To my family…

ENGINEERING OF PROBIOTIC *SACCHAROMYCES BOULARDII* AS A HOST FOR LIVING THERAPEUTIC APPLICATIONS

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE

OF BILKENT UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF

MASTER OF SCIENCE

IN

MATERIALS SCIENCE AND NANOTECHNOLOGY

By

ORHAN NEDİM KURT

SEPTEMBER 2022

ENGINEERING OF PROBIOTIC *SACCHAROMYCES BOULARDII* AS A HOST FOR LIVING THERAPEUTIC APPLICATIONS

By Orhan Nedim Kurt

September 2022

We certify that we have read this dissertation and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Urartu Özgür Şafak Şeker (Advisor)

Ceren Özkul Koçak

Aykut Erbaş

Approved for the Graduate School of Engineering and Science:

Orhan Arıkan

Director of the Graduate School

ABSTRACT

ENGINEERING OF PROBIOTIC *SACCHAROMYCES BOULARDII* AS A HOST FOR LIVING THERAPEUTIC APPLICATIONS

Orhan Nedim Kurt

MSc in Materials Science and Nanotechnology

Advisor: Urartu Özgür Şafak ŞEKER

September, 2022

Therapeutic molecules or biologically active agents used in the treatments of disorders have been manufactured employing chemical synthesis methods with high costs and limited accessibility. Even though this approach to producing therapeutic molecules has enabled life-changing substances to be produced, there are still countless diseases waiting for a cure. Therefore, it would not be possible to meet the treatment needs via just conventional manufacturing approaches.

Living therapeutics are programmed cells for the production and direct delivery of therapeutic molecules to the human body. Indeed, biologically engineered cells have been used for a long time to manufacture biomolecules in industrial settings. Also, there have been many studies that designed and programmed living organisms to cure diseases such as cancer, diabetes, inflammatory bowel syndrome, ulcer, and colitis.

Saccharomyces boulardii CNCM I-745 is a yeast strain used as a probiotic for a very long time in humans. *S. boulardii* is the first and the only yeast strain approved to be

used in human medicine so far. It has been shown that *S.boulardii* has many benefits to its host, including restoring the microbiome and competing with pathogens. Also, *S.boulardii* is a promising organism to be engineered and programmed as a living therapeutic factory due to its great compatibility with the human body and its metabolic features enabling us to manufacture complex molecules. Aiming for this, we created a TRP1 auxotroph *S.boulardii* strain using synthetic biology tools to eliminate the use of antibiotic resistance genes in the further steps since it would be problematic for the spread of antibiotic resistance. To do that, we employed the CRISPR CAS-9 system and a donor DNA harboring a stop codon to be inserted into the target gene (TRP1). After verifying the construction of the TRP1 auxotroph strain, we designed an episomal expression vector comprising a strong, constitutive promoter, extracellular signal sequence (Alpha sequence), and a terminator (CYC1). Next, we inserted the Intrinsic Factor protein (the key protein responsible for vitamin B12 absorption) sequence into the vector to recombinantly produce it and secrete it into the gut. However, according to our preliminary data, we could not observe an expression of the protein of interest, suggesting that the system needs further optimization and investigation to work properly. Considering the lack of auxotrophic yeast strains available to be engineered for therapeutic purposes, our engineered *S.boulardii* strain can be employed in yeast-based living therapeutics applications against different disorders waiting for their treatment.

Keywords: Living therapeutics, Synthetic Biology, *Saccharomyces boulardii*, CRISPR Cas-9, B12 Deficiency, Intrinsic Factor

ÖZET

PROBİYOTİK *SACCHAROMYCES BOULARDİİ*'NİN YAŞAYAN TERAPÖTİK UYGULAMALARINDA KONAK OLARAK KULLANILMAK ÜZERE GELİŞTİRİLMESİ

Orhan Nedim Kurt

Malzeme Bilim ve Nanoteknoloji, Yüksek Lisans

Tez danışmanı: Urartu Özgür Şafak ŞEKER

Eylül, 2022

Hastalıkların tedavisinde kullanılan terapötik moleküller veya biyolojik olarak aktif maddeler, yüksek maliyetli ve sınırlı erişilebilirlik ile kimyasal sentez yöntemleri kullanılarak üretilmektedirler. Terapötik moleküller üretmeye yönelik bu yaklaşım, yaşamı değiştiren maddelerin üretilmesini sağlasa da, hala tedavi bekleyen sayısız hastalık vardır. Bu nedenle çok sayıdaki bu hastalıkların tedavi ihtiyaçlarını sadece konvansiyonel üretim yaklaşımları ile karşılamak mümkün olmayacaktır.

Canlı terapötikler, terapötik moleküllerin üretimi ve insan vücuduna doğrudan verilmesi için programlanmış hücrelerdir ve endüstriyel ortamlarda biyomoleküller üretmek için uzun süredir kullanılmaktadırlar. Ayrıca, kanser, diyabet, inflamatuar bağırsak sendromu, ülser ve kolit gibi hastalıkları tedavi etmek için canlı organizmaları tasarlayan ve programlayan birçok çalışma yapılmıştır.

Saccharomyces boulardii CNCM I-745, insanlarda çok uzun süredir probiyotik olarak kullanılan bir maya suşudur. *S. boulardii*, bugüne kadar insan tıbbında kullanılması onaylanan ilk ve tek maya suşudur. *S.boulardii*'nin mikrobiyomu

yenilemek ve patojenlerle rekabet etmek de dahil olmak üzere konakçısına birçok faydası olduğu gösterilmiştir. Ayrıca, *S.boulardii*, insan vücudu ile büyük uyumluluğu ve karmaşık moleküller üretmemizi sağlayan metabolik özellikleri nedeniyle canlı bir terapötik fabrika olarak tasarlanıp programlanacak umut verici bir organizmadır. Bunu hedefleyerek, antibiyotik direncinin yayılması için sorun yaratacağından ileriki adımlarda antibiyotik direnç genlerinin kullanımını ortadan kaldırmak için sentetik biyoloji araçlarını kullanarak bir TRP1 auxotroph *S.boulardii* suşu oluşturduk. Bunu yapmak için, CRISPR CAS-9 sistemini ve hedef gene (TRP1) eklenecek bir durdurma kodonunu barındıran bir donör DNA'yı kullandık. TRP1 oksotrof suşunun yapısını doğruladıktan sonra, güçlü, kurucu bir promotör, hücre dışı sinyal dizisi (Alfa dizisi) ve bir sonlandırıcı (CYC1) içeren bir epizomal ekspresyon vektörü tasarladık. Daha sonra, rekombinant olarak üretmek ve bağırsağa salgılamak için Intrinsic Factor proteini (B12 vitamini emiliminden sorumlu anahtar protein) dizisini vektöre yerleştirdik. Ancak, ön verilerimize göre, ilgilenilen proteinin bir ifadesini gözlemleyemedik, bu da sistemin düzgün çalışması için daha fazla optimizasyon ve araştırmaya ihtiyaç duyduğunu gösteriyor. Terapötik amaçlar için tasarlanabilecek oksotrofik maya suşlarının eksikliği göz önüne alındığında, mühendislik ürünü *S.boulardii* suşumuz, tedavisini bekleyen farklı bozukluklara karşı maya bazlı canlı terapötik uygulamalarda kullanılabilir.

