IRT	4IT3, HyTest Ltd
Anti-Mouse Polyvalent Immunoglobulins (G,A,M)−Alkaline Phosphatase antibody produced in goat	Mouse Immunoglobulins	A0162-1ML, Sigma-Aldrich

3.4. Equipment

Table 3.6: List of equipment.

Electroporator	4309000019, Eppendorf AG, Germany
Microplate Reader	7091000, Bio Tek, USA
Microplate Washer	4071000, Bio Tek, USA 7310040, Bio Tek, USA
Laminar Flow Cabinet	Hera-Safe Class II Cabinet, Thermo Scientific, USA
Electrophoresis System	1640301, Bio-Rad, USA
Centrifuges	Eppendorf, USA Thermo Fisher Scientific, USA LMS Harmony, USA

Table 3.6: Continuation of the table.

Power Supply	Bio-Rad, USA
NanoDrop	Thermo Fisher Scientific, USA
Vortex	Scientific Industries, USA
TransBlot Transfer System	1704155, Bio-Rad, USA
Ice Machine	AF100, Scotsman, USA
15 ml, 50 ml Falcon Tubes	TPP, Switzerland
Petri	NUNC, Denmark
Water Bath	Nüve, Turkey
Spectrophotometer	Biochrom Libra, UK
Ultrasonic Bath	Bandelin, Germany
pH Meter	Mettler Toledo, USA
Medium-Pressure Liquid Chromatography System	NGC™, Bio-Rad, USA
Autoclave	Sistek, Turkey
Magnetic Stirrer	Sigma-Aldrich, USA
Lyophilizator	Martin Christ, Germany
Analytical Balance	Precisa Gravimetrics AG, Switzerland
Shaker	BioSan, Latvia
37^{o} C Incubator	Nüve, Turkey
Bench Top Shaking Incubator NB-205L	N-BIOTEK, South Korea
+4 ^{o} C, -20 ^{o} C Incubator	Bosch, Germany
ThermoMixer® Temperature Controller and Mixer	5382000015, Eppendorf, USA
Gel Documentation System	Bio-Rad, USA
UV Transilluminator	Bio-Rad, USA
Peristaltic Pump P-1	Pharmacia, USA
Fraction Collector	Bio-Rad, USA
CO_2 Incubator	Thermo-Scientific, USA

4. METHODS

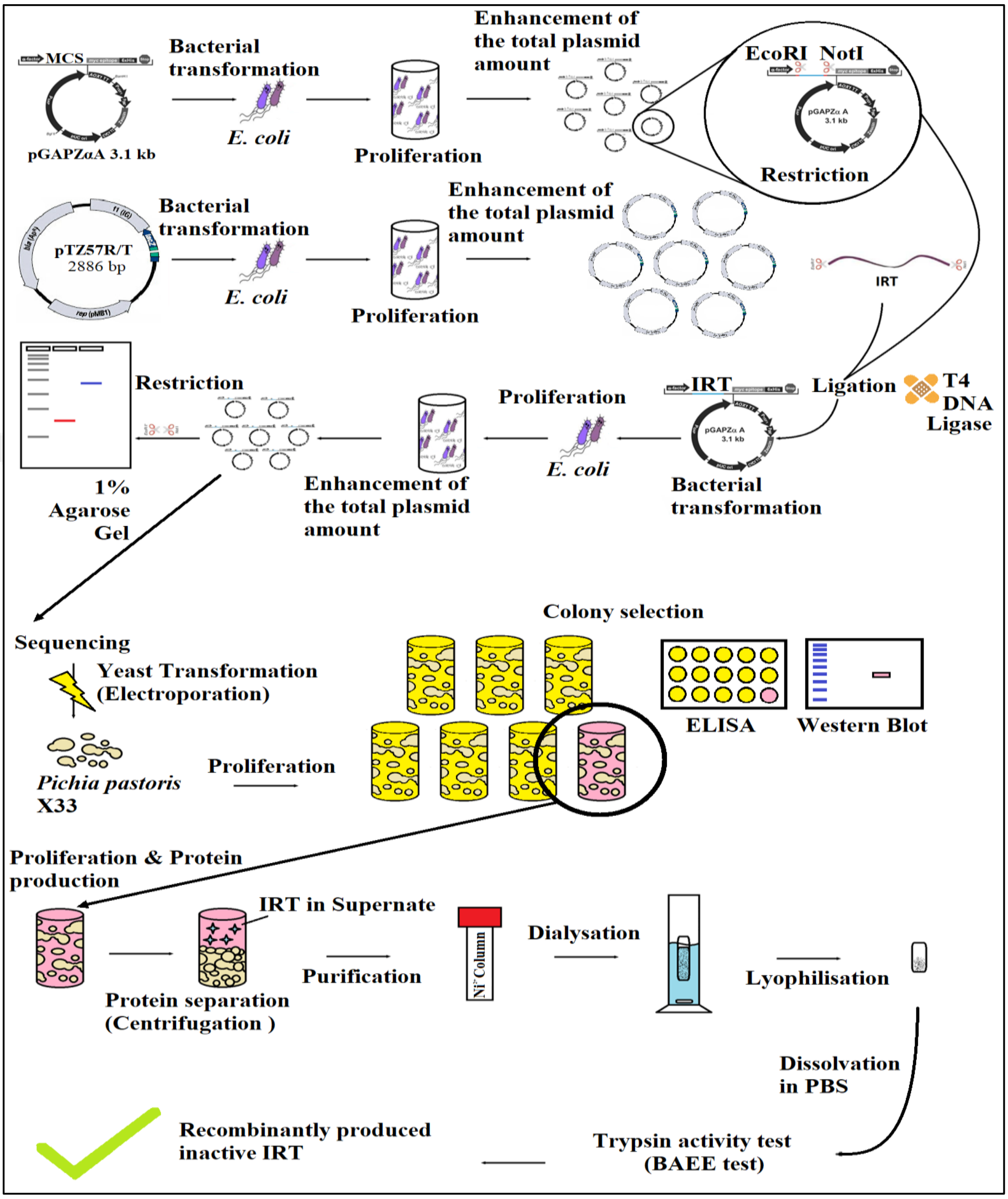

Figure 4.1: General scheme.

In order to produce inactive IRT, firstly IRT1 and IRT2 genes were designed and bought in pTZ57R/T bacterial vector. Also, pGAPZαA vector was purchased, and all of them are multiplied in E. coli. Both plasmids were isolated and cut by restriction endonuclease enzymes. IRT genes and pGAPZαA vector were reunited via ligation process. After that, the total amount of ligated plasmids were amplified by transforming into E. coli. Multiplied plasmids were isolated and controlled. Plasmids containing desired genes were transformed into Pichia pastoris X33. Integrated IRT genes that have alpha factor signal sequence makes yeast to secrete IRT1 and IRT2 protein into the culture medium. IRT1 and IRT2 proteins were isolated from the medium by using nickel column. Isolated proteins were dialyzed and lyophilized. IRT1 and IRT2 protein powders were dissolved to check whether it has trypsin-like catalytic activity. Non-catalytic IRTs are the ones being looked for. The general scheme is shown in Fig. 4.1. Each step of the figure is explained in detail below regarding titles.

4.1. Sequence Optimization

Nucleotide sequences of both IRT isoforms were reached via UniProt database (P07477, P07478). Their amino acid sequences accessed using the UniProt accession code also contains the signal peptide that regulates the intracellular trafficking of the protein produced in the cell. Typically, this signal peptide is not present in its mature form of two proteins. As the yeast was used in the protein production, the original signal peptide, which has a role in the mammalian cell, was not added and two sequences were designed by beginning from the 16th amino acid.

IRT, which is a protease, is in the form of trypsinogen when it was first produced. It cannot show enzymatic activity in this form. However, after the activation peptides between amino acids 16 and 23 were cleaved, they show protease activity. This activation peptide can be cleaved by enterokinases and trypsin itself. This poses a risk both of reducing production efficiency due to uncontrolled protease activity during protein production, and of cutting off the antibody in the environment in kit studies to be performed. For this reason, it was aimed to prevent activation of trypsinogen by changing Lysine to Aspartic acid that is located at 23^{rd} amino acid of the activation peptide of both two isoforms, thus eliminating the potential risks encountered in protein production, kit studies and antigen storage.

Another critical consideration in recombinant antigen production, codon optimization, was achieved using the IDT Codon optimization tool, which is available online (http://eu.idtdna.com/CodonOpt). Subsequently, the sequences that shown in results were obtained by adding sequences specific to EcoRI (GAATTC) and NotI

(GCGGCCGC) restriction enzymes, respectively, at the beginning and end of IRT1 and IRT2 sequence.

Since this vector was not a yeast expression vector, it will not provide protein expression when transformed directly into yeast. For this reason, it was necessary to transfer the designed genes to a yeast expression vector. So that pGAPZ αA, which is a yeast expression vector, was chosen for this purpose.

4.2. Bacterial Transformation

The modified sequence has to be multiplied. Consequently, newly designed DNAs were transferred into *E. coli* Top 10. *E. coli* Top 10 was chosen because is easy to use, well known and fast-reproducing bacteria strain.

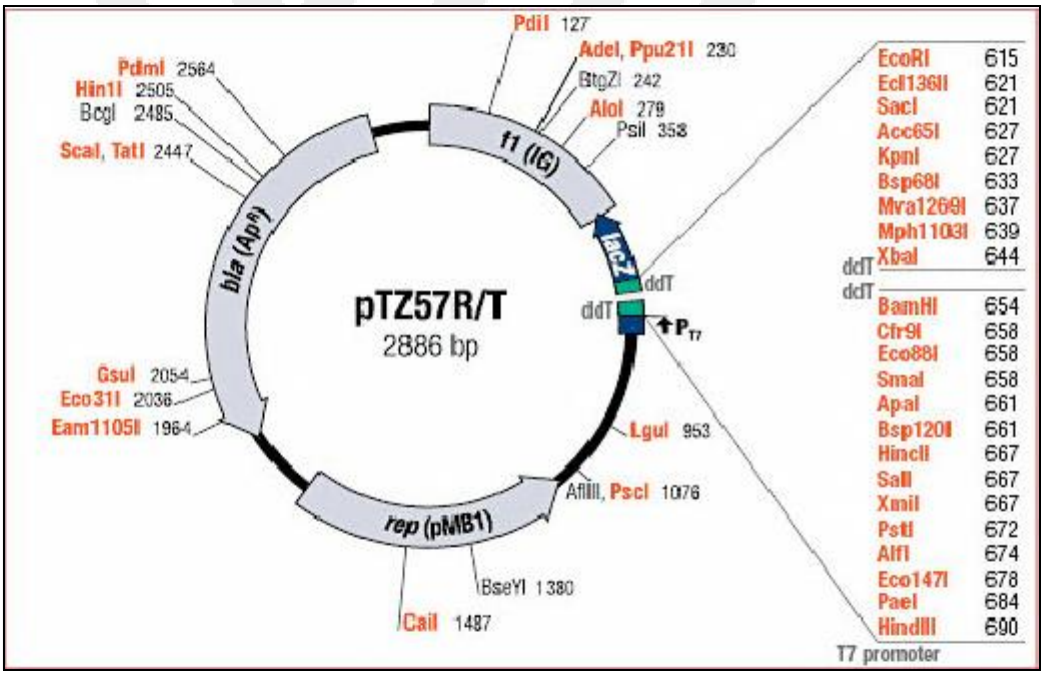

Figure 4.2: Map of the PTZ57R / T vector.

As seen on the map in Fig. 4.2, there is an α-secretory signal that allows the proteins produced in the yeast to be secreted into the culture supernatant before the multiple cloning sites [Web 2, 2018]. After multiple cloning sites, there are myc epitope and his tag sequences for facilitating the purification of the protein that produced. The following steps were followed in the transfer of the genes contained in the pTZ57R / T vector to the pGAPZαA vector [Web 3, 2018].

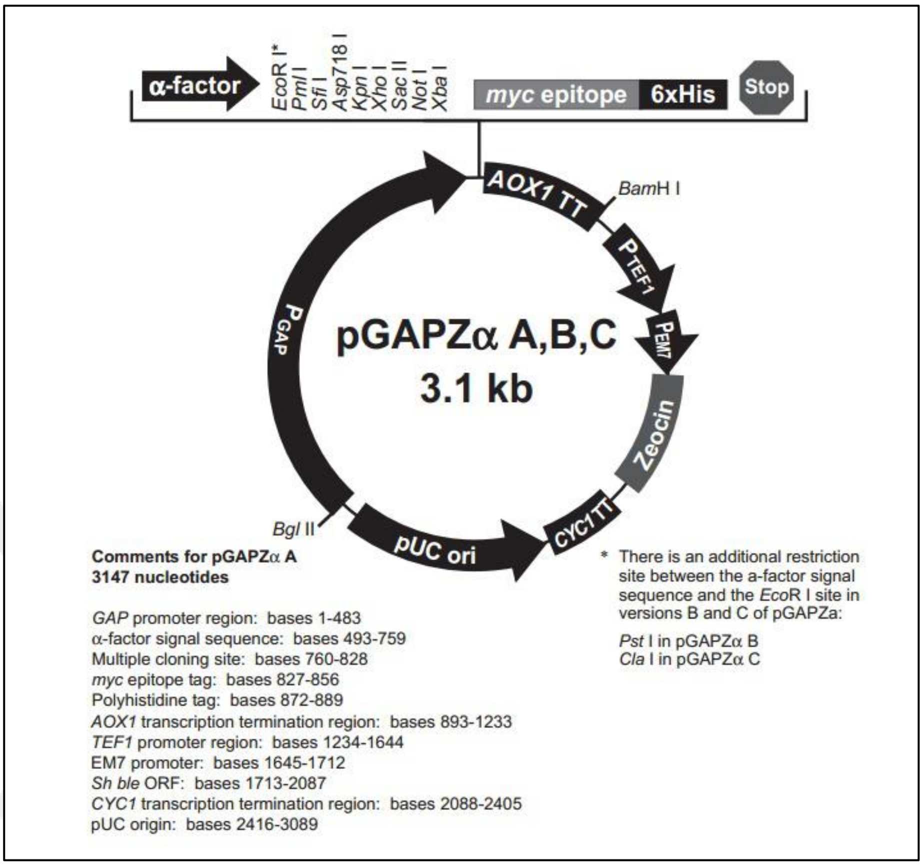

Figure 4.3: Map of the pGAPZ αA vector.

Bacterial transformation and molecular cloning were carried out in 8 steps, as seen below.

- Amplification of plasmids in *E. coli*.
- Isolation of plasmids from *E. coli*.
- Restriction of plasmids with EcoRI and NotI.
- Ligation of restricted IRT gene and pGAPZαA plasmid.
- Transformation of ligation reaction into *E. coli*
- Amplification of the colonies generated, in a liquid medium, and plasmid isolation.
- Restriction of isolated plasmids and testing with agarose gel size assessment.
- Sequence analysis.

4.2.1. Amplification of Plasmids in *E. coli*

Both IRT1, IRT2, and pGAPZαA cloning processes were the same, except pGAPZαA transformed *E. coli* incubated in Low Salt LB agar and Zeocin antibiotic (0.25µl/ml). In order to transfer plasmids into *E. coli*, firstly competent *E. coli* cells were prepared. For this, Top10 *E. coli* cells were grown in agar LB plates at 37°C overnight. The next day, a colony from LB agar was grown in 5 ml of liquid LB broth by shaking at 220 rpm at 37°C overnight. The following day, 5 ml of liquid bacterial culture was grown by adding 50 ml of LB and shaking at 220 rpm at 37°C until OD600 reached 0.48-0.75 in 2 hours. The culture reached the desired range of OD600 value and centrifuged at 4100 rpm for 7 min + 4°C by transferring to a 50 ml falcon tube. The supernatant was removed, and the pellet was dissolved in 15 ml 0.1 M sterile $CaCl_2$ and left on ice for 15 min. Subsequently, centrifugation was repeated at 4100 rpm for 7 min +4°C. The supernatant was removed, and the pellet was dissolved in 0.1M sterile CaCl2 + 15% glycerol solution and 100 µl of sterile Eppendorf tubes were dispensed. Aliquots were first frozen in liquid nitrogen. It was then transferred to -80°C.

For plasmid transformation into *E. coli*:

- The tubes were taken from -80°C and left on ice for about 10 min.
- The plasmids were placed on *E. coli* and incubated for 30 minutes on ice.
- The tubes were held for 1 min at 42°C and then placed on the ice again for 5 min.
- 800 µl LB heated to 37°C was added on the heat-shock exposed *E. coli* and incubated for 1 hour at 37°C.
- *E. coli* were centrifuged at 6000 rpm for 3 min, 600 µl of supernatant was discarded and the pellet resolved in the remaining LB.
- Resuspended *E. coli* cells were spread on the Ampicillin (µl/ml) agar LB plates allowed to stand overnight at 37°C.
- *E. coli* that can survive and grow on Ampicillin agar plates were *E. coli* that have acquired antibiotic resistance as a positive result of plasmid transformation.

4.2.2. Isolation of Plasmids from *E. coli*

Two single colonies for each IRT isoforms selected from Ampicillin LB agar plates were incubated in 5 ml of liquid LB with Ampicillin and incubated at 37°C overnight at 220 rpm. The following day, plasmids grown in E. coli were isolated as pointed out in the insert of the Thermo Scientific GeneJet Miniprep Plasmid Kit. Isolated IRT plasmids named as IRT1-pTZ57R/T for IRT1 and IRT2-pTZ57R/T for IRT2.

4.2.3. Digestion of Plasmids with EcoRI and NotI

In order to be able to perform the cloning process, both the cloning vector and genes in pTZ57R plasmids must be cleaved with the same restriction enzymes. For this, three reactions were set up, consisting of 50 µl of pGAPZαA, 50 µl of IRT1-pTZ57R / T and 50 µl of IRT2-pTZ57R / T, each containing approximately 2.5 µg of DNA and incubated at 37°C for 3 hours. Reaction conditions are shown in Table 4.2.3.

Table 4.1: Restriction reaction conditions.

	pGAPZαA	IRT1-pTZ57R/T	IRT2-pTZ57R/T
DNA	3,49 µl (~=2500ng)	3,77 µl (~=2500ng)	3,03 µl (~=2500ng)
CutSmart®	5 µl	5 µl	5 µl
EcoRI	0,5 µl	0,5 µl	0,5 µl
NotI	0,5 µl	0,5 µl	0,5 µl
ddH_2O	40,51 µl	40,23 µl	40,97 µl

After 3 hours of incubation of the reaction prepared using these conditions, 10 µL of 6X loading was loaded onto them and the assay was carried out by loading into a 1% agarose gel. The final volume was set to 50 µl because adding only 10 µl of 6x loading dye is easy to calculate.

After that, QIAquick Gel Extraction Kit’s manual was followed in order to isolate restricted genes and plasmids.

4.2.4. Ligation of the IRT Gene and pGAPZαA Plasmid

"Insilico Ligation Calculator" (http://www.insilico.uni-duesseldorf.de/Lig_Input.html) web tool was used to calculate the ligation reaction.

The following reaction was established in the light of the results of "Insilico Ligation Calculator" web tool. The prepared reactions according to Table 4.2 and Table 4.3 were set up and incubated overnight at 18°C.

Table 4.2: Reaction mixture for the ligation of IRT1 into pGAPZ αA.

IRT1	3,03 µl (85,89 ng)
pGAPZ αA	2,6 µl (75 ng)
T4 DNA Ligase	1 µl
T4 DNA Ligase Reaction Buffer	1,2 µl
Nuclease-free ddH_2O	4,17 µl
Total volume	12 µl

Table 4.3: Reaction mixture for the ligation of IRT2 into pGAPZ αA.

IRT2	2,37 µl (85.89 ng)
pGAPZ αA	2,6 µl (75 ng)
T4 DNA Ligase	1 µl
T4 DNA Ligase Reaction Buffer	1.2 µl
Nuclease-free ddH_2O	4,83 µl
Total volume	12 µl

4.2.5. Transformation of Ligation Reaction nto *E. coli*

The next day, the ligation reaction was transferred to the previously prepared competent *E. coli* cells. Genes transfer to the competent cells were the same as the previous procedure (4.2.1.), except the media. At this stage, mediums were prepared with a zeocin antibiotic, because of the existence of the Zeocin resistance gene in the pGAPZαA vector. This way performed Antibiotic-selection. Also, low-salt LB (LS-

LB) was prepared in order to prevent deactivation of Zeocin. Because the salt concentration of standard LB medium can deactivate Zeocin.
For IRT-pGAPZ αA gene transformation into *E. coli*:

- The tubes were taken from -80°C and left on ice for about 10 min.
- The plasmids were placed on the *E. coli* and incubated for 30 minutes on ice.
- The tubes were held for 1 min at 42°C and then placed on the ice again for 5 min.
- *E. coli* exposed to the heat-shock were placed on LS-LB heated to 37°C by placing 800 µl for 1 hour at 37°C.
- *E. coli* were centrifuged at 6000 rpm for 3 min, 600 µl of supernatant was discarded and the pellet resolved in the remaining LS-LB.
- The Zeocin-agar LS-LB plates, previously brought to room temperature, were allowed to spread their cells and were allowed to stand overnight at 37°C.
- *E. coli* that can survive and grow on antibiotic agar plates were bacteria that have acquired antibiotic resistance as a positive result of plasmid transformation.

4.2.6. Amplification of the Colonies Generated, in a Liquid Medium, and Plasmid Isolation

Growth colonies on Zeocin containing agar plates were incubated by, adding 25 µg/ml Zeocin containing low salt LB, and shaking at 220 rpm at 37°C overnight. Plasmids were isolated following the instructions of the Thermo Scientific GeneJet Miniprep plasmid isolation kit.

4.2.7. Restriction of Isolated Plasmids and Testing with Agarose Gel Size Assessment.

Isolated plasmids were restricted with the only EcoRI in order to linearise. Thereby length of the plasmid has been observed. Restriction reaction was set up same for both IRT1 and IRT2 as Table 4.4.

Table 4.4: Restriction mixture of the linearization reaction.

Plasmid	5 µl
Cut Smart Buffer	3µl
EcoRI	0,5 µl
Nuclease-free ddH_2O	21,5 µl
Total volume	30 µl

Prepared two reactions were incubated for 1 hour at 37°C. After 1 hour, 5 µl of each reaction were taken, mixed with 1 µl of 6X loading dye, and run in a 1% agarose gel.

4.2.8. Sequence Analysis

Sequence analysis was performed via the procurement of services. DNA samples were sent to Dr. ZEYDANLI Hayat Bilimleri Tic. Ltd. Şti. for sequencing. Sequencing results were examined via "BLAST" (https://blast.ncbi.nlm.nih.gov/Blast.cgi) web tool. For each IRT isoforms, pGap forward primer (GTCCCTATTTCAATCAATTGAA) was used as the former primer, and aox1 primer (GCAAATGGCATTCTGACATCC) was used as the reverse primer. 9 colonies have sent for IRT1, and 7 colonies sent for IRT2.

4.3. Production and Purification of IRT in *Pichia pastoris* X33

4.3.1. Transformation of the IRT Gene, Which Transferred to the Yeast Expression Vector, into the Yeast

Pichia pastoris X33 strain cells to be used in transformation were grown in 10 mL YEPD overnight at 30°C. The *P. pastoris* culture grown overnight was diluted using a total of 100 mL volume YEPD, with an A_{600} of approximately 0,3. The prepared culture was allowed to grow at 30°C for 4-5 hours. A_{600} value of the culture should be around 1-2. The concentration of the culture which measured was adjusted to be $8x10^8$ cells for each transformation. The cells in this concentration were precipitated at room temperature for 5 minutes at 4000 rpm. The supernatant on the cells was removed, and the cells washed with sterile water. The cells were again resuspended at room temperature for 5 minutes at 4000 rpm, poured out and resuspended in a mixture of 8 mL 100 mM LiAc, 10 mM DTT, 0.6 M sorbitol and 10 mM Tris-HCl pH 7.5 at room temperature for 30 minutes. After this time, cells were precipitated at room temperature for 5 minutes at 4000 rpm, removed from the supernatant and washed in 50 mL of 1 M sorbitol at 0°C. Finally, the cells were resuspended to a final concentration of 10^{10} cells / mL by repeated washing with 25 mL, 10 mL 1 M sorbitol. *Pichia* cells thus prepared were mixed with 8 µL of linearized DNA at a concentration of 3 ng / µL and incubated for 5 minutes on a 0.2 cm tube of ice. After waiting, the cells were electroporated at 2 kV. The time constant was greater than 5 ms. Following electroporation, 1 M sorbitol was added at 1 ºC at 0 ºC in the cuvette. The mixture was stirred at 30°C for 1 hour in a shaking incubator. YEPD containing 1 M sorbitol was added followed by 1 M in 30°C incubator and agitated by stirring for 1 hour. The whole mixture was grown in Petri dishes containing 100µg / mL Zeocin and allowed to grow at 30°C for 2-4 days.

4.3.2. Selection of IRT-Producing Yeast Colonies

The IRT gene transformed colonies were resuspended in 100 µL of water on a 96-well plate with sterile pipette tips after growing. 5 µl of resuspended yeast was added to Zeocin YEPD plates, and the remaining 95 µl was grown in 5 mL of liquid YEPD for 96 h at 30°C in a shaking incubator. Untransformed strain X33 was also incubated as a negative control. The yeast supernatants obtained from 24 colonies were checked by sandwich ELISA, for the elimination of the colonies that were not producing IRT1 and IRT2 proteins. Each antibody used for ELISA can recognize both IR1 and IRT2 isoforms.

For the sandwich ELISA, 100 ng/well of mouse-originated IRT antibody 2C4 (HyTest Ltd 4IT3) was first coated onto the plate bottom and incubated for 1 hour at room temperature. The antibody-coated wells were then washed with 0,5% Tween 20 in PBS and blocked with 1% Hammerstein Casein, 1% PVP (Polyvinylpyrrolidone) and 30 µl / ml 1M NaOH overnight at + 4°C. Because of the lower background signal, this mixture was used as the blocking solution in all sandwich ELISA applications performed within the scope of this study. Blocked wells were washed the next day.

After washing, 100 µl of the supernatant was added to the wells and incubated for 1 hour at 37°C. 50 ng of Human Trypsin 1 Recombinant (MCLAB, HTR1-200) was used for positive control of IRT1, and 50 ng of Trypsin-2 Human Recombinant (RayBiotech, 228-13214-3) was used for positive control of IRT2. Subsequently, IRT supernatants were washed and incubated for 1 hour at 37°C with the addition of 100 ng / well mouse-derived Biotin-labeled IRT antibody 4A7 (HyTest Ltd 4IT3) and the plate was washed 5 times. The plate was then stained with HRP labeled 4A7 TMB substrate. After color formation, the reaction was stopped with 2M H_2SO_4. The resulting color was measured at 450 nm.

Western blotting and silver staining methods were also used as a selection method. For this purpose, SDS-PAGE was performed firstly. 2 same 12% 10ml separating SDS-PAGE gels (Table 3.3) were prepared in a falcon tube and poured between 0,75 mm glasses which were placed in the gel casting system. 2-propanol was layered top of the gels to make the top of the gels to enable polymerization of the gel. After separating gels become solid, stacking gels were prepared (Table 3.3) and combs were inserted on the gel. Polymerization of gels take approximately 30 minutes, then combs were removed, and the gels were washed with dH_2O and put in the vertical electrophoresis system which was filled with 1X running buffer (Table 3.2). Protein samples were span to prevent losing some of them. 20 µl of samples were loaded to each well of the gel one by one. First and the second gels were prepared and loaded entirely same. 500 ng IRT1 (MCLAB, HTR1-200) was used as a positive control in the germs, and Thermo prestained protein marker (26616) was used as a marker. 20 µl of final volume was used because 0,75 mm glasses were used. Each 5 µl of 4x Laemmli Sample Buffer was used for each 15 µl of sample.1X SDS Running Buffer was used for electrophoresis via diluting with dH_2O from 10X Stock Solution. The running process was started with 80V. The proteins were run at 80V along the stacking gel until to reach to the separating gel portion. After arriving at the separating gel, the process was started to be carried out at 120V, and the execution was continued until the loading dye was out of the gel (Takes approximately 90 minutes). One of the gels was used for Western Blotting, and the other one was spared for Silver Staining.

For Western Blot a PVDF and 6 blotting papers with the same size with SDS PAGE gel was cut. The PVDF membrane should be activated before use, so the membrane was bathed with methanol for 30 seconds and then washed 3 times with water. Then the membrane was incubated in Shaker (at 60 rpm) for 10-20 min in

Transfer Buffer. The blotting paper and the PVDF membrane were held in 1X transfer buffer. Then 3 blotting papers, the PVDF membrane, the SDS gel, another 3 blotting papers were stacked respectively on the cassette of Trans-blot Turbo Transfer System (Biorad). For each layer, except the membrane, a roller was used to remove air bubbles. The transfer was actualized in 20 minutes with 20V and 2.0 A. After transferring, the membrane was blocked overnight at + 4°C in 1X TBS containing 5% skimmed milk powder to avoid non-specific bands. The following day the blocked membrane was washed with TBST (Table 3.2) for 10 minutes 3 times. Then 13C9 antibody (Table 3.3), which is an antibody developed in TÜBİTAK MAM GMBE TTL, was used in 1:300 dilution with PBS (Table 3.2). The PVDF membrane was incubated in 13C9 antibody solution for an hour at room temperature. Subsequently, the membrane was washed with TBST (Table 3.2) for 15 minutes 3 times. After that, Alkaline Phosphatase linked Anti-Mouse Polyvalent antibody (Table 3.3.) was used in 1:3000 dilution with PBS (Table 3.2). The incubation takes 1 hour at room temperature. The membrane was washed with TBST (Table 3.2) for 15 minutes 3 times again. One NBT/BCIP Ready-to-Use Tablet was dissolved in 10 ml dH_2O and poured on the membrane. The membrane was incubated in the dark and after approximately 5 minutes of results were observable. After the bands were observed, the membrane was washed with dH_2O and dried. Dry membrane was scanned in the scanner.

The other gel that was reserved for Silver Staining method was being also done. Silver Staining is a method that nonspecifically stains all proteins after separation of proteins according to the difference in mass sizes. In this staining method, all solutions were prepared with ultrapure water, and the gel was placed in a plastic container. In the beginning, the gel was kept in the Fix buffer (Table 3.2) overnight. Then, the gel was washed in the Wash buffer (Table 3.2) 3 times for 20 minutes each. The gel was then washed 3 times for 20 seconds with dH_2O. It was then shaken in a band-fix buffer containing silver nitrate (Table 3.2) for 20 minutes. Subsequently, it was washed twice with dH_2O for 20 seconds. The development buffer (Table 3.2) was agitated for 10 minutes for imaging. The gel was then rinsed with dH_2O for 2 minutes 2 times. It was shaken for 10 minutes with stopping buffer (Table 3.2) so that the bands could not continue to develop. The resulting gels were then scanned on the scanner.