Anahtar kelimeler: canlı terapötikler,sentetik biyoloji, *Saccharomyces boulardii*, CRISPR CAS-9, B12 eksikliği, İntrinsik Faktör

ACKNOWLEDGEMENTS

First, as a biologist, it has always been a privilege for me to become a member of the Synthetic Biosystems Laboratory, Seker Lab. Here, in Dr. Şeker's group, I had an opportunity to learn and apply the fundamentals of synthetic biology and molecular biology and how to conduct scientific research, and I experienced being an independent researcher taking responsibility for his own study, which significantly improved my self-confidence and provided me strong skills for the future steps in my career. Thus, I would like to express my great appreciation and thanks to Dr. Urartu Şeker as he offered me this valuable chance and guided me as a mentor throughout my research, supporting me whenever I needed help.

I would like to present my cordial thanks to Dr. Burcu Gündüz Ergün for her valuable contributions to my research. She has always been helpful and guiding when I faced an experimental issue.

I would also like to thank my jury members, Dr. Aykut Erbaş and Dr. Ceren Özkul Koçak, for their time and their valuable comments and evaluations regarding my study.

I feel incredibly lucky to have my lovely friends Cemile Elif Özçelik, Ahmet Hınçer and Cemre Araz. I could not imagine a time there without them. Elif was always around whenever I needed support, an idea, or advice about anything related to my life. Ahmet has the greatest laugh I have ever seen, and he has been supportive of me during my journey here, and he asked me great questions making me think very hard. Cemre has infinite positive energy and love that gave me the power to move on when I felt tired and was about to give up. Again, I am happy to know them and have them as my best friends.

It was quite challenging for me to adapt to the studies conducted in the lab and learn the basics of techniques we used in the lab. I spent my first months in the lab working with Recep Erdem Ahan and learned almost everything I experienced thanks to his help and guidance. He shared his ideas and recommendations with me whenever I had a question about my research, even in the last days of my thesis. Also, he is a great friend. We spent numerous times together drinking coffee and talking about life, science, society, politics, and the issues we had in our life. Therefore, I could not express my thanks enough for his help and friendship.

I want to especially thank Gizem Makas, our laboratory manager, for her great kindness and help during my time in Seker Lab and for our super fun conversations. She is one of the secret heroes of the team.

All my friends from laboratory have always helped me, especially during my thesis writing process, and offered me to do whatever they can for me to ease my tasks. Therefore, I would like to thank all current and former Synthetic Biosystems Laboratory group members; Ahmet Hınçer, Aslı Semerci, Anooshay Khan, Dr. Behide Saltepe, Dr. Burcu Gündüz Ergün, Dr. Ceren Özkul Koçak, Büşra Merve Kırpat, Cem Dirse Polat, Cemile Elif Özçelik, Çisil Köksaldı, Doğuş Akboğa, Dr. Ebru Aras, Dr. Ebru Şahin Kehribar, Ece Avcı, Dr. Esra Yuca, Eray Ulaş Bozkurt, Gökçe Özkul, Gozeel Binte Shadid, Julian Ostaku, Mehmet Emin Bakar, Melis Karaca, Merve Erden Tüçer, Merve Yavuz, Murat Alp Güngen, Nedim Hacıosmanoğlu, Recep Erdem Ahan, Senem Şen, Sıla Köse, and Suat Tüçer.

I would also love to thank my dear girlfriend, Serra Yılmaz, for her support and for being with me always, especially during the tiring and stressful times of writing.

Lastly, I would like to state my love and thanks to my family and my childhood friend Ömer Egemen Gül. I could not achieve anything without having their belief in me and their love.

.

TABLE OF CONTENTS

List of Tables

List of Figures

CHAPTER 1

INTRODUCTION

1.1. Living Therapeutics

The development process of novel therapeutic molecules that would be used as medicine has been made possible widely via synthetic chemistry methods over the last century. Indeed, a great number of drugs and molecules, many of them are life-changing, have been discovered and explored thanks to this approach. However, countless human diseases and disorders still await new solutions. It is, therefore, necessary to extend the conventional way of designing, optimizing, and manufacturing new therapeutic molecules[1]. Recently, biological molecules, including peptides, antibodies, cytokines, and enzymes, have become reliable and powerful agents for fighting diseases. On the other hand, most of these biologically active molecules must be produced in living cells in settings with strict production and control steps, increasing the cost of the final product and thus limiting the accessibility of these drugs. Hence, strategies that will decrease the costs, increase the availability of the therapeutic molecules, and enable more effective agents to be improved are necessary[2].

Living therapeutics can be described as organisms engineered to fix and cure disorders by producing and (or) delivering a molecule in the human body[3]. Producing and delivering bioactive molecules through the engineered cells to the body has significant advantages over conventional methods. First, expected side effects are less likely since they are administrated in non-invasive ways, which is painful, and locally rather than being applied systemically. Second, the cost to produce, purify, and deliver a compound in a facility is much higher than employing

microorganisms to produce the compound in the body[4]. Additionally, it is possible to express more complex compounds, or more than one compound, using the features of the cells enabling us to control the timing of the production and the properties of the molecule[3].

At the beginning of the 2000s, *Lactobacillus plantarum* (*L. plantarum*) and *L. casei* were engineered to express and deliver the tetanus toxin fragment C (TTFC), a non-toxic fragment, into the human body by presenting the relevant antigen on the cell surface with the aim of triggering the immune system[5]. More recently, *Bifidobacterium longum* has been engineered to target solid tumors by secreting an immune modulator molecule, *Listeria monocytogenes* has been designed to target cervical cancers by means of expressing an antigen; *Lactococcus lactis* has been engineered against oral mucositis[6], and these organisms are currently in phase trials[7].

In addition to engineered bacteria being used as living factories in the body, mammalian cells have long been studied to be programmed as therapeutic devices. Engineered T cells carrying chimeric receptors (CAR-T cells) are one of the most known types of mammalian living therapeutics and have been approved, for the first time, to be used in the treatment of lymphoma[8].