4.3.3. Medium Optimization

After determination of IRT producing colonies, optimum medium conditions for protein expression experimented. As mentioned Table 3.2 original YPD includes 2% glucose. Apart from that colonies were incubated in 0%, 0,25%, 0,5%, 1% and 2% glucose inclusive YPD broth medium. IRT1 colonies were incubated for 96 hours while IRT2 colonies held for 72 hours according to optimum incubation period that detected in 4.3.2. After incubation ELISA, SDS-Gel, Western Blot, and Silver Staining methods have performed as same as 4.3.2. except SDS-Gel's loading dye was GangNam-STAIN, not BLUeye Prestained Protein Ladder.

4.3.4. IRT Purification

A 6x His-tag sequence was added in order to make purification easier when the both IRT1 and IRT2 sequences were designed. For this reason, a nickel column, HisTrap HP (GE Healthcare, 17-5247-01), was used for IRT purification. A peristaltic pump (Pharmacia, P-1) was set to 1 ml/min flow rate and fractions collected in 1,5 ml tubes via Bio-Rad 2110 fraction collector. The nickel column purification procedure is a method based on the affinity of nickel to imidazole. Sodium phosphate buffer (20 mM sodium phosphate, 0,5 M NaCl, pH 7.4) can be used as binding or elution buffer depending on imidazole concentration since imidazole is a molecule that can be found in histidine structure and can compete with histidines. Both of them have affinity against nickel. Depending on the concentration of imidazole, the binding affinity of the column can be adjusted. Thus only poly-histidine tailed proteins are allowed to bind because of their high affinity instead of weak natural histidine-containing proteins. Elution buffer contains 500 mM of imidazole because free imidazoles replace themselves with proteins that have with a poly-histidine tail. The used column can be reused by washing with water at the end of the process. Because of the conformational structure of each protein is different, their optimal imidazole concentration of binding and elution buffers are different also. So, a buffer optimization step was required firstly, to perform successful nickel column purification. Optimization of the imidazole concentration was critical at this stage, since increasing the amount of binding by reducing the level of imidazole may also increase the non-specific binding of the colony and result in a lower purity product. For this reason, the sample was first passed through the column with different binding buffers, and each tube was read at OD 280 in a spectrophotometer (Biochrom LibraDuring the purification process, the buffer contained in each tube was read, and the collected fractions were recorded. Distilled water (5 ml), binding buffer (5 ml), sample (5 ml sample + 5 ml binding buffer), resuspension buffer, elution buffer, distilled water were passed sequentially from the column. The sample was transferred to the desired volume, then resuspended in the binding buffer and flow continued until the OD 280 value fell below 0.05. After unwanted molecules were washed away and the HisTagged proteins were bound to the column, elution buffer with high imidazole concentration was applied to the column. By this, imidazole is attached to the nickel, and HisTagged proteins were purified. Lastly, the column was washed with 10 ml of distilled water.

After the binding buffer optimization for each IRT isomers, the same process was repeated. Then IRT containing fractions were dialyzed against distilled water and lyophilized. After lyophilization, IRT that resuspended in PBS were ready for experimental tests.

4.4. Characterization of IRT1 and IRT2

In order to reveal specific characteristics of mutant IRT1 and IRT2 and compare with wild-type forms, characterization studies were carried.

4.4.1. ELISA Test

For the sandwich ELISA of IRT1, 400 ng/well of mouse-originated IRT antibody 4A7 (HyTest Ltd 4IT3) was first coated onto the plate bottom and incubated for 1 hour at room temperature. The same process was performed with 400 ng/well of mouse-originated IRT antibody 11F9 (TÜBİTAK MAM) for IRT2. After this point, the process is the same for both IRT1 and IRT2. The antibody-coated wells were then washed with 0,5% Tween 20 in PBS and blocked with 1% Hammerstein Casein, 1% PVP (Polyvinylpyrrolidone) and 30 µl / ml 1M NaOH overnight at + 4°C. In order to lower the background signal, this mixture was used as the blocking solution in all sandwich ELISA applications performed within the scope of this study. Blocked wells were washed the next day. After washing, serial diluted mutant IRT1 (IRT1MAM), mutant IRT2 (IRT2MAM), IRT1 positive control (Human Trypsin 1 Recombinant, MCLAB, HTR1-200) without inhibitor (Aprotinin), IRT1 positive control (Human Trypsin 1 Recombinant, MCLAB, HTR1-200) with inhibitor in 1:1000 dilution (Aprotinin), IRT2 positive control (Trypsin-2 Human Recombinant, RayBiotech, 228-13214-3) without inhibitor (Aprotinin) and IRT2 positive control (Trypsin-2 Human Recombinant, RayBiotech, 228-13214-3) with inhibitor in 1:1000 dilution (Aprotinin) was used. Positive control samples that include aprotinin was dispersed in 50mM Tris-HCl pH7.2 150mM NaCl, 1 mM EDTA buffer, instead of PBS. Serial dilution was set up to detect mutant IRTs antigenic activity around CF detection threshold (70 ng/ml blood) as mentioned in Table 2.1. Subsequently, IRT samples were washed and incubated for 1 hour at 37°C with the addition of mouse-derived Biotin-labeled IRT antibody 5B1 (HyTest Ltd 4IT3) in 1:4000 concentration and the plate was washed 3 times. After that, the plate was incubated with Streptavidin-POD conjugate in 1:20000 concentration and washed 5 times. The plate was then stained with HRP labeled 4A7 TMB substrate. After color formation, the reaction was stopped with 2M H_2SO_4. The resulting color was measured at 450 nm.

4.4.2. Western Blot Test

Western blotting was used for the identification of mutant IRT1 and mutant IRT2 and compare them with their wild-type forms. Steps were similar to section 4.3.2. This time PageRuler™ Prestained Protein Ladder was used as the ladder, and 500 ng of proteins were loaded to each well.

4.4.3. IRT Proteolytic Activity Test

IRT (Immunoreactive Trypsinogen) is a protease as mentioned earlier and has low stability. While the recombinant IRT proteins were being designed, the mutation carried out in the direction of proteolytic activity avoidance has been described previously. IRT proteolytic activity test was performed by using a trypsin substrate BAEE (Nα-Benzoyl-L-arginine ethyl ester hydrochloride) (Sigma-Aldrich, 2645-08-1). to measure the effect of the mutation made in this direction. The reaction is shown in Fig. 4.4. was applied, in order to understand whether the recombinant mutant protein could or could not avoid Trypsinogen-Trypsin transformation. The experimental protocol, the steps followed during the experiment and the test results were as follows.

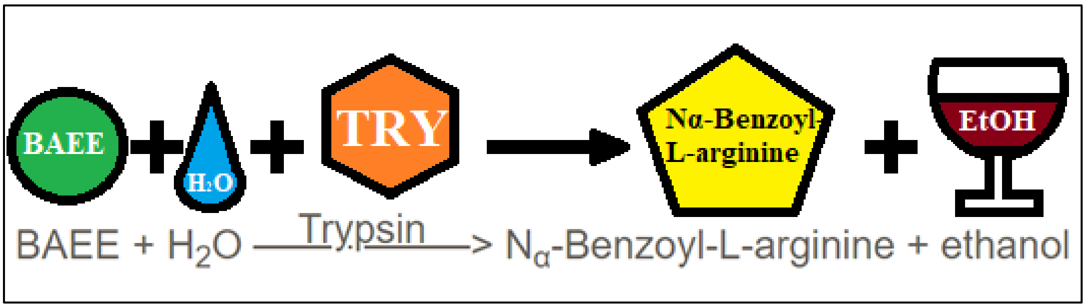

Figure 4.4: BAEE - Trypsin reaction scheme.

For the BAEE test, the solutions were first prepared at room temperature using ultrapure water. The recipes of the solutions prepared:

- Buffer (67 mM Sodium Phosphate Buffer, pH 7,6 at 25°C) – Prepare an 8,04 mg/ml solution using sodium phosphate, monobasic in ultrapure water. Adjust to pH 7,6 at 25°C with 1 M NaOH solution.
- Substrate Solution (0,25 mM Nα-Benzoyl-L-arginine ethyl ester) – Prepare a 0,086 mg/ml solution using Nα-Benzoyl-L-arginine ethyl ester in Buffer.
- HCl Solution (1 mM Hydrochloric Acid) – Prepare a 1,000-fold dilution of 1 M Hydrochloric acid solution in ultrapure water.
- Enzyme Solution (Trypsin) – Immediately before use, prepare a solution containing 425-575 units/ml of Trypsin in cold (2-8°C) HCl Solution.

After the solutions were prepared, 3 ml of Buffer was mixed with 200 µl of HCl solution for reference and read in spectrophotometer readings at 253 nm absorbance (A_{253}) for one minute on quartz cuvettes for 6 times.

5. RESULTS

5.1. Sequence Optimization

Newly designed IRT sequences that contain EcoRI (GAATTC) and NotI (GCGGCCGC) restriction sites were designed. As figured out at methods, with the help of biocomputational tool the following modified sequences were obtained.

5.1.1. IRT1 Sequence

Optimized IRT1 sequence is used as follows. Restriction sites are shown in red, and the K23D mutation is shown in blue.

GAATTCGCACCTTTTGATGATGATGAC**GAT**ATTGTGGGCGGATACAACT
GTGAGGAAAATTCAGTTCCATACCAGGTTTCTCTTAATAGTGGGTACCAC
TTCTGTGGTGGTTCTCTTATAAATGAACAGTGGGTTGTGTCTGCTGGGCAT
TGCTACAAGTCACGAATCCAAGTCAGGTTAGGCGAACACAATATTGAGG
TGTTGGAGGGAAACGAGCAGTTTATAAACGCAGCTAAGATCATCAGGCA
TCCACAGTACGATCGAAAGACTTTGAATAATGACATAATGCTTATTAAGT
TGTCAAGTCGTGCTGTTATTAATGCCAGGGTTTCTACTATTTCTCTGCCTA
CCGCTCCCCCTGCTACGGGCACAAAGTGCCTTATTTCAGGGTGGGGAAAT
ACTGCTTCCTCAGGCGCTGACTACCCAGACGAGCTACAATGTCTAGATGC
TCCTGTATTGAGTCAAGCCAAATGTGAGGCCAGTTACCCAGGAAAGATTA
CCTCCAACATGTTCTGCGTTGGTTTTCTGGAGGGTGGAAAGGATAGTTGT
CAGGGAGATTCTGGTGGGCCAGTTGTCTGTAACGGTCAACTGCAAGGTGT
AGTGTCATGGGGCGACGGTTGCGCTCAAAAAAACAAACCTGGTGTGTAT
ACAAAGGTATACAATTACGTGAAGTGGATTAAGAATACCATTGCCGCTA
ATTCT**GCGGCCGC**

5.1.1. IRT2 Sequence

Optimized IRT2 sequence is used as follows. Restriction sites are shown in red, and the K23D mutation is shown in blue.

GAATTCGCCCCTTTTGATGATGATGACGATATCGTCGGTGGCTATATATG
CGAGGAGAACTCAGTTCCATACCAGGTTTCCCTTAACTCCGGTTATCATT
TCTGTGGCGGAAGTTTGATATCAGAACAGTGGGTCGTTTCAGCTGGCCAT
TGCTATAAGTCACGAATTCAAGTAAGACTAGGAGAACACAACATAGAAG
TCTTGGAGGGTAATGAGCAATTCATTAACGCAGCCAAGATAATCAGACA
CCCAAAGTATAATAGTCGTACTCTGGATAATGACATACTTTTGATTAAAT
TGTCCTCTCCCGCAGTTATAAATTCCCGTGTCTCTGCTATCAGTCTGCCTA
CTGCCCCTCCAGCTGCCGGAACCGAGTCATTGATCTCCGGTTGGGGTAAT
ACATTGTCCTCTGGCGCTGACTATCCTGACGAACTGCAATGCTTAGATGC
TCCTGTCCTAAGTCAGGCCGAGTGTGAGGCATCCTATCCTGGAAAAATCA
CCAACAACATGTTTTGTGTAGGGTTTTTAGAAGGCGGAAAGGATTCATGC
CAAGGTGATTCTGGTGGGCCTGTGGTTTCAAACGGTGAGTTGCAAGGTAT
CGTTTCTTGGGGATATGGTTGTGCCCAGAAGAACAGGCCAGGCGTCTATA
CAAAGGTCTATAATTACGTGGACTGGATCAAAGATACCATCGCTGCTAAT
AGT**GCGGCCGC**

5.2. Bacterial Transformation

5.2.1. Amplification of Plasmids in *E. coli*

Transformation of the ampicillin resistance gene including plasmids provides survival of colonies on LB plates that have ampicillin. There were some colonies survived on the plate, indicating that the transformation was successful. For each IRT isoform survivor colonies were obtained.

5.2.2. Isolation of Plasmids from *E. coli*

In order to increase the total amount of IRT genes and pGAPZαA plasmid, the bacterial transformation was made. After that, a colony selected from antibiotic LB agar plates and incubated overnight. The next day, plasmids were isolated, and the concentration of plasmids was measured in Nanodrop. Results show that isolation was successful. 661,8 ng/µl of IRT1, 822.7 ng/µl of IRT2 and 716,0 of pGAPZαA plasmids were isolated.

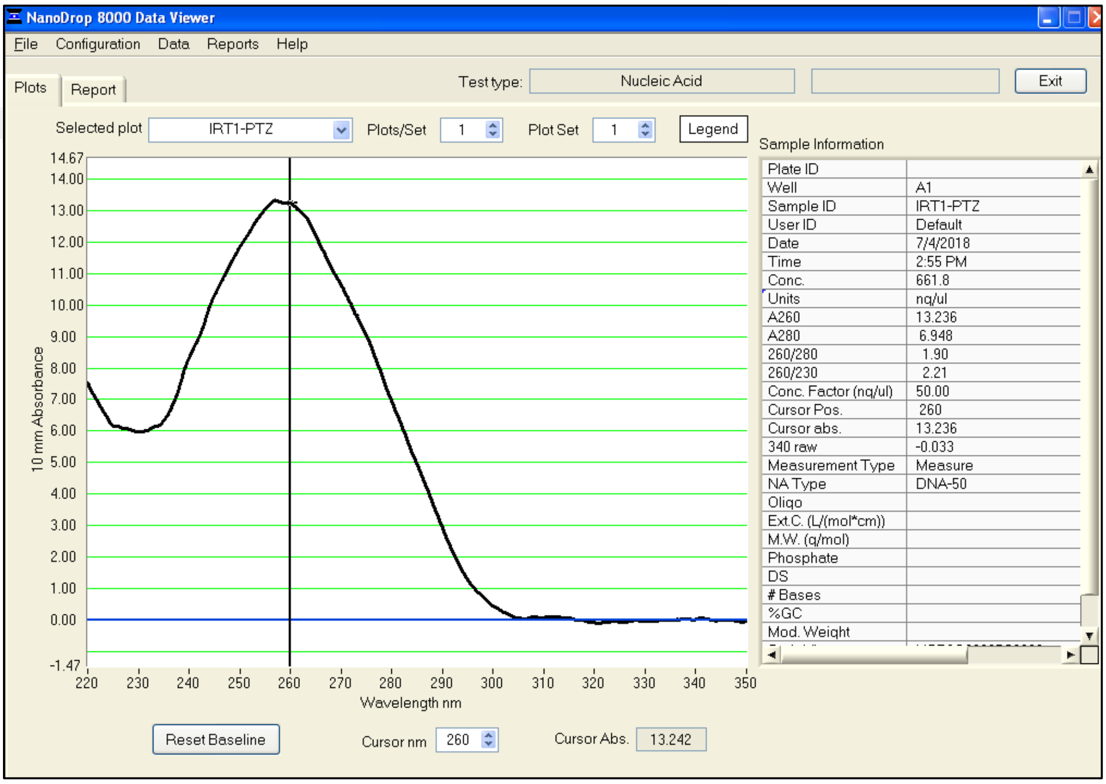

Figure 5.1: Concentration of IRT1, measured by Nanodrop.