1.2. Probiotics as Living Therapeutics

According to the Food and Agricultural Organization (FOA) and the World Health Organization (WHO), probiotics are defined as "Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host."[9]. The probiotics commonly used include *Bacillus spp.*, *Bifidobacterium spp.*, *Enterococcus spp*. *Lactobacillus spp.*, and *Streptococcus spp*.[10]. Although probiotics have

achieved significant improvements against some certain diseases, such as phenylketonuria, diabetes, and inflammatory bowel disease (IBD), there is no one type of probiotic that works for everyone and every condition. But, it is possible to program and engineer probiotics for specific tissues, tracts, or cells via metabolic engineering techniques and synthetic biology tools[11].

Infection diseases might be targeted by benefiting from probiotics. For example, in a previous study, an *Escherichia coli* strain was engineered using a gene circuit to detect and combat *Pseudomonas aeruginosa* infection[12]. Moreover, disorders caused by enzyme deficiency can be fixed through programmed probiotics. In another study, *E. coli Nissle* 1917 was designed to cure phenylketonuria by secreting two enzymes functioning in phenylalanine metabolism when the cell is triggered via anoxic environment[13]. In more recent studies, a novel EcN strain was developed to treat inflammatory bowel disease (IBD), secreting an immunoregulatory protein[14]; and another EcN strain was engineered to secrete catalase to enhance the radiosensitivity of the tumors[15].

In addition to secreting curing molecules, probiotics can also be programmed to detect the biomarkers of a certain condition and give signals that can be observed easily. With this aim, in a study, a bacterial probiotic was genetically programmed to detect a molecule produced during liver metastasis and create a substance that will change the color of the urine, thus enabling the naked-eye detection of the related biomarker[16].

1.2.1. *Saccharomyces boulardii* CNCM I-745 as a Living Therapeutics

Saccharomyces boulardii (S.boulardii) is a yeast cell with a genetic similarity of more than 99% with *S. cerevisiae*[17]. *Saccharomyces boulardii* CNCM I-745 is the first yeast that has been investigated to be used in humans as a probiotic agent[18].

Compared to *S.cerevisiae*, *S.boulardii* is more resistant to acidic conditions[19] and temperature and grows better at 37 ºC, which is the average human body temperature[20]. *S.boulardii* is also durable against harsh conditions of the stomach and the bile salts in the intestinal tract and is capable of colonizing at the intestine[21].

As a probiotic agent, *S.boulardii* provides a large number of benefits. It has been shown that *S.boulardii* competes with bacterial pathogens, including *Clostridium difficile, Escherichia coli, Shigella, and Salmonella*[22]. Also, it can be applied to people receiving antibiotics since *S.boulardii* has a natural resistance to antibiotics[23]. Additionally, it was shown that *S.boulardii* enhanced the intestinal microbiota's regeneration[24].

Considering these features of *S.boulardii*, it is a wise choice for a chassis organism to be engineered as a living therapeutic device. In recent years, *S. boulardii* was engineered to secrete and deliver a molecule to the bloodstream following colonization in the gut, and it successfully induced an immune response in mice against the molecule recombinantly produced by *S.boulardii*[25]. Another study designed a *S.boulardii* strain by engineering a synthetic promoter and a transactivation system through which different heterologous therapeutic proteins can be expressed in a more effective and precise way[26].

Importantly, *S.boulardii* has already been used in the biotechnology industry to manufacture many compounds, including insulin, growth factor derivative, granulocyte-macrophage colony-stimulating factor, and hepatitis B antigen[27]. Also, *S.boulardii* is capable of synthesizing complex molecules requiring post-translational modifications following the expression[28], which offers us a crucial advantage in designing and improving novel therapeutics.

1.3.Pernicious Anemia and Intrinsic Factor

Pernicious anemia (PA)is a type of megaloblastic anemia that is caused by vitamin B12 (cobalamin) deficiency[29], [30], manifesting symptoms including fatigue, pallor, impaired red blood cell formation, and jaundice[31]. PA is the most common reason for cobalamin deficiency over the world[32]. Also, pernicious anemia is considered an autoimmune disease in which autoantibodies against the protein (Intrinsic Factor), responsible for B12 absorption, and gastric parietal cells producing Intrinsic Factor[33]. Parietal cell antibodies retain the parietal cells from producing Intrinsic Factor; Intrinsic factor antibodies, on the other hand, interfere with the interaction of IF with the cobalamin molecule in the intestine[34], [35] Figure 1. Depending on the cobalamin deficiency rate, the consequences of the condition might vary from mild clinical features to serious neurological complications. Additionally, some other diseases, including thyroid disorders, vitiligo, and diabetes mellitus, were found to be associated with pernicious anemia[36]. The current approach to controlling pernicious anemia is cobalamin replacement therapy[32].

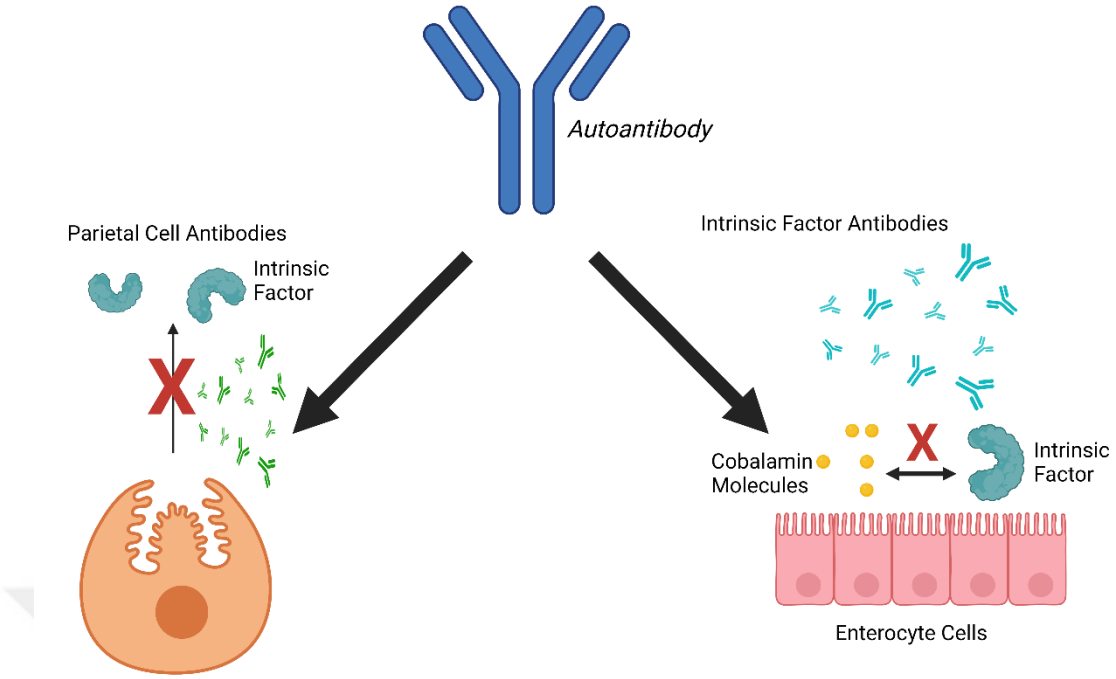


Figure 1. Schematic illustration of autoantibodies produced against parietal cells and Intrinsic Factor.