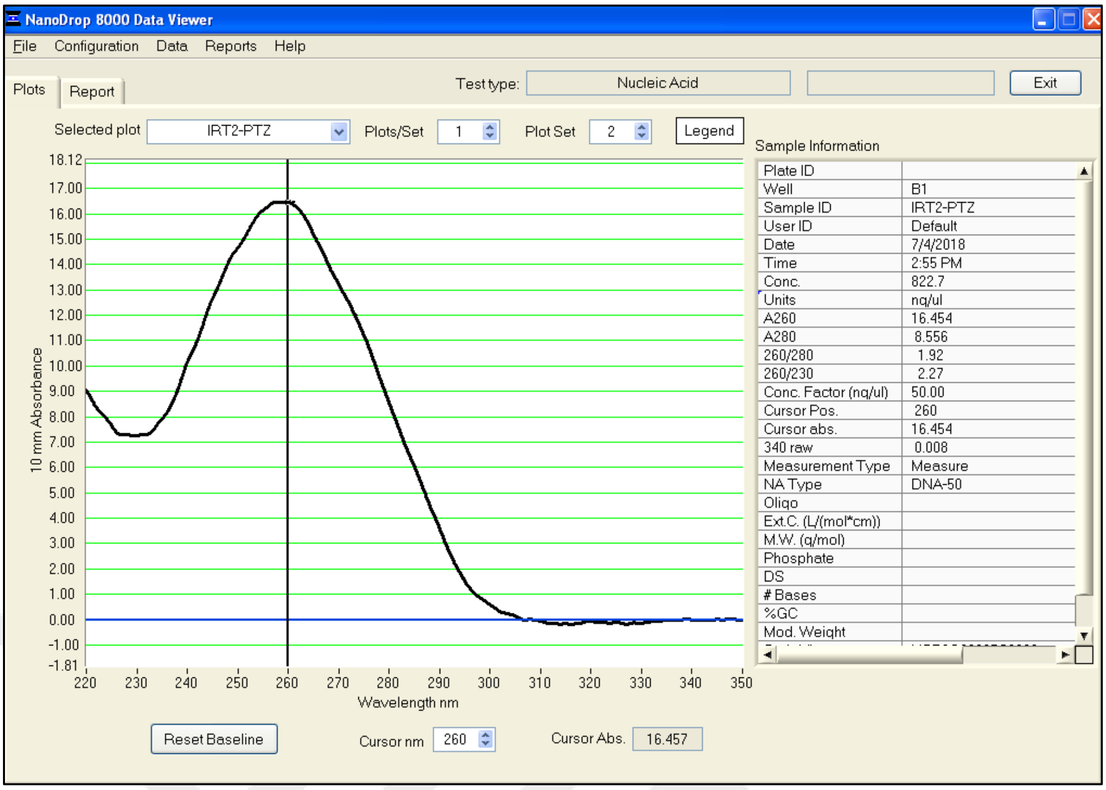

Figure 5.2: Concentration of IRT2, measured by Nanodrop.

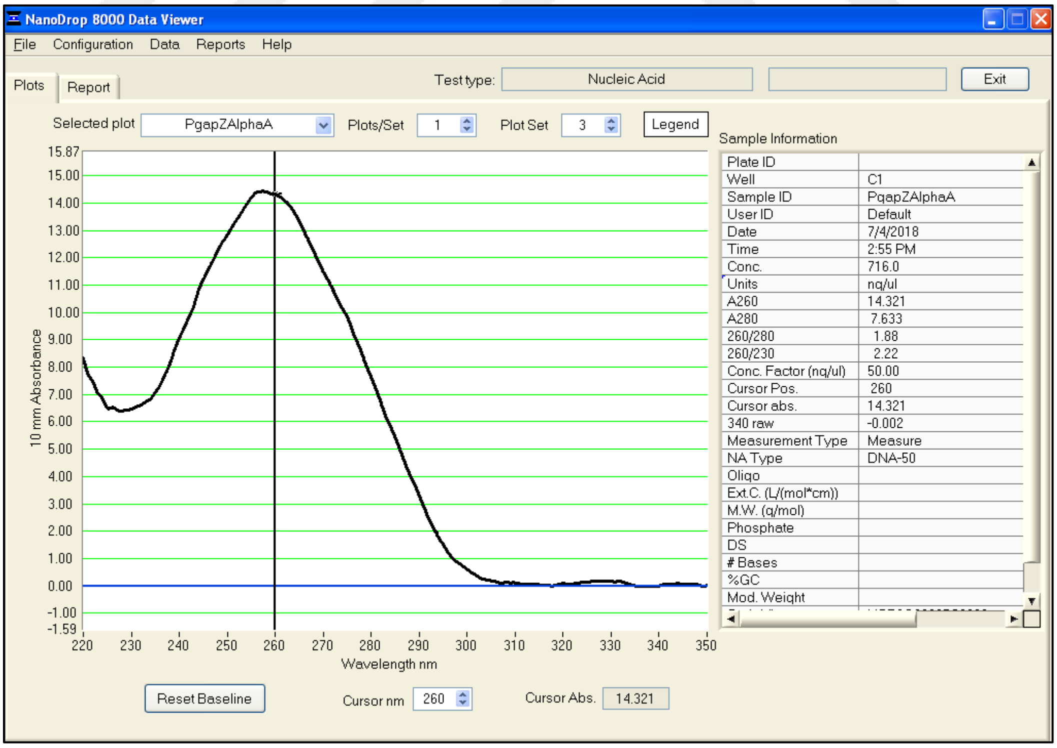

Figure 5.3: Concentration of pGAPZαA, measured by Nanodrop.

5.2.3. Restriction of Plasmids with EcoRI and NotI

At this step, pTZ57R/T plasmids that include IRT1 and IRT2 gene and pGAPZαA vector was restricted by the same restriction endonuclease couple, as shown in Fig. 5.4 and Fig. 5.5.

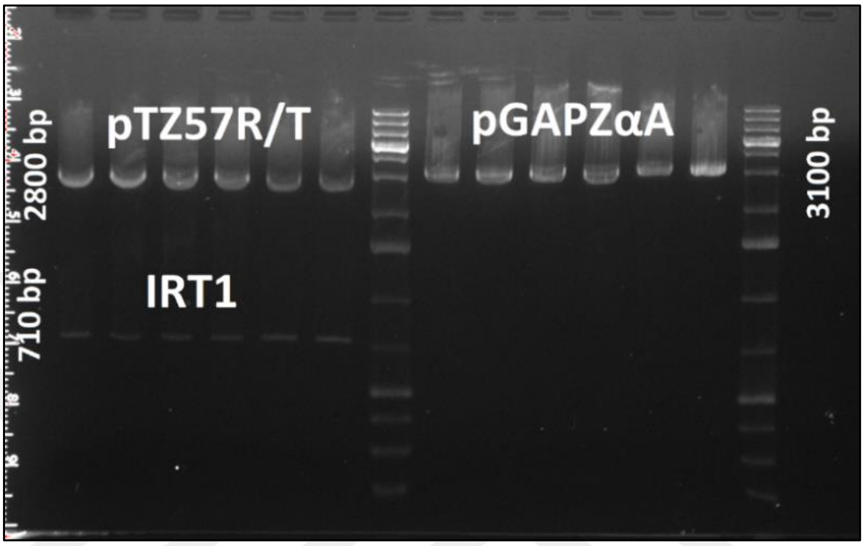

Figure 5.4: Agarose gel (1%) image of pGAPZαA and restricted pTZ57R/T that including IRT1.

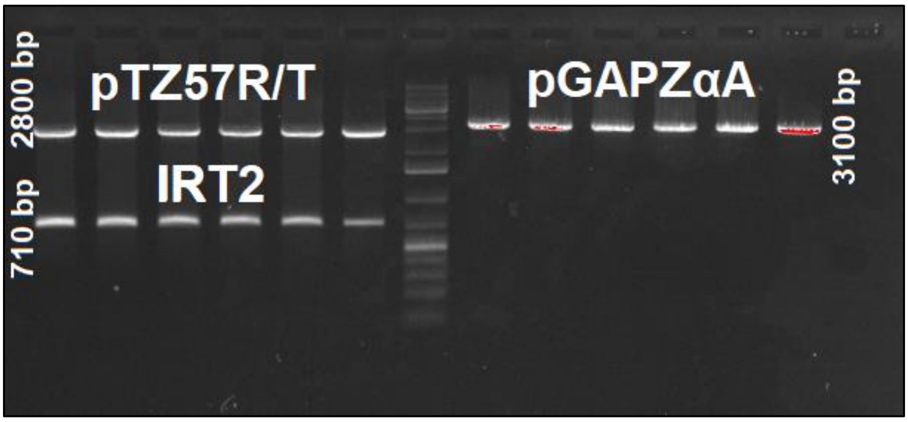

Figure 5.5: Agarose gel (1%) image of pGAPZαA and restricted pTZ57R/T that including IRT2.

Achievement of restriction reactions was proven via agarose gel results. Two separate clear bands show restriction of the gene including plasmid was successful. If both the gene and the target plasmid has correct length, it means they were suitable for the next step, ligation. A photograph of the gel showing that pGAPZαA and IRT1 and

IRT2 were cut as desired, is shown in Fig. 5.4 and Fig. 5.5. Figures also show that their lengths were right. In this way, they were appropriate for ligation.

IRT and pGAPZαA, which were proven to be restricted correctly in the gel image, were excised from the gel by following the steps explained in QIAquick Gel Extraction Kit's manual.

5.2.4. Ligation of the Restricted IRT Gene and pGAPZαA Plasmid

After isolation of IRT and pGAPZ αA samples, they were measured with Nanodrop. Both have approximately 30 ng/μl. So the following calculation was made with the help of "Insilico Ligation Calculator" (http://www.insilico.uni-duesseldorf.de/Lig_Input.html) (Fig. 5.6 and Fig. 5.7). It indicates 85.9 ng of insert DNA should be used for 75 ng of vector. Ligation was made in order to this results. For both IRT1 and IRT2 reactions, conditions were the same, except the concentration of IRT1 and IRT2.

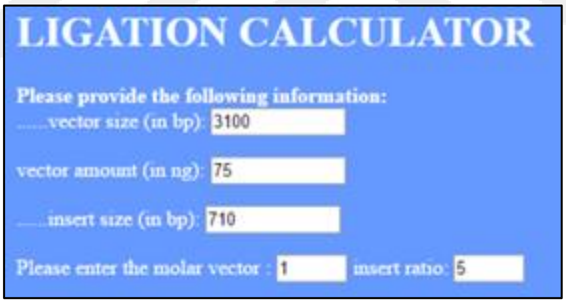

Figure 5.6: The input of Insilico Ligation Calculator.

Figure 5.7: The output of Insilico Ligation Calculator.

5.2.5. Transformation of Ligation Reaction into *E. coli*

Colonies grown on Zeocinized low salt LB plate shows ligation was successful. Because Zeocin is an antibiotic that prohibits the existence of bacteria. An Ampicillin resistance gene, which stays in pGAPZ αA vector, allows bacteria to survive on this kind of a plate.

5.2.6. Amplification of the Colonies and Plasmid Isolation

Colonies grown on Zeocin-low salt agar plates were inoculated in 25 μg / ml Zeocin containing low salt LB and incubated by shaking at 220 rpm at 37°C overnight. Plasmids were isolated following the instructions of the Thermo Scientific GeneJet Miniprep plasmid isolation kit.

5.2.7. Restriction of Isolated Plasmids and Testing with Agarose Gel Size Assessment.

Photographs of the gels showing that the ligations both of IRT-pGAPZαA were successful as shown in Fig. 5.8 and Fig. 5.9.

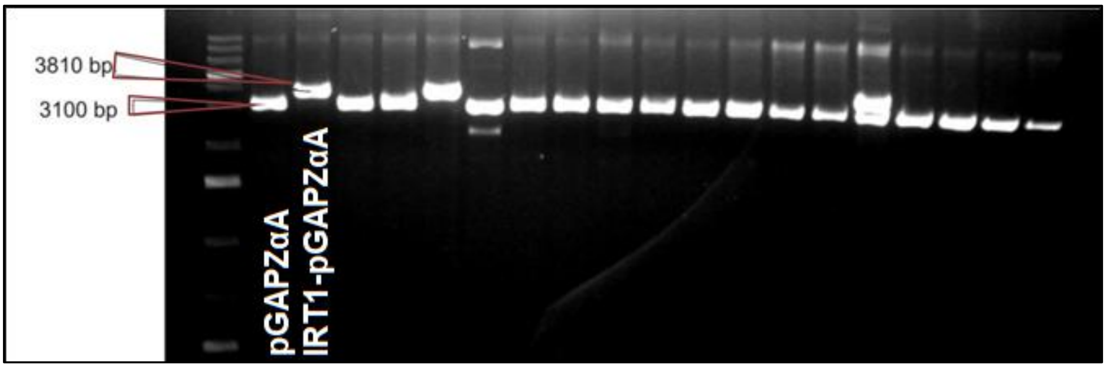

Figure 5.8: Length of IRT1-pGAPZαA plasmids points out positive colonies.

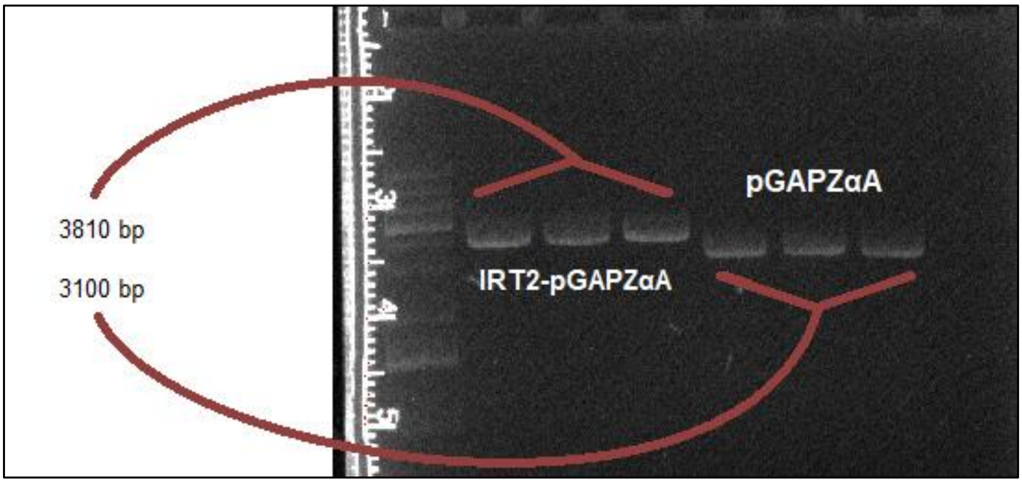

Figure 5.9: Length of IRT2-pGAPZαA plasmids points out positive colonies.

DNAs that were found to be of the correct length were sent to the sequence to be verified after their concentration were measured in Nanodrop.