Parietal cell antibodies block the functional Intrinsic Factor production, while Intrinsic Factor antibodies detain IF proteins interacting with the cobalamin molecule, failing the B12 absorption mechanism.

There are three essential proteins involved in the Cobalamin absorption process; haptacorrin, transcobalamin II, and Intrinsic Factor[37]. Intrinsic factor, a glycoprotein that is produced by gastric parietal cells in the stomach and functions a crucial role in the absorption and transportation of vitamin B12[38]. Normally, dietary B12 supplement binds to haptacorrin molecule, which protects B12 from the harsh conditions of the stomach at the superior gastric part. Then, the haptacorrins are degraded at the ileum, resulting in the release of B12. Following degradation of

haptacorrins and release of B12, B12 molecules bind to the Intrinsic Factor to be transported to the enterocyte cells, where absorption is established[39] Figure 2.

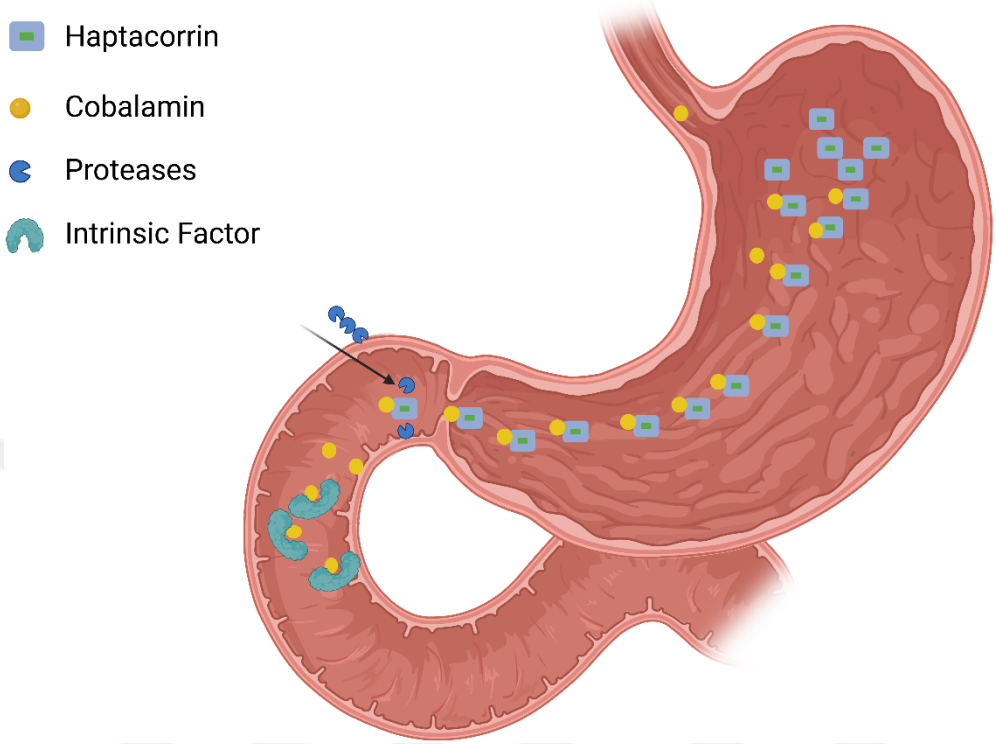


Figure 2. Schematic Illustration of the Cobalamin Uptake Pathway.

Haptacorrins are the first proteins that Cobalamin molecules encounter within the stomach. Then, haptacorrins are degraded by proteases, and the Intrinsic Factor bind to Cobalamin to transport them to the enterocyte cells to be absorbed.

1.4. Objective of the Study

Living cells have long been used to manufacture biologically active compounds in industrial settings. Additionally, a great effort has been made to engineer microorganisms to cure diseases by secreting therapeutic molecules within the human body, starting the era of living therapeutics. Probiotics enable safer, cheaper, and more reliable ways of producing and delivering the molecules into the human body since they are already being used in human medicine and since we have a great

body of knowledge about their physiological and genetic properties. Although many probiotics have been designed to overcome various conditions, numerous disorders are still waiting for more effective treatments. In this study, we aim to engineer *Saccharomyces boulardii* CNCM I-745 as a chassis cell through which recombinant therapeutic proteins can be expressed in the body. For this purpose, we knocked out the TRP1 gene of the *S.boulardii* employing CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) CAS-9 system by inserting a stop codon into the coding sequence of the TRP1 gene via homologous recombination (Figure 3, and Figure 4.). Thus, we established an auxotrophic TRP1Δ *S.boulardii* strain, enabling us to proceed without antibiotic selection strategies, which would be problematic to be used in humans due to the spreading probability of antibiotic resistance. Then, we constructed an episomal expression vector comprising a constitutive TEF1 promoter and CYC1 terminator and the Alpha secretion signal in which the therapeutic protein can be inserted and thus be produced extracellularly in the gut. Next, we inserted the Intrinsic Factor (IF) protein sequence into this vector to combat Pernicious Anemia caused by IF-related B12 deficiency (Figure 3.).

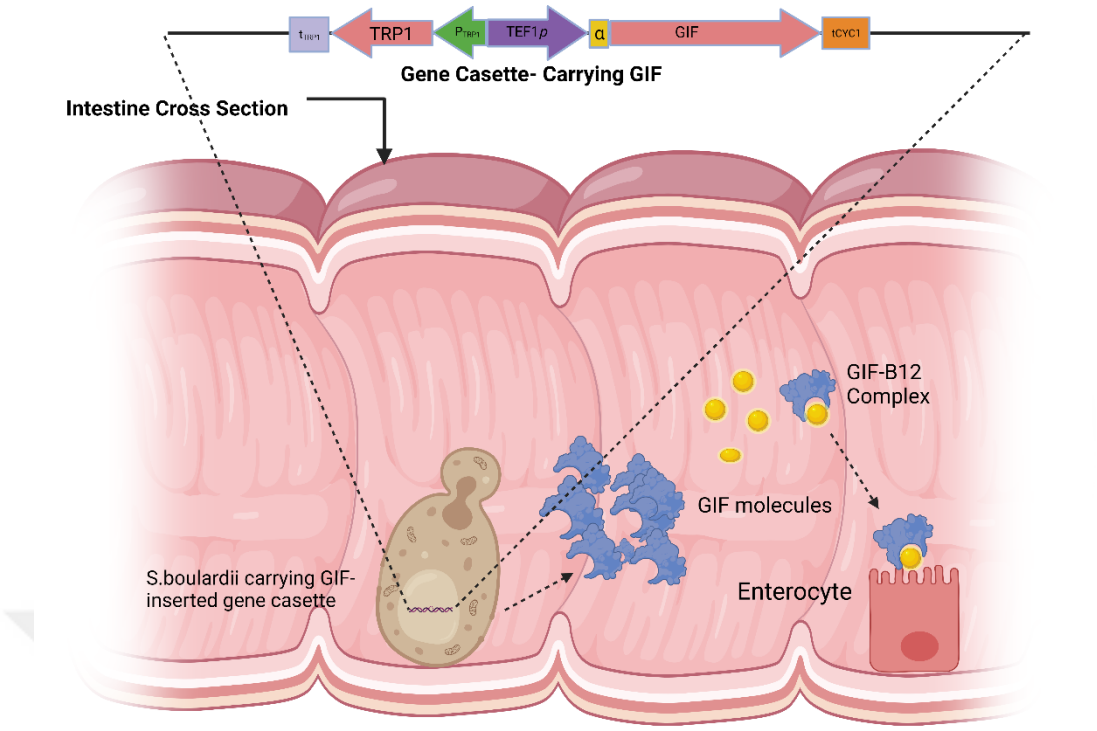


Figure 3. Schematic Illustration of TRP1 Auxotroph *S.boulardii* Carrying GIF-Inserted Expression Plasmid and Secreting the GIF in the Gut.

The secreted GIF proteins bind to Cobalamin molecules and ensure they are absorbed by the enterocyte cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Revival, Growth, and Cell Stock Preparation

Saccharomyces Boulardii (CNCM-I745®) used in this study was obtained from a pharmacy in lyophilized form (Biocodex, Reflor® 250 mg). Lyophilized *S.boulardii* was revived by resuspending one capsule of Reflor in 10mL of YPD medium (Tryptone 2% (w/v), Yeast Extract 1% (w/v), D-Glucose 2% (w/v)) and grown at 30ºC, 200 rpm overnight. Next day, the revived *S.boulardii* was inoculated onto YDP agar media using a loop, (Agar 2% (w/v), Tryptone 2% (w/v), Yeast Extract 1% (w/v), Glucose 2% (w/v)) and grown at 30ºC for 2 days. Then, a single colony was picked up and inoculated in 5 mL of YPD medium at 30ºC overnight. For cell stock preparation, 500 µL of an overnight culture of *S.boulardii* was added to 500 µL of sterile glycerol (30% (v/v)) in cryotubes, resulting in a 15%(v/v) glycerol ratio, and stored at -80 ºC.

2.2. Donor DNA, Plasmids Used in The Study, and Their Construction

Initially, p414-TEF1p-Cas9-CYC1t-NAT1 (Addgene number, 64329) and pRS42H-gRNA-trp-HYB plasmid with TRP1 disruption gRNA sequence (Addgene number, 64331) were obtained from the Addgene. The ordered plasmids, p414-TEF1p-Cas9-CYC1t-NAT1 and pRS42H-gRNA-trp-HYB, were delivered in *E. coli* DH5α cells in a stub culture format. The *E. coli* DH5α cells carrying the plasmids were inoculated on LB agar plates with corresponding antibiotics overnight. Next day, a single colony from the Cas-9 plasmid plate and a single colony from the gRNA plasmid

were picked up and grown in 3 mL of LB medium supplied with the relevant antibiotics overnight. Following incubation, cultures were pelleted by centrifugation at 8000 x g for 5 minutes and the supernatant was discarded. Plasmid isolation was performed using Thermo Scientific GeneJet Plasmid Miniprep Kit according to the manufacturer's instructions. The isolated Cas-9 and gRNA plasmids were loaded in 1% agarose gel, run at 125 V for 30 min, and cleaned up from the gel through NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel).

The pETcon-TEF1p-Alpha-His-GS-6His/GIF-CYC1t-TRP plasmid was constructed as follows: First, the empty backbone, pETcon(-)(Addgene number,41522) plasmid was simultaneously digested with KpnI-HF (NEB) and then BamHI-HF(NEB), according to the double digestion protocol suggested on the NEB's digestion tool. The resulting digestion product was loaded in 1% agarose gel containing SYBR™ Safe DNA gel stain, and electrophoresis was performed at 125 V for 30 minutes. The expected gel band was cut and cleaned up from the agarose gel via NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Primer pairs used to amplify the parts of the gene insert were designed to harbor 30 base pairs of overlapping sequences with each other according to their order. Additionally, the ONK-25 primer contains a KpnI cut site to be fused at the 5' of the TEF1 promoter, and the ONK-34 primer has a BamHI cut site to be added at the 5' of the CYC1 terminator. Then, the TEF1 promoter and CYC1 terminator were amplified from the p414-TEF1p-Cas9-CYC1t-NAT1 (Addgene number, 64329) plasmid using ONK-25/ONK-32 and ONK-27/ONK-34 primer pairs, respectively. The Alpha secretion signal sequence was amplified from the pGAPZ A plasmid (Thermo Fisher Scientific, Catalog No: V20520)) with the ONK-29/ONK-30 primer pair. The GIF protein sequence amplified from a gene construct synthesized by Genewizz, using ONK-24/ONK-31

primer pair designed to add GS-Linker and 6His-Tag to the N terminus of the protein. All PCR amplifications were established using Q5 polymerase. The resulting PCR products were loaded in 1% agarose gel with SYBR™ Safe DNA gel stain, and electrophoresis was conducted at 125V for 30 minutes. Amplified fragments with expected lengths were cut and cleaned up from the gel using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Next, the TEF1 promoter and Alpha secretion signal were fused via overlap extension PCR using Q5 DNA polymerase and ONK25/ONK30 primer pair, resulting in the TEF1-Alpha fragments. The GIF-coding sequence and CYC1 terminator were fused via overlap extension PCR using Q5 DNA polymerase and ONK-31/ONK-34 primer pair, resulting in the GIF-CYC1 fragment. Overlap extension PCR products were loaded in 1% agarose gel with SYBR™ Safe DNA gel stain, and electrophoresis was conducted at 125V for 30 minutes. Fused fragments were cut and cleaned up from the gel using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Then, TEF1-Alpha and GIF-CYC1 fragments were fused using ONK-25/ONK-34 primer pair through overlap extension PCR protocol, and the PCR product was loaded in agarose gel, followed by cut and clean-up steps. The gene insert containing the TEF1 promoter, the Alpha secretion signal, the GIF sequence with GS-Linker and 6His-Tag, and the CYC1 terminator was digested with KpnI and BamHI enzymes simultaneously by following the manufacturer's instructions. The digestion reaction mix containing the final insert with KpnI and BamHI cut sites was loaded in 1%(w/v) agarose gel and run at 125V for 30 min. Next, the corresponding fragment was cut and cleaned up from the gel. The vector and the gene cassette were assembled using T4 DNA ligase(reaction recipe is given in Appendix E, Table E.12). The plasmid map is shown in Figure C.3.