5.2.8. Sequence Analysis

Sequence analysis of selected clones was performed. Results obtained from sequencing analysis was compared with desired IRT sequences, via BLASTn web tool. IRT1 sequence analysis was compared with the desired sequence to detect the results shown in Figures 5.10, 5.11 and 5.12.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
170215-068_E21_IRT1_5-2_pGap_forward.ab1	2639	2639	100%	0.0	100%	Query_63256
170324-084_C01_IRT1_Y3_pGap_forward.ab1	2196	2196	91%	0.0	97%	Query_63250
170215-068_G21_IRT1_1_5_-_1_pGap_forward.ab1	2178	2178	94%	0.0	96%	Query_63257
170324-084_I01_IRT1_Y6_pGap_forward.ab1	2169	2169	92%	0.0	97%	Query_63253
170324-084_G01_IRT1_Y5_pGap_forward.ab1	2163	2163	92%	0.0	96%	Query_63252
170324-084_E01_IRT1_Y4_pGap_forward.ab1	2148	2148	94%	0.0	96%	Query_63251
170324-084_K01_IRT1_Y7_pGap_forward.ab1	1114	1114	47%	0.0	96%	Query_63254
170324-084_M01_IRT1_Y8_pGap_forward.ab1	885	885	38%	0.0	96%	Query_63255
170324-084_A01_IRT1_Y2_pGap_forward.ab1	505	505	33%	1e-145	86%	Query_63249

Figure 5.10: Forward sequence analysis results of IRT1 clones.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
170215-068_I21_IRT1_5-2_aox1.ab1	2621	2621	99%	0.0	100%	Query_97600
170324-084_G03_IRT1_Y6_aox1.ab1	2204	2204	97%	0.0	95%	Query_97596
170324-084_A03_IRT1_Y3_aox1.ab1	2198	2198	97%	0.0	95%	Query_97593
170215-068_K21_IRT1_1_5_-_1_aox1.ab1	2189	2189	96%	0.0	96%	Query_97601
170324-084_C03_IRT1_Y4_aox1.ab1	2137	2137	96%	0.0	95%	Query_97594
170324-084_E03_IRT1_Y5_aox1.ab1	2106	2106	99%	0.0	93%	Query_97595
170324-084_K03_IRT1_Y8_aox1.ab1	2080	2080	93%	0.0	95%	Query_97598
170324-084_I03_IRT1_Y7_aox1.ab1	2054	2054	85%	0.0	97%	Query_97597
170324-084_O01_IRT1_Y2_aox1.ab1	531	531	23%	2e-153	96%	Query_97599

Figure 5.11: Reverse sequence analysis results of IRT1 clones.

Both forward and reverse sequence analysis of IRT1 point out clone named 5-2 has perfect similarity. Results Show that cloning of IRT1 was successful.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
170630-069_E01_6_pGap_F.ab1	1205	1205	99%	0.0	98%	Query_169185
170630-069_K01_11_pGap_F.ab1	1000	1000	87%	0.0	96%	Query_169188
170630-069_C01_5_pGap_F.ab1	963	963	83%	0.0	97%	Query_169184
170630-069_G01_9_pGap_F.ab1	931	931	89%	0.0	94%	Query_169186
170630-069_I01_10_pGap_F.ab1	374	374	31%	1e-106	97%	Query_169187
170630-069_A01_2_pGap_F.ab1	361	361	45%	9e-103	88%	Query_169183
170630-069_M01_12_pGap_F.ab1	294	294	25%	9e-83	97%	Query_169189

Figure 5.12: Forward sequence analysis results of IRT2 clones.

	Description	Max score	Total score	Query cover	E value	Ident	Accession
	170630-069_G03_10_aox_R.ab1	1280	1280	100%	0.0	99%	Query_221189
	170630-069_A03_5_aox_R.ab1	1275	1275	100%	0.0	99%	Query_221186
	170630-069_I03_11_aox_R.ab1	1251	1251	100%	0.0	99%	Query_221190
	170630-069_E03_9_aox_R.ab1	1083	1083	93%	0.0	97%	Query_221188
	170630-069_C03_6_aox_R.ab1	1027	1027	93%	0.0	95%	Query_221187
	170630-069_K03_12_aox_R.ab1	998	998	91%	0.0	94%	Query_221191
	170630-069_O01_2_aox_R.ab1	852	852	72%	0.0	97%	Query_221185

Figure 5.13: Reverse sequence analysis results of IRT2 clones.

170630-069_G03_10_aox_R.ab1

Sequence ID: Query_221189 Length: 1510 Number of Matches: 1

Range 1: 160 to 855 Graphics ▼ Next Match ▲ P

Score	Expect	Identities	Gaps	Strand
1280 bits(693)	0.0	695/696(99%)	0/696(0%)	Plus/Minus

```
Query  1    GCCCCTTTTGATGATGATGACGATATCGTCGGTGGCTATATATGCGAGGAGAACTCAGTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  855  GCCCCTTTTGATGATGATGACGATATCGTCGGTGGCTATATATGCGAGGAGAACTCAGTT  796

Query  61   CCATACCAGGTTTCCCTTAACTCCGGTTATCATTTCTGTGGCGGAAGTTTGATATCAGAA  120
            |||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||
Sbjct  795  CCATACCAGGTTTCCCTTAATTCCGGTTATCATTTCTGTGGCGGAAGTTTGATATCAGAA  736

Query  121  CAGTGGGTCGTTTCAGCTGGCCATTGCTATAAGTCACGAATTCAAGTAAGACTAGGAGAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  735  CAGTGGGTCGTTTCAGCTGGCCATTGCTATAAGTCACGAATTCAAGTAAGACTAGGAGAA  676

Query  181  CACAACATAGAAGTCTTGGAGGGTAATGAGCAATTCATTAACGCAGCCAAGATAATCAGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  675  CACAACATAGAAGTCTTGGAGGGTAATGAGCAATTCATTAACGCAGCCAAGATAATCAGA  616

Query  241  CACCCAAAGTATAATAGTCGTACTCTGGATAATGACATACTTTTGATTAAATTGTCCTCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  615  CACCCAAAGTATAATAGTCGTACTCTGGATAATGACATACTTTTGATTAAATTGTCCTCT  556

Query  301  CCCGCAGTTATAAATTCCCGTGTCTCTGCTATCAGTCTGCCTACTGCCCCTCCAGCTGCC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  555  CCCGCAGTTATAAATTCCCGTGTCTCTGCTATCAGTCTGCCTACTGCCCCTCCAGCTGCC  496

Query  361  GGAACCGAGTCATTGATCTCCGGTTGGGGTAATACATTGTCCTCTGGCGCTGACTATCCT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495  GGAACCGAGTCATTGATCTCCGGTTGGGGTAATACATTGTCCTCTGGCGCTGACTATCCT  436

Query  421  GACGAACTGCAATGCTTAGATGCTCCTGTCCTAAGTCAGGCCGAGTGTGAGGCATCCTAT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  435  GACGAACTGCAATGCTTAGATGCTCCTGTCCTAAGTCAGGCCGAGTGTGAGGCATCCTAT  376

Query  481  CCTGGAAAAATCACCAACAACATGTTTTGTGTAGGGTTTTTAGAAGGCGGAAAGGATTCA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375  CCTGGAAAAATCACCAACAACATGTTTTGTGTAGGGTTTTTAGAAGGCGGAAAGGATTCA  316

Query  541  TGCCAAGGTGATTCTGGTGGGCCTGTGGTTTCAAACGGTGAGTTGCAAGGTATCGTTTCT  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  TGCCAAGGTGATTCTGGTGGGCCTGTGGTTTCAAACGGTGAGTTGCAAGGTATCGTTTCT  256

Query  601  TGGGGATATGGTTGTGCCCAGAAGAACAGGCCAGGCGTCTATACAAAGGTCTATAATTAC  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255  TGGGGATATGGTTGTGCCCAGAAGAACAGGCCAGGCGTCTATACAAAGGTCTATAATTAC  196

Query  661  GTGGACTGGATCAAAGATACCATCGCTGCTAATAGT  696
            ||||||||||||||||||||||||||||||||||||
Sbjct  195  GTGGACTGGATCAAAGATACCATCGCTGCTAATAGT  160
```

Figure 5.14: Sequence analysis results of IRT2 clone 10. The point mutation is shown in blue.

Results of IRT2 sequence analysis were shown in Figures 5.12, 5.13 and 5.14. Forward analysis results showed that the DNA sequence of the 10th clone had 97% similarity to the desired sequence, indicating that there was a single nucleotide mutation in the DNA vector (Fig. 5.14). In order to see whether this single nucleotide mutation is affecting the protein sequence, the resulting DNA sequence was translated into the protein sequence and compared with the target protein sequence. As a result of this comparison, it was shown that the single nucleotide mutation did not affect the protein structure (Fig. 5.15), and yeast transformation studies were able to be started.

EMBOSS_001_2

Sequence ID: Query_141387 **Length:** 40 **Number of Matches:** 1

Range 1: 1 to 40 Graphics ▼ Next Match ▲ Previous

Score	Expect	Method	Identities	Positives	Gaps
83.2 bits(204)	1e-29	Compositional matrix adjust.	40/40(100%)	40/40(100%)	0/40(0%)

```
Query  1    APFDDDDDIVGGYICEENSVPYQVSLNSGYHFCGGSLISE   40
            APFDDDDDIVGGYICEENSVPYQVSLNSGYHFCGGSLISE
Sbjct  1    APFDDDDDIVGGYICEENSVPYQVSLNSGYHFCGGSLISE   40
```

Figure 5.15: The first 120 base pair of the IRT2 clone 10 was translated into the peptide sequence, indicating that the point nucleotide mutation does not affect the protein sequence.

5.3. Production and Purification of IRT in *Pichia pastoris* X3

5.3.1. Transformation of the IRT Gene, which Transferred to the Yeast Expression Vector, into the Yeast

Transformed yeasts survived on the YPD plate with Zeocin. Both IRT isoform transformations show successful results (Fig 5.16) (Fig. 5.17).

Figure 5.16: Surviving pGAPZ αA-IRT1 colonies. Zeocin resistance gene found on pGAPZ αA shows achievement of the transformation.

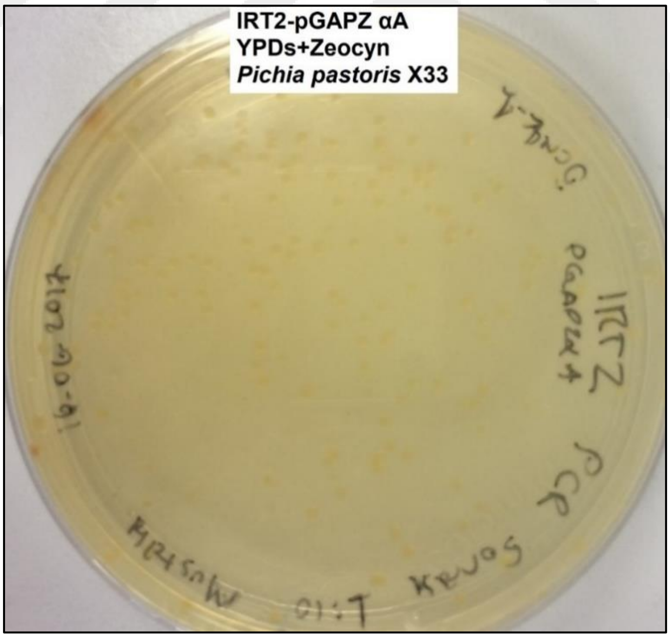

Figure 5.17: Surviving pGAPZ αA-IRT2 colonies. Zeocin resistance gene found on pGAPZ αA shows achievement of the transformation.

5.3.2. Selection of IRT-Producing Yeast Colonies

5.3.2.1 Selection of IRT1-Producing Yeast Colonies

It was observed that 23 of the 24 colonies examined in the test produced functional IRT1 (Figure 5.18).

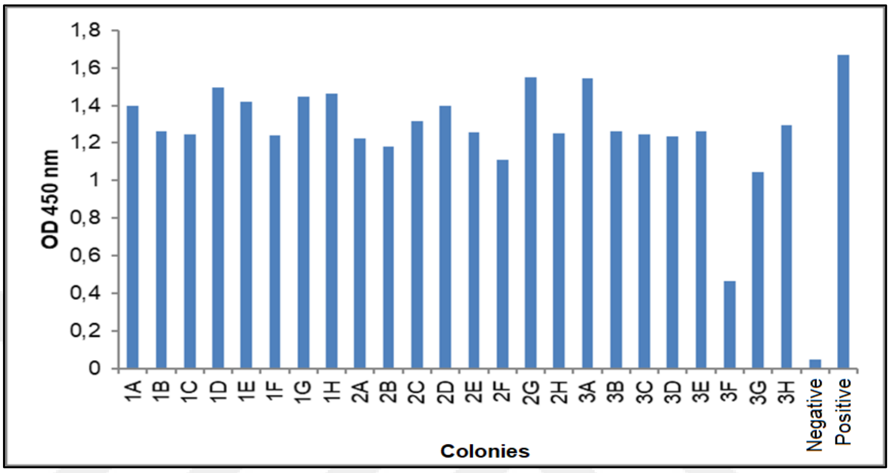

Figure 5.18: Sandwich ELISA results from the IRT1 broad transformed yeast culture supernatants showed OD450 values.

Two of the 23 colonies were selected which gave the highest signal in the ELISA, and a subculture was performed every 24 hours for 96 hours to determine the optimum culture times of the IRT1 proteins that were identified to be functional. At the end of 4 days, the supernatants were checked by SDS-PAGE and western blot. SDS-PAGE and western blot analyzes were performed using 12% SDS-PAGE gels. 15µl of the prepared gels were mixed with 5µl of loading dye. 500 ng IRT1 (MCLAB, HTR1-200) was used as a positive control, and Thermo prestained protein marker (26616) was used as a marker. After the proteins were run at 120 V for about 90 min, one of the gels was stained with the silver staining protocol, and the other gel was transferred to the PVDF membrane at 20 V for 20 min. The membrane was blocked with 1% milk powder. After blocking, imaging was performed by adding mouse anti-IRT, AP labeled anti-mouse IgG and BCIP / NBT substrate, respectively.

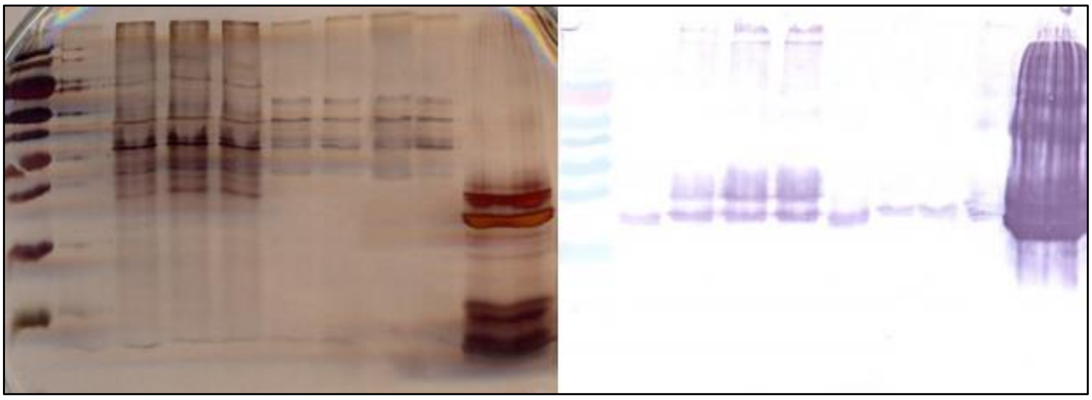

Figure 5.19: 12% SDS Gel Image (Silver Color on Left, Western Blot on Right). Same samples were loaded into the two gels in the same order. The wells are loaded like that: Marker (Thermo prestained protein marker 26616), 2G - 24 hours, 2G - 48 hours, 2G - 72 hours, 2G - 96 hours, 3A - 24 hours, 3A - 48 hours, 3A – 72 hours, 3A – 96 hours, Positive Control IRT1 (10µg/well) (MCLAB, HTR1-200).