The wholeT4 ligation reaction mixture was then transformed into chemically competent *E. coli* DH5α cells. Chemically component *E. coli* DH5α cell preparation is as follows: DH5α cells were inoculated from -80ºC cell stock and cultured overnight in LB medium with proper antibiotics. The next day, the cell culture was diluted 1:100 in LB with appropriate antibiotics and grown until OD_{600} value of 0.2–0.5. Then cells were chilled on ice for 10 minutes, followed by centrifugation at 1000 x g for 10 minutes at 4ºC. The supernatant was discarded, and the cell pellet was resuspended in Transformation and Storage Buffer (TSS) (PEG 8000 20% (w/v), DMSO 10% (w/v), $MgCl_2$ 100mM, pH 6.5 in LB) with a ratio of 1:10 of the original volume. Chemically competent cells were aliquoted into 1.5 mL microcentrifuge tubes as 100µL volumes and stored at -80 ºC until use. After 16 hours from the transformation of the T4 ligation mix into the component *E. coli* DH5α cells, different colonies were selected from the LB agar plate, and the presence of the gene insert in the colonies was checked by performing colony PCR using ONK24/ONK-29 primer pair. Positive colonies harboring the pETcon-TEF1p-Alpha-His-GS-6His/GIF-CYC1t-TRP plasmid were grown overnight in LB supplied with the relevant antibiotics. After overnight incubation, plasmid isolation was performed on the cultures of all positive colonies to be verified via Next Generation Sequencing. Sequencing data were analyzed by Geneious Prime® (2021.1.1) software. The final alignment is given in Figure D.2

Donor DNA used to insert a stop codon into the TRP1 gene of *S.boulardii* through homologous recombination was created using ONK-5/ONK-6 primer pairs via overlap extension PCR. ONK-5/ONK-6 primers were designed to have 30 overlapping base pairs.

2.3. Development of Auxotrophic *S.boulardii* Mutant

To create the TRP1Δ auxotroph *S.boulardii* strain used in this study, p414-TEF1p-Cas9-CYC1t-NAT1 (Addgene number, 64329) plasmid was transformed to wild-type *Saccharomyces Boulardii* (CNCM-I745®) via electroporation method (details of the protocol is given below). Then, transformed cells were inoculated on YPD agar plates with the proper antibiotic and grown at 30 ºC for 3 days. At the end of the 3 days, colonies were screened for harboring the Cas-9 plasmid via colony PCR using p22seq(DNA)/ORI-F(DNA) primers. Positive colonies were further grown on YPD agar with the relevant antibiotic at 30 ºC for 2 days to be transformed with pRS42H-gRNA-trp-HYB plasmid, carrying TRP1 disruption gRNA sequence (Addgene number, 64331). *S.boulardii* cells containing Cas-9 plasmid were co-transformed with TRP1-disrupting gRNA plasmid, and the TRP1 donor DNA, by electroporation. Transformed cells were plated on YPD agar with two proper antibiotics, the first is for Cas-9 plasmid, and the second is for gRNA plasmid. Then, cells were grown at 30 ºC for 4 days. Colonies that grew on the YPD agar plates supplied with two antibiotics were investigated via the negative selection method explained below. The colonies that grew on the double-antibiotic plates but did not grow on synthetic drop-out media without tryptophan (SDO w/o Trp) were grown in 5mL of YPD medium at 30 ºC overnight. Next, the TRP1 gene sequence of the transformed *S.boulardii* cells was amplified using Q5 DNA polymerase with ONK-7/ONK-8 primer pair and sent to NGS to be analyzed. Following the verification of TRP1 knocked-out strain via NGS, the proper colonies were sequentially cultured on YPD media to remove the plasmids from the cells (the plasmid removal step is described below).

2.4. Electroporation of *Saccharomyces boulardii*

First, *S.boulardii* cells to be electroporated were inoculated from -80 ºC on YPD agar at 30 ºC for 2 days to prepare fresh plates. At the end of the 2 days, a single colony was picked up and grown in 5mL of YPD at 30ºC, 200 rpm overnight. The next day, overnight culture was added in 50 mL of YPD at 30ºC, 200 rpm until the OD_{600} reached 0.8, which took approximately 9 hours. Then, the whole culture was transferred to a 50mL sterile falcon and pelleted by centrifugation at 1100 x g at 4 ºC for 5 minutes. By this step, all the next steps were carried out on ice. The supernatant was decanted and the pellet was resuspended with 20 mL of sterile water previously chilled on ice. The suspended cells were centrifugued again and the supernatant discarded. The cell pellet was treated with 20 mL of 0.1 M LiAc buffer (16mL of 1 M Sorbitol, 2mL of 10 X Tris-Eda Buffer(pH 7.5, 100 mM Tris-HCl, 10 mM EDTA), 2 mL of 1M LiAc (pH 7.5)) and waited at 30 ºC for 30 minutes. Next, 0.2 mL of 1 M DTT was added to the culture, and the cells were kept at 30 ºC for 15 minutes. Then, cells were centrifuged at 1100 x g at 4 ºC for 5 minutes and washed twice with 20 mL of ice-cold sorbitol (1M) by resuspending and recentrifuging at 1100 x g at 4 ºC for 5 minues. The resulting pellet was resuspended in 200µl of 1M ice-cold sorbitol and aliquoted as 50µL volumes in 1mL of microcentrifuge tubes on ice. Then, 1µg of plasmid, and 3µg of donor DNA (if required) was added onto the cells and mixed with pipetting. Cells treated with DNA samples were kept on ice for 15 minutes. All cells were transferred into electroporation cuvette (green cap) and the cuvette set in the electroporation device, Bio-Rad Gene Pulser Xcell Electroporation System. 1.5kV voltage was applied to the cuvett and 1mL of ice-cold YPD containing 1M sorbitol was added on cells after the pulse. The suspension was mixed by pipetting and incubated at 30 ºC for 3 hours. Incubated cells were

centrifuged at 3000 x g for 1 minute and resuspended in 150 µL of sterile water. The resuspended cells were inoculated on YPD agar plates or Synthetic Drop-Out Agar with the proper antibiotics, depending on the plasmid sent into the cells. Transformed colonies were checked via colony PCR or negative selection plates (for the TRP1 gene knock-out experiment).

2.5. Negative Selection of TRP1Δ Auxotroph *S.boulardii* Colonies

To perform negative selection of *S.boulardii* colonies lacking a functional TRP1 gene synthetic drop-out agar medium without tryptophan was prepared(1.92g/L synthetic drop-out mix w/o trp, 6.7g/L Yeast Nitrogen Base without amino acids, 20g D-Glucose, 20g/L Agar). The colonies that grew on YPD agar plates with double antibiotics, one for Cas-9 plasmid and the second for gRNA plasmid, were spread on either SDO w/o trp or YPD agar plates simultaneously and grown at 30 ºC for 2 days. At the end of the two days, the colonies that grew on the YPD agar plate but did not grow on SDO w/o trp plates were picked up from the YPD plate and further inoculated in 5mL of SDO w/o trp medium at 30 ºC, at 200 rpm overnight. Then, the gene sequence of the TRP1 gene was amplified using Q5 DNA polymerase with ONK-7/ONK-8 primer pair and sent to NGS for analysis. The NGS data were analyzed with Geneious Prime® (2021.1.1) software.

2.6. Removal of Plasmids with Antibiotic Resistance Gene from Yeast Cells

The colonies verified as TRP1 auxotrophs via NGS data were inoculated on YPD agar plates as follows: First, the colonies were inoculated on a YPD agar plate with the Nourseothricin antibiotic at 30 ºC for 2 days. Meanwhile, control groups were inoculated on YPD agar plates with two antibiotics, Nourseothricin (NAT) and Hygromycin B (HyB), for the Cas-9 plasmid and the gRNA plasmid, respectively. The exact process was repeated two times by picking up single colonies each time.

At the end of the 6th day (following the 3rd inoculation), colonies were selected from the YPD plate with NAT antibiotics and simultaneously spread on either YPD agar plates with NAT or YPD agar plate with NAT and HyB antibiotics and grown at 30 ºC for 2 days. Then, the colonies that grew on the YPD agar plate with only NAT but did not grow on the two antibiotics plate were selected and inoculated on the YPD agar plate without antibiotics at 30 ºC for 2 days. At the end of the 2nd day, colonies were further inoculated to a fresh YPD agar plate without antibiotics. This process was repeated one more time at the end of the second day. Lastly, the single colonies were picked up and spread either on a YPD agar plate with two antibiotics or a YPD agar plate without antibiotics at 30 ºC for 2 days. The colonies that survived on the YPD agar plate without antibiotics but could not survive on the YPD agar plate with double antibiotics were selected and inoculated in 5mL of YPD medium at 30 ºC, at 200 rpm overnight to prepare at -80 ºC cell stocks.

2.7. Chemical Transformation of *Saccharomyces boulardii*

The chemical transformation of yeast cells was performed using LiAc/Single-Stranded Carrier DNA-PEG method. First, a fresh culture of *S.boulardii* was prepared from -80 ºC cell stock by inoculating on a YPD agar plate and growing at 30 ºC for 2 days. Then, a single colony was picked from the fresh YPD agar plate and inoculated in 10mL of 1 X YPAD medium (1% Yeast Extract, 2% Tryptone, 80 mg/L Adenine Hemisulfate, 2% D-Glucose) at 30 ºC at 200 rpm overnight. Meanwhile, 50 mL of 2 X YPAD medium (2% Yeast Extract, 4% Tryptone, 80 mg/L Adenine Hemisulfate, 4% D-Glucose) was put at 30 ºC in a 250 mL flask. The whole culture was diluted in mL of pre-warmed 2 x YPAD medium when the OD_{600} value reached 2.0 (0.1 OD_{600} was considered to contain $1x10^6$ cells/mL). According to this calculation, the total number of cells added to the 50 mL of 2 x YPAD was

approximately 200 million. Then, the culture was incubated at 30 ºC at 200 rpm for around 4 hours until the OD_{600} of the suspension reached 2.0. The while, carrier DNAs stored at -20 ºC were denatured in a heat block at 98ºC for 5 minutes, chilled on ice, and kept in ice until use. The preparation of single-stranded carrier DNA is as follows: The ultra-pure salmon sperm DNA solution was purchased from Thermo Fisher Scientific (Catalog Number: 15632011). Each tube in the box contained 1mL of salmon sperm DNA (10mg/mL). Then, salmon sperm DNA was 1:5 diluted in TE buffer (pH 8.0, 10 mM Tris-HCl, 1 mM Na_2EDTA) to a concentration of 2mg/mL. The diluted DNAs were stored at -20 ºC until use.

Once the 50 mL culture reached the 2.0 of OD_{600} value, cells were harvested at 3000 x g for 5 minutes at room temperature. Harvested cells were resuspended with 25 mL of sterile water and centrifuged at 3000 x g for minutes at 20 ºC. The wash step was repeated one more time, and cells were pelleted by centrifugation. The pelleted cells were resuspended in 1 mL sterile water and transferred to a 1.5 mL microcentrifuge tube. The cell suspension was centrifuged at 13000 x g for 30 seconds, and the supernatant was removed. Cells were resuspended in 1 mL sterile water, mixed vigorously, and aliquoted in 100 µL volumes into 1.5 mL microcentrifuge tubes. Each aliquot contained 100 million cells approximately. Cells were centrifuged at 13000 x g for 30 seconds, and the supernatant was discarded. The resulting cell pellet was treated with the 326 µL transformation buffer(240µL PEG 4000 (50% (w/v)), 36 µL 1 M LiAc, 50 µL Single-stranded carrier DNA (2mg/mL)) and vortexed. Then, the plasmid was added to the cells, treated with transformation buffer, and mixed by pipetting. Following adding the plasmid, sterile water was added to the mix to a 34 µL final volume (plasmid volume plus water volume). The cell suspension in the transformation buffer was incubated at 42 ºC for 40 minutes. At the end of the 40

minutes, cells were pelleted by centrifugation at 13000 x for 30 seconds and resuspended in 500 µL sterile water. Depending on the plasmids applied, the resulting suspensions were inoculated on YPAD agar plates or Synthetic Drop-Out agar plates with or without antibiotics.