As shown in Figure 5.19., both the 2G and 3A cells showed maximum IRT production after 96 hours, and the bands were found to be around 24 kDa molecular weight as it should be. The lack of proteolytic cleavage bands seen in commercial IRTs suggests that consistent results with the desired results have been obtained since this cut-off activity is desired to be avoided when designing recombinant IRT. Considering the density of the bands obtained as a result of the western blot study, it was decided to continue the studies with 2G-encoded yeast.

5.3.2.2 Selection of IRT2-Producing Yeast Colonies

Colonies selected from YPDS plate (Fig 5.17) and seven of them were incubated in YPD medium. They were tested in Western blot and Silver staining method. Five of them produce IRT2 as seen in Fig. 5.20. They were tested in the ELISA method (Fig. 5.21). Two of the five colonies giving the highest signal in the ELISA were selected, and a subculture was performed every 24 hours for 96 hours to determine the optimum culture times of the IRT2 proteins determined to be functional and produced. At the end of 4 days, the supernatants were checked by western blot. Western blot analyzes were performed using 12% gels. 15µl of the prepared gels were mixed with 5µl of loading dye. 500 ng IRT2 (RayBiotech, 228-13214-3) was used as a positive control in the gels, and BLUeye Prestained Protein Ladder (PM007-0500) was used as a marker. After the proteins were run at 120 V for about 90 min, the gel was transferred to the PVDF membrane at 20 V for 20 min. The membrane was blocked with 1% milk powder. Following blocking, mouse anti-IRT, AP labeled anti-mouse IgG, and BCIP / NBT substrate was used, respectively, followed by imaging (Figure 5.22).

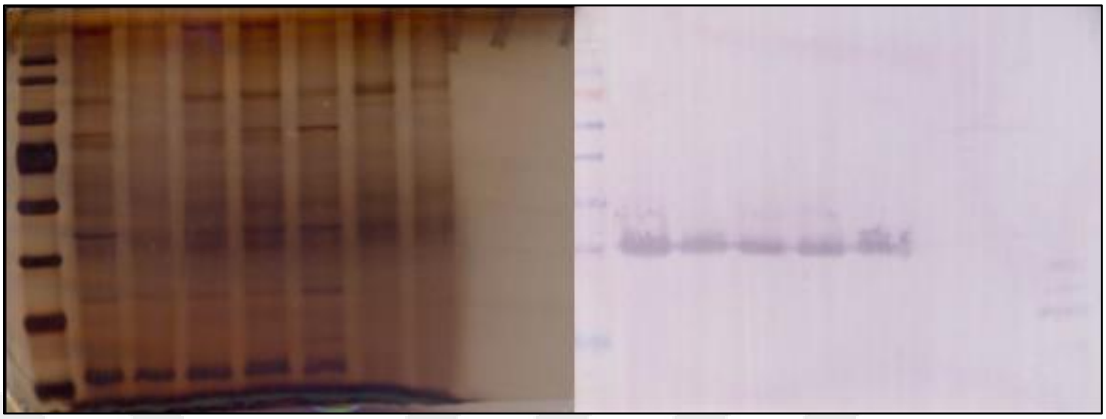

Figure 5.20: 12% SDS Gel Image (Silver Staining on the left, Western Blot on the right). The two gels were loaded in the same order. The loads are respectively; Marker (Thermo prestained protein marker 26616), 1A, 1B, 1C, 1D, 1E, 1F, 1G, Positive Control (IRT2 RayBiotech).

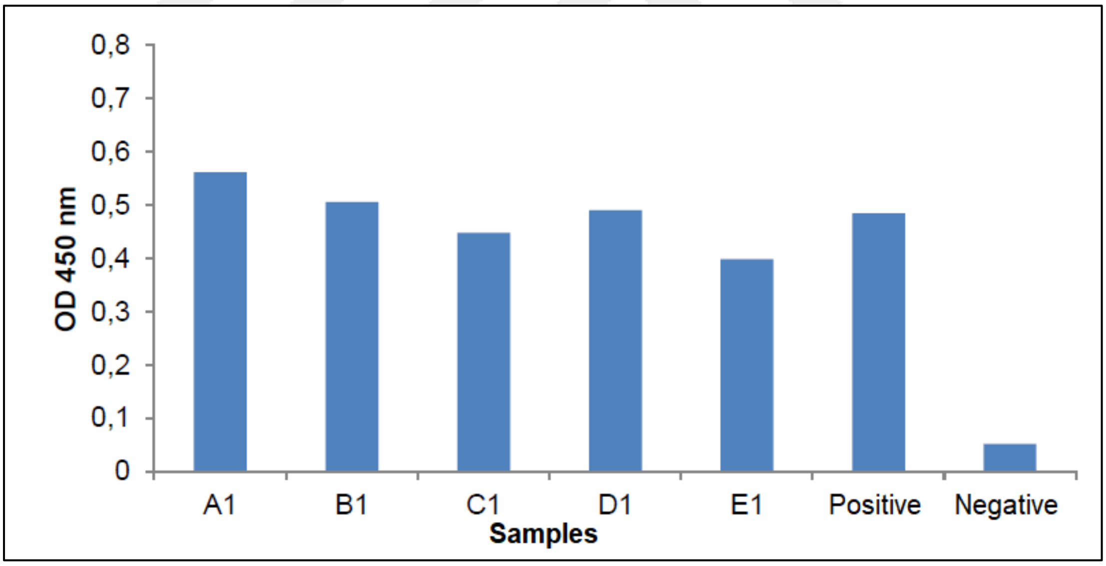

Figure 5.21: Sandwich ELISA results from the IRT2 broad transformed yeast culture supernatants showed OD_{450} values.

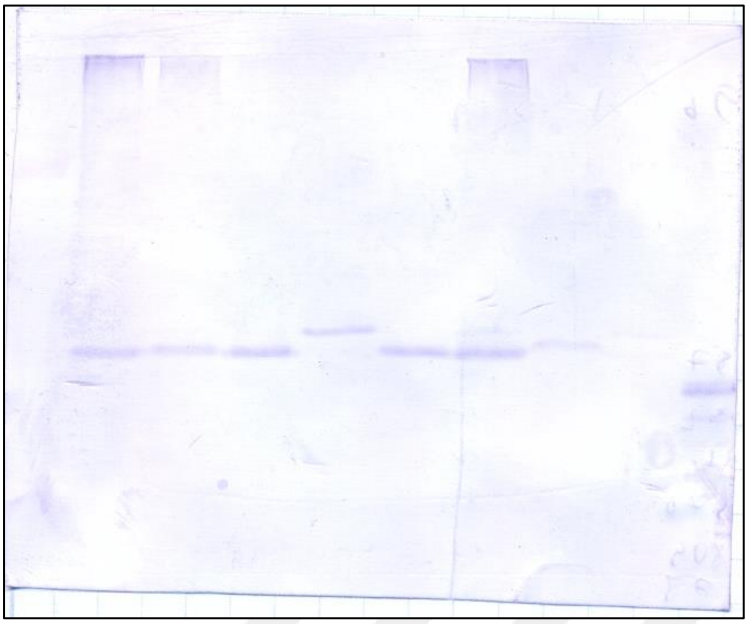

Figure 5.22: Western Blot result of IRT2. Respectively; Marker (BLUeye Prestained Protein Ladder PM007-0500), 1A - 24h, 1A - 48h, 1A - 72h, 1A - 96h, 1B - 24h, 1B - 48h, 1B - 72h, 1B - 96h , Positive Control (IRT2 RayBiotech, 228-13214-3).

5.3.3. Medium Optimization

ELISA results of incubated colonies point out that 2% glucose included YPD has the best condition for recombinant mutant IRT production. For both IRT isotypes the tendency is similar (Fig. 5.23).

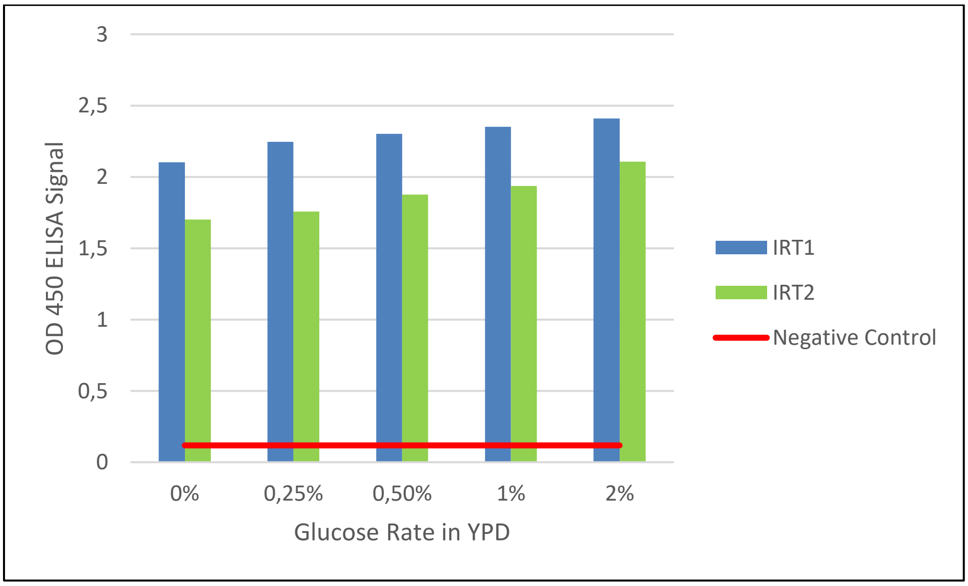

Figure 5.23: ELISA Results of YPD Glucose optimization.

Additionally, Western Blot results also show 2% Glucose conditions is the optimum for not only IRT1 but also IRT2 production (Fig. 5.24 and Fig. 5.25).

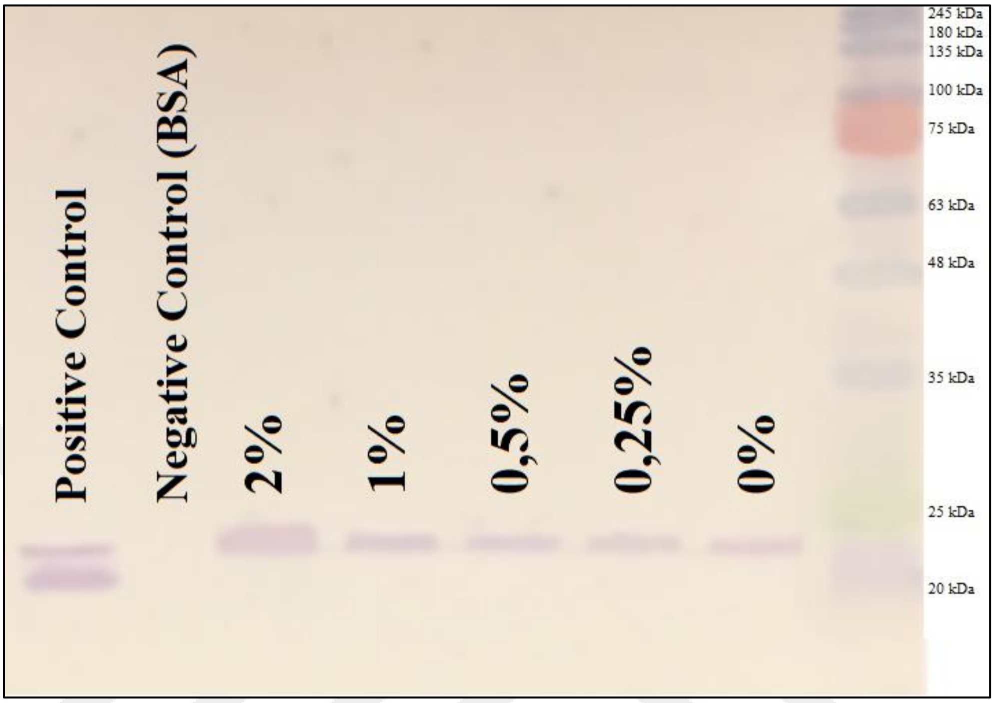

Figure 5.24: Western Blot results of IRT1 incubation in different glucose conditions.

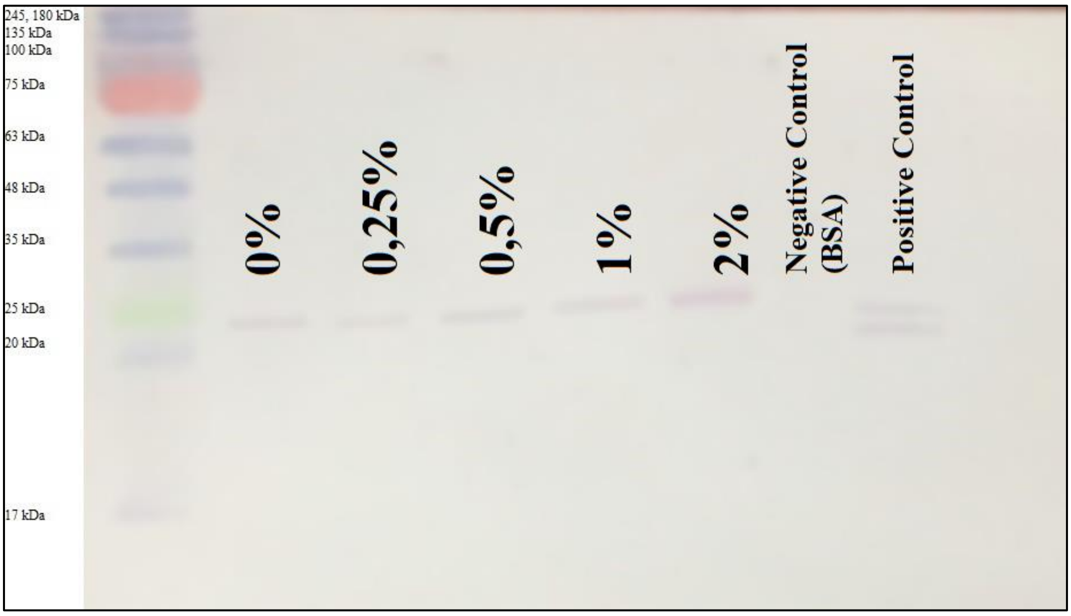

Figure 5.25: Western Blot results of IRT2 incubation in different glucose conditions.

YPD glucose optimization results were revealed that 2% glucose condition was the optimal state for IRT production.

5.3.4. IRT Purification

During the purification process, the buffer contained in each tube was read concerning the buffer (Binding or Elution), and the collected fractions were recorded. Distilled water (5 ml), binding buffer (5 ml), sample (5 ml sample + 5 ml binding buffer), resuspension buffer, elution buffer, distilled water were eluted sequentially from the column. The sample was transferred to the desired volume, then resuspended in the binding buffer and flow continued until the OD_{280} value fell below 0.05, then buffered until the OD_{280} was again below 0.05 by reference to the elution buffer, it was passed through 10 ml of distilled water to wash the last column. The purification results were shown in Fig. 5.26 for IRT1 and Fig. 5.27 for IRT2.

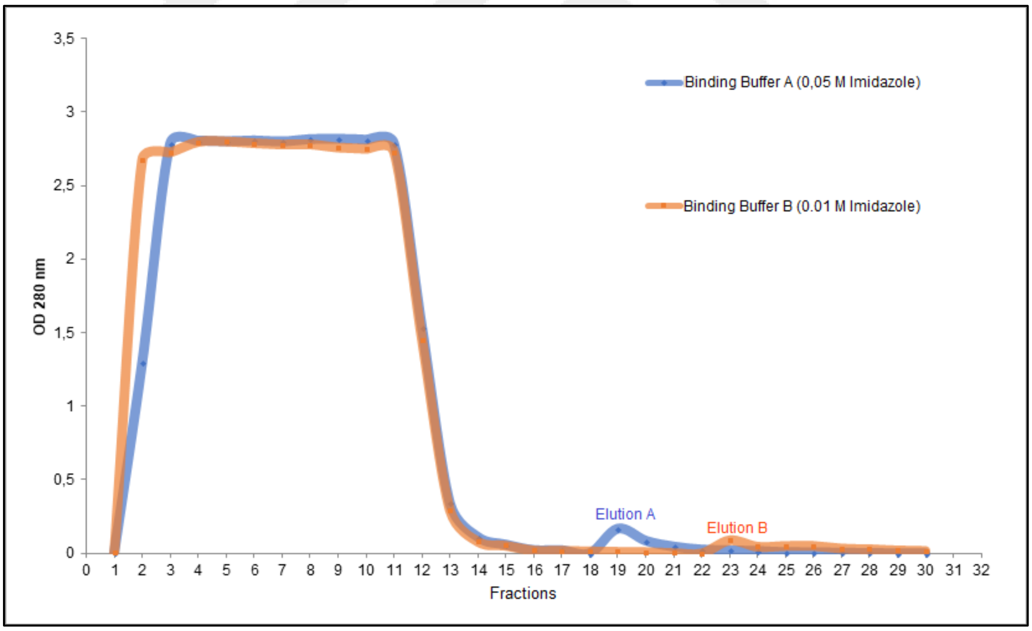

Figure 5.26: OD_{280} results of binding buffer optimization study for IRT1 binding to nickel column.

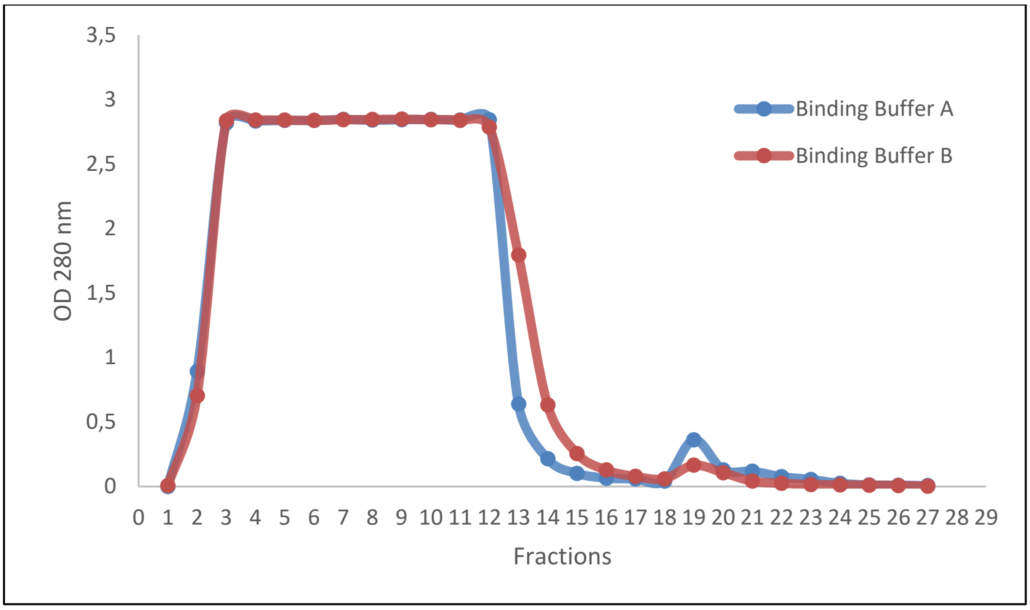

Figure 5.27: OD_{280} results of binding buffer optimization study for IRT2 binding to nickel column.

These results both show that better binding can be achieved with 20 mM NaPO4, 0.5 M NaCl, 0.01 M imidazole pH: 7.4 buffer. For the elution, 20 mM NaPO4, 0.5 M NaCl, 0.5 M imidazole pH 7.4 buffer was used. The optimized procedure was repeated to obtain pure IRT fractions in the most suitable elution buffer. As examples of standard studies, OD_{280} values of 3 purifications named H1 (IRT1), H2 (IRT1) and M1 (IRT2) were shown in Figure 5.28 and ELISA results were shown in Fig. 5.29. Sandwich ELISA applied for fraction selection. For the sandwich ELISA, 100 ng / well of mouse-originated IRT antibody 2C4 (HyTest Ltd 4IT3) was first coated onto the plate bottom and incubated for 1 hour at room temperature. The antibody-coated wells were then washed with 0.5% Tween 20 in PBS and blocked with 1% Hammerstein Casein, 1% PVP (Polyvinylpyrrolidone) and 30 µl / ml 1M NaOH overnight at + 4°C. Because of the lower background signal, this mixture was used as the blocking solution in all sandwich ELISA applications performed within the scope of this study. Blocked wells were washed the next day. After washing, 100 µl of each fraction and 50 ng of WT IRT1 (MCLAB, HTR1-200) and 50 ng of Trypsin-2 Human Recombinant (RayBiotech, 228-13214-3), as a positive control, was added to the wells and incubated for 1 hour at 37°C. Subsequently, IRT supernatants were washed and incubated for 1 hour at 37°C with the addition of 100 ng / well mouse-derived Biotin-labeled IRT antibody 4A7 (HyTest Ltd 4IT3) and the plate was washed 5 times. The plate was then stained with HRP labeled 4A7 TMB substrate. After color formation, the reaction was stopped with 2M H_2SO_4. The resulting color was measured at 450 nm, and the results showed that our purification was successful (Fig. 5.29).

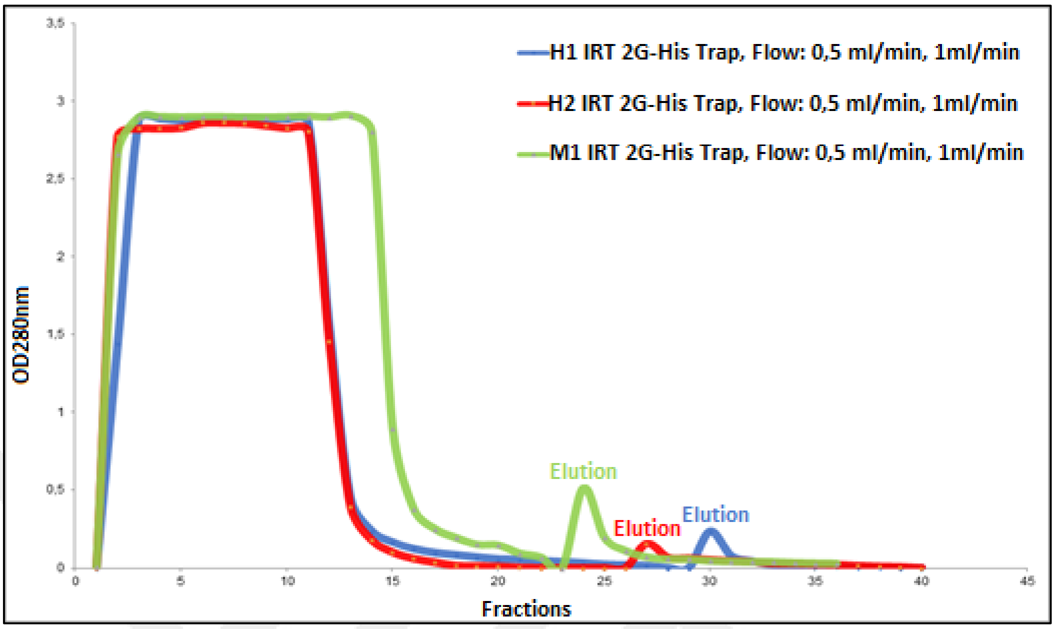

Figure 5.28: OD_{280} results of His-Tag nickel column purification for IRT. The peaks observed after the elution buffer show the pure IRT obtained from each sample.

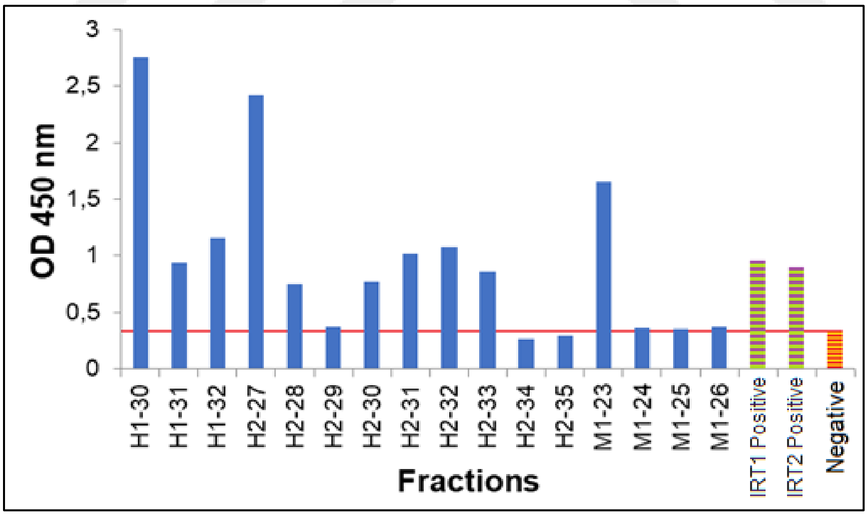

Figure 5.29: The OD_{450} nm values obtained as a result of the ELISA run after purification.

5.4. Characterization of IRT1 and IRT2

5.4.1. ELISA Test

ELISA tests were applied to find out the immune activity of K23D mutated IRT1 and IRT2 (Fig. 5.30 and Fig. 5.31). A comparison between the wild-type form and mutant form has done. Additionally, wild-type form that supported by an inhibitor (Aprotinin) was also compared with mutant form.

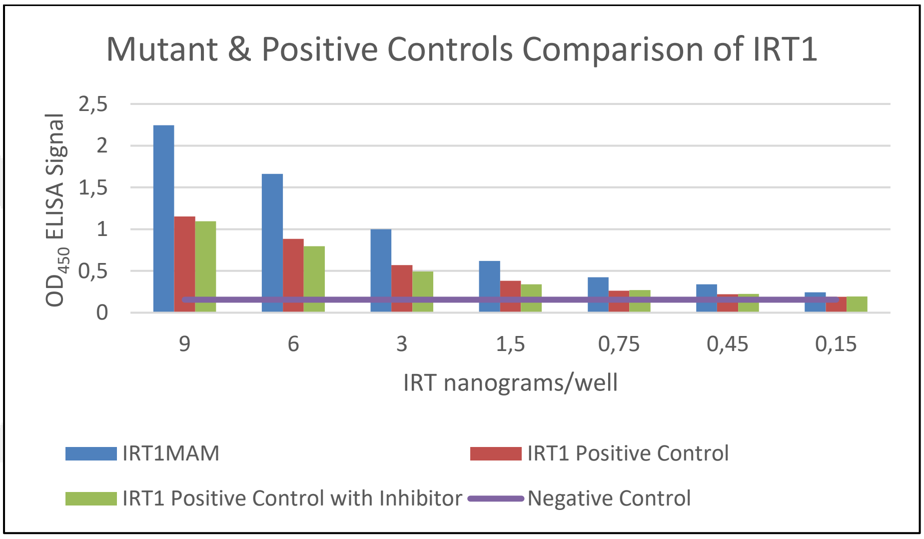

Figure 5.30: Mutant & Positive Controls Comparison of IRT1.

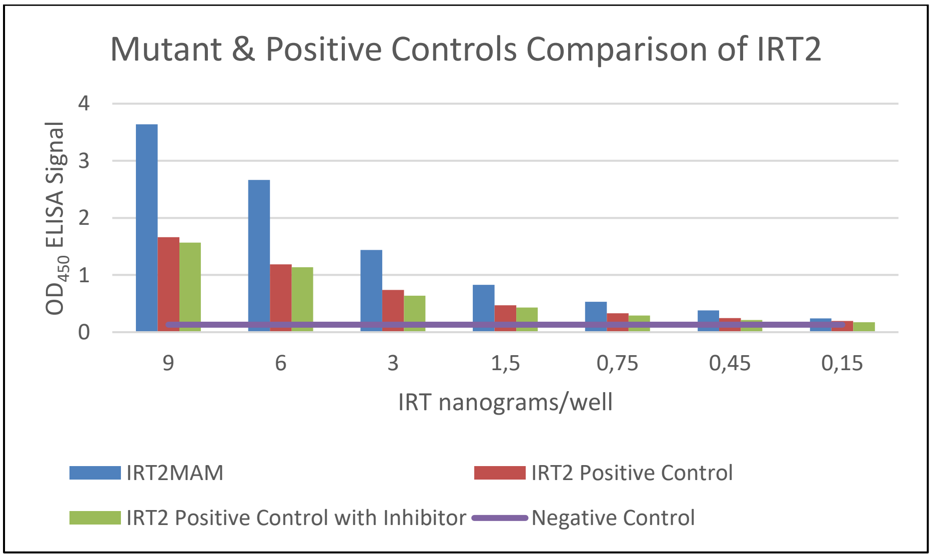

Figure 5.31: Mutant & Positive Controls Comparison of IRT2.

Sandwich ELISA results show that mutant IRT1 (IRT1MAM) and IRT2 (IRT2MAM) has higher immunogenic activity than wild-type forms (IRT1 Positive Control, IRT2 Positive Control). Also, positive controls with inhibitor (Aprotinin) have less immunogenic activity then K23D mutated IRTs.

5.4.2. Western Blot Test

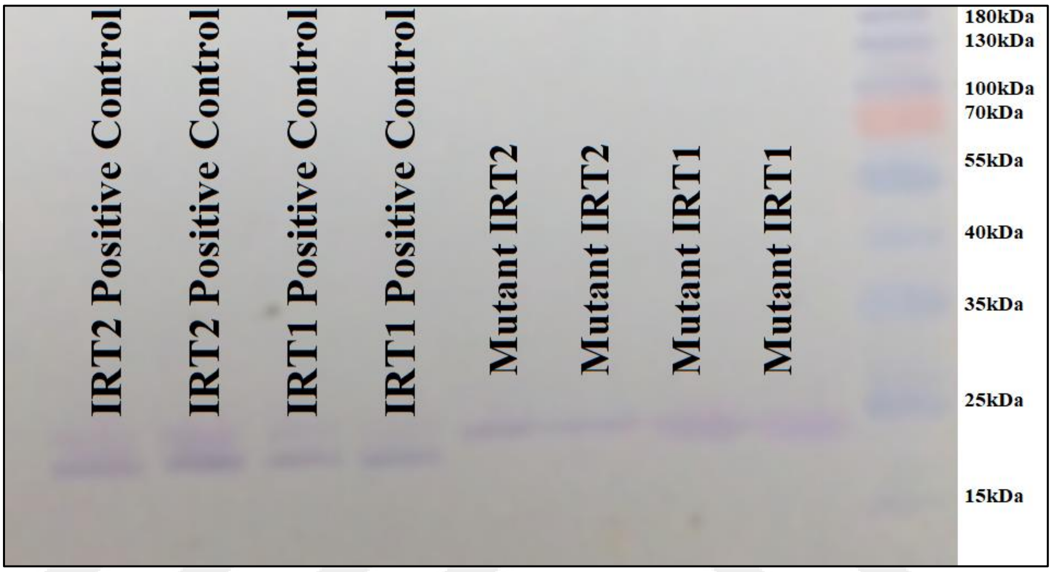

Figure 5.32: Western Blot results of IRT Characterization.

Western blot results suggest two remarkable results. First of all anti IRT antibodies correctly recognizes mutant IRTs as same as wild-type for both ELISA and western blot. Secondly, Mutant IRT shows that they can protect their structure because they give one clear band while positive controls have two bands. Moreover, wild-type form's above bands were so weak; it means they cut themselves.

5.4.3. IRT Proteolytic Activity Test

BAEE test was carried out for both IRT1 and IRT2. Wild Type (WT) IRT1 and Wild-Type (WT) IRT were used as positive control. The values of the reference were stable as shown in Table 5.1 Then, 75 µl of WT-IRT1 (mg/ml) was mixed with 3 ml of substrate buffer and 125 µl of HCl solution, the mixture was transferred into a quartz cuvette and read in OD_{253} for a minute, and the results were recorded. Results were used as positive control. The same procedure was repeated for purified recombinant IRT1 (mg/ml) and IRT2 (mg/ml) the results were quite low and stable as shown in the Fig. 5.34. The values of both mutant IRT1 and IRT2 were not significantly changed as shown in Fig. 5.34.

Table 5.1: Comparison of IRT catalytic activity with Wild-Type (WT) IRT1 and IRT2.

Reference	WT-IRT1	WT-IRT2	IRT1	IRT2	Minutes
0,143	0,068	0,056	0,005	0,008	0
0,143	0,126	0,095	0,006	0,009	1
0,144	0,168	0,126	0,007	0,009	2
0,144	0,207	0,186	0,007	0,009	3
0,143	0,214	0,192	0,009	0,011	4
0,144	0,214	0,192	0,011	0,013	5
0,143	0,214	0,192	0,013	0,013	6

To interpret the results obtained, the equation in Figure 5.4. 2., which shows Trypsin activity over the BAEE decrease, was used.

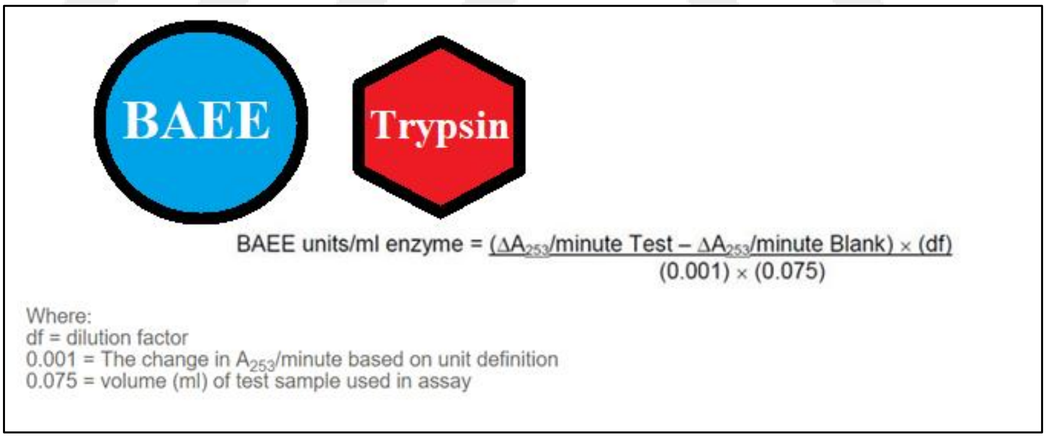

Figure 5.33: The formula that compares the amount of BAEE to the amount of Trypsin according to the test results.

As seen in Fig. 5.33, the result of Fig. 5.34 means Units of BAEE/mL Trypsin, because as pointed out in Fig. 4.4 and Fig. 5.33 the total amount of BAEE was the only variable of the equation. BAEE, which is a trypsin substrate, was decreasing when incubated with wild-type while recombinant IRT was not triggering this kind of a reaction.

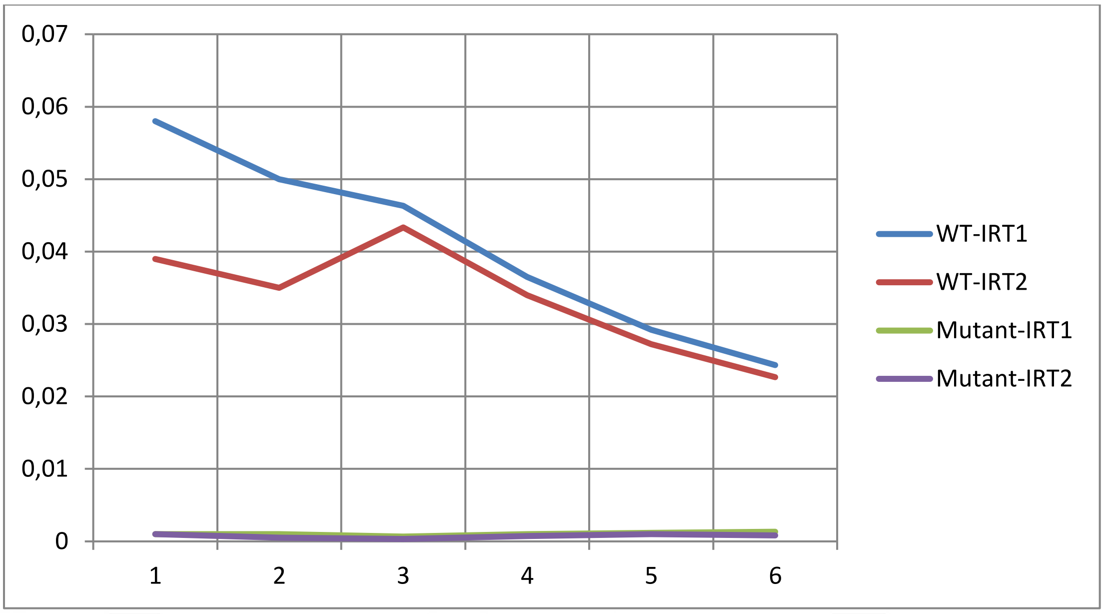

Figure 5.34: Graph showing the amount of BAEE / Trypsin. Starting from higher rates and decreasing means consumption of BAEE.

As seen from the results above, recombinantly produced IRT1 and IRT2 have no proteolytic activity as aimed.

6. DISCUSSION

The main objective was generating an immunoassay to detect CF patients from their dried blood samples. The study was based on producing recombinant Immunoreactive Trypsinogen (IRT) isoform as a biomarker as described in the literature [Therrell et al., 2012; Li et al., 2006].

Cystic fibrosis is a disease in which epithelial cells are caused by different mutations, resulting in the same results [Kleven et al., 2008; Ratjen et al., 2015]. In this disease, epithelial cells, which have no capability to stabilize ions, cannot keep the mucosa moist and the drying mucosa hardens. Hardening does not allow the cilium to move and mucociliary clearance process stops [Robinson et al., 1997]. Thus, pathogens begin to recede in the environment. The solidification of the mucous membrane, which is also found in the narrow opening of the pancreas, narrows this opening [Skach et al., 2002; Robinson et al., 1997]. The affected pancreas cannot release the enzymes into the small intestine, and the accumulated proteases cause the pancreas to begin to self-digest [Whitcomb et al., 1996]. Digestion causes injury in the blood vessels. The pancreatic enzymes, like IRT, pass through the blood circulation from the wound. Because of that IRT is essential for CF diagnosis [Therrell et al., 2012; Li et al., 2006]. The background of the study is based on IRT detection in CF patients, as mentioned before. IRT in Trypsinogen form is an enzyme found naturally in the pancreas, not in the bloodstream [Paju et al., 2000]. They are released into the intestine for protein digestion, where they turn into trypsin [Sahin-Toth, 2005]. In the usual absorption process trace amount of IRT can be pass into the bloodstream. So that in small quantities of IRT is normal in the bloodstream but in Trypsin form. However, it is unusual to have trypsinogen in the blood [Therrell et al., 2012; Li et al., 2006]. Trypsinogen is only found in the pancreas, and its involvement in the bloodstream can take place directly in the bloodstream of the pancreas. This happens with the injury of the pancreas [Cabrini et al., 1990; Voet et al., 1995]. Pancreatic injuries are either caused by an external physical factor (such as a knife or a strike), or by natural reasons. For example, in the case of Cystic Fibrosis (CF) or autoimmune disorders, the body may lose its integrity. [Therrell et al., 2012] In the case of Autoimmune Pancreatitis (AIP), IRT can also be transmitted in the form of Trypsinogen from the injured tissue to the bloodstream, and detection of these IRTs can make a difference in the diagnosis of these diseases. [Magnani et al., 2010] So using Trypsinogen form as a biomarker in CF patients is more sensible. [Andersén et al., 2001] The challenge of the satiation is storage of Trypsinogen before it transforms in Trypsin form. Therefore, the development of anti-Trypsinogen antibody could be harder than the development of anti-Trypsinogen antibody. The study describes a method to produce a hassle-free human recombinant immune reactive trypsinogen pro-enzyme which has the similar antigenic properties with the native form but does not exhibit proteolytic activity for the development of durable detection kits with longer shelf life. It has been proven with BAEE test and supported by Western Blot results. In ELISA results K23D mutated IRTs also demonstrated not only having immunoassay response below CF patient detection threshold but also having a higher signal than Wild-Type forms. Having higher signals in ELISA around 0,21 ng/well (equals with 70 ng/ml sample in dried blood spot) than Wild-type form is remarkable for CF diagnosis.

Detection and quantification of protease enzymes in protein-based assays is a challenge since the analyte may cleave assay components. [Farady et al., 2014; Farady et al., 2008; Wu et al., 2007] Immunoreactive Trypsinogen (IRT) is a protease, so the problem had to be overcomed. For the purpose of avoiding proteolytic activity, K23D mutation has done. Mutations that inhibit the proteolytic activity of IRTs have been performed successfully before, but in the study of Siriwardena et al., Glutamine was used instead of Aspartic acid. However, they have targeted the 23rd amino acid like our study. [Siriwardena et al., 2012] In another IRT research that aims activation inhibition, IRT3 was targeted as distinct from our study because of its natural inhibitor resistance. [Oiva et al., 2011]

Our study was considerably parallel with Siriwardena’s research. Our mutant IRT1MAM and IRT2MAM proteins conserved their conformations more than wild-type forms as demonstrated in western blot results. Siriwardena et al. were suggested their mutant form was more stable than wild-type [Siriwardena et al., 2012], we did not examine our mutant IRTs in the same way. However, western blot results give us an idea about there could be a similarity between our mutant form’s stability and Siriwardena’s mutants. It can be researched deeper in the possible future studies.

Another remarkable point of ELISA results of K23D mutated IRTs have a higher immune response then not only Wild-type but also even Wild-type with inhibitor. Extra compounds are undesired elements in optimal ELISA studies. [Gan et al., 2013;

Voller et al., 1976] Although, K23D mutated IRTs decrease the number compounds, give a higher response. The situation may reduce expenses for ELISA studies that detecting Immunoreactive Trypsinogens.

Experiments show that K23D mutated IRTs were also detected by IRT antigens that developed against commercial IRTs. It may be difficult to denote the difference between trypsin and trypsinogen since recombinant IRT in trypsinogen form, and wild-type IRT in trypsin form contains many common epitopes, except the activation peptide. [Richárd Szmola, Zoltán Kukor, 2003] In the future experiments, antibody development studies aiming to detect only Trypsinogen or Trypsin may positively be affected by K23D mutated IRTs via using them as the immunization molecule or the antigen standard. Beyond Cystic Fibrosis (CF), K23D mutated IRT proteins can be used in many fields, such as diagnosis of Autoimmune Pancreatitis (AIP) [Guy-Crotte et al., 1992; Korvuo et al., 1996]

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BIOGRAPHY

Mustafa Divyapıcıgil was born in 9th of January 1992 in İstanbul. He went to İstanbul Köy Hizmetleri Anadolu Lisesi (İKHAL) for his high school education. He received his B.Sc. Degree in Molecular Biology and Genetics Department at Gebze Institute of Technology in 2016. He started his M.Sc. education in Gebze Technical University, Graduate School of Natural and Applied Sciences, Molecular Biology and Genetics Department. In the same time, he started to be a scholar in TÜBİTAK MAM Genetic Engineering and Biotechnology Institute, and he performed his M.Sc. thesis studies in there, under the supervisions of Prof. Tamer YAĞCI and Dr. Esin AKÇAEL. He started to be a project assistant as a scholar in the project named "Development of Immunoreactive Trypsinogen (IRT) and Pancreatitis Related Protein (PAP) ELISA Kits for Early Diagnosis of Cystic Fibrosis in Newborn Screening." under the supervision of Assoc. Prof. Dr. Fatıma YÜCEL and Dr. Esin AKÇAEL. He graduated with an M.Sc. degree in 2018.

APPENDICES

Appendix A: Protein and DNA Markers

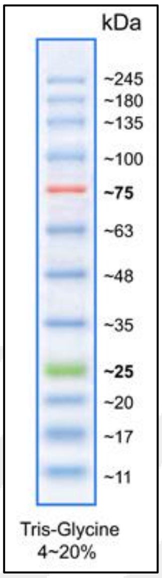

Figure A 1.1: Protein Ladder
(BLUeye Prestained Protein Ladder)
PM007-0500

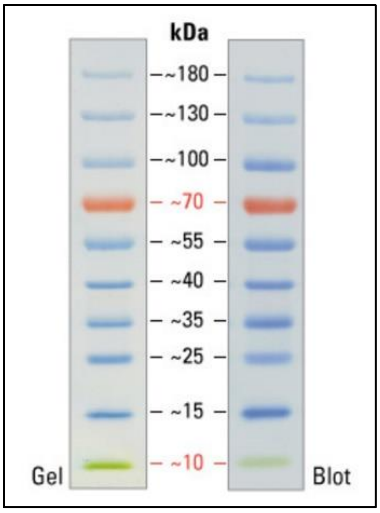

Figure A 1.2: Protein Ladder
(PageRuler™ Prestained Protein Ladder)
26616

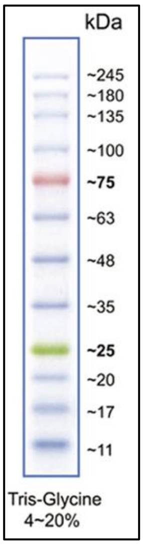

Figure A 1.3: Protein Ladder
(GangNam-STAIN™ Prestained Protein Ladder)
24052-3

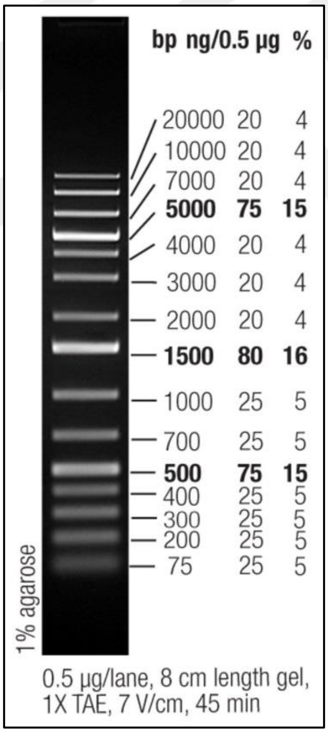

Figure A 1.4: DNA Molecular Weight Marker
(1 Kb Plus DNA Ladder)
10787018