2.8. Next Generation Sequencing and Alignment of the Results

After constructs were established and confirmed via colony PCR, each colony carrying the proper plasmid was inoculated in the appropriate media. The constructed plasmids were isolated using Thermo Scientific GeneJet Plasmid Miniprep Kit, applying the instructions of the manufacturer. Concentrations of the isolated plasmids were measured through Thermo Scientific NanoDrop™ 2000 Spectrophotometer. Depending on the plasmid DNA concentration, 2000 ng of plasmid DNA in total was prepared for NGS analysis by mixing the corresponding amount of the DNA solution with the proper amount of sterile water up to 20 µL. NGS results were downloaded in FASTA format from the file sent by the sequencing company, and the plasmid maps were downloaded as .gb files from the Benchling tool. The sequencing result data and the reference map downloaded from the Benchling were aligned using Geneious Prime® (2021.1.1) software

2.9. Recombinant Production of Intrinsic Factor

To express extracellular recombinant Intrinsic Factor having a GS linker and 6xHistag, pETcon-TEF1p-Alpha-His-GS-6His/GIF-CYC1t-TRP plasmid whose sequence was verified by NGS (Figure D 2.) was transformed into TRP1 auxotrophic *Saccharomyces boulardii* cells via chemical transformation as mentioned in section 2.7. Following the transformation, the transformed cells were inoculated on synthetic drop-out agar media plates w/o tryptophan at 30 °C for 2 days until single colonies became visible. Next, colonies transformed with pETcon-TEF1p-Alpha-His-GS-

6His/GIF-CYC1t-TRP plasmid were inoculated in synthetic drop-out media w/o tryptophan at 30 °C and grown for 60 hours. At the end of the 60th hour, 1 mL of the culture was taken from the flask, and cells were pelleted by centrifugation at 5000 x g for 5 minutes. The 1 mL supernatant was transferred to a 15 mL falcon tube. The acetone precipitation method was applied to the supernatant before SDS-Page and Western Blow were performed (Acetone precipitation is described in section 2. 10.). Cell pellets were stored at -20 °C until use. The supernatant taken and cell pellet held at -20 °C were used in SDS-Page and Western Blot assays. No external induction was applied to the cells during the 60 hours of inoculation since the production of the recombinant Intrinsic Factor is controlled by a constitutive promoter, TEF1, and its extracellular release is established by the Alpha secretion signal.

2.10. SDS-PAGE and Western Blot

SDS-PAGE and Western Blot techniques were employed to confirm that the Intrinsic Factor is produced recombinantly by *S.boulardii*. First, TRP1Δ *S. boulardii* cells transformed with pETcon-TEF1p-Alpha-His-GS-6His/GIF-CYC1t-TRP plasmid were grown at 30 °C for 60 hours. Following the 60th hour, 1 mL of the culture was centrifuged at 5000 x g for 5 minutes, and 1 mL of supernatant was transferred into a 15 mL falcon tube. The remaining cell pellet was stored at -20 °C until the SDS-Page was performed. Next, the four times volume of the supernatant (4 mL) cold acetone (-20 °C) was added to the supernatant sample. The mixture was held at -20 °C overnight. The next day, the liquid phase of the mix was discarded neatly and properly without resuspending the pellet at the bottom. The resulting pellet was evaporated at room temperature for 30 minutes. After that, the pellet obtained from acetone precipitation and the cell pellet kept at -20°C were dissolved in 1 X SDS

loading dye and put at 95°C for 5 minutes before loading to the gel. SDS-Page gel with a concentration of 10% was prepared. Separating gel was prepared using 2.2 mL of dH_2O, 2.6 mL of 1.5 M Tris-HCl (pH 8.8), 100 µL of 10% (w/v) SDS, 5 mL of Acrylamide/Bisacrylamide (30%/0.8% w/v), 100 µL of 10% (w/v) APS, and 10 µL of tetramethylethylenediamine (TEMED). Stacking gel was prepared by mixing 2.975 mL of ddH_2O, 1.25 mL of 0.5 M Tris-HCl (pH 6.8), 50 µL of 10% (w/v) SDS, 0.67 mL of Acrylamide/Bisacrylamide (30%/0.8% w/v), 50 µL of 10% (w/v) APS, and 5 of µL TEMED. After sample loading, the gel was run at 100V in 1X SDS Running Buffer for 45 minutes and then at 140V until the end of the run. Then, the gel was stained using Coomassie staining solution (3 g/L Coomassie Brilliant Blue R -250, 45% methanol, 10% acetic acid), followed by a destaining step in destaining buffer (30% methanol, 10% acetic acid) to clean the Coomassie Brilliant Blue from the gel.

Western Blot analysis was conducted as the recombinant Intrinsic Protein is his-tagged. To perform Western Blot, another SDS-Page gel with 10% concentration was prepared as described above, and samples were run in 1 X SDS running buffer for 45 minutes at 100 V and then at 140V until the end of the run. Following the end of the run, samples found on the gel were transferred to a Polyvinylidene fluoride (PVDF) membrane (Thermo Scientific) employing the Transblot Transfer System (Biorad). Then, the PVDF membrane was blocked with 5% skimmed-milk powder (prepared in 1X TBST) at 4°C overnight on a rotator. After the blocking step, the PVDF membrane was incubated with the anti his-tag primary antibody solution, dilution rate of 1:10000, for 1.5 hours at room temperature on a rotator. Next, the membrane was washed on the rotator with 1X TBST at room temperature for 5 minutes, 10 minutes, and 10 minutes respectively. The membrane was treated with

the anti-mouse secondary antibody that is conjugated with horseradish peroxidase (HRP), dissolved in 1X TBST with a dilution rate of 1:10000, for 1.5 hours at room temperature on the rotator. Subsequently, the membrane was washed on the rotator by using 1X TBST for 5, 10, and 10 minutes respectively. Lastly, the membrane was incubated with ECL substrate (Biorad) following the manufacturer's protocol and visualized via the Fusion® Software of Vilber Lourmat imaging system.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Development of TRP1 Auxotroph *Saccharomyces boulardii* Strain

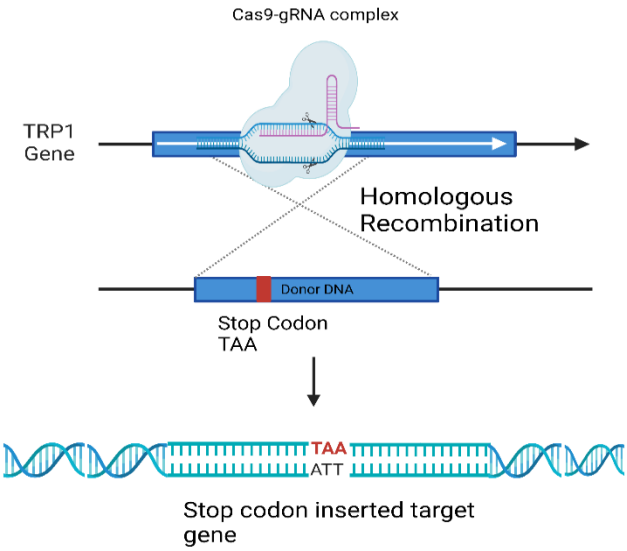
To create an auxotrophic *S.boulardii* strain, the pRS414-TEF1p-Cas9-CYC1t plasmid (Addgene number, 64329) carrying human-optimized *Streptococcus pyogenes* Cas-9 enzyme sequence as the gene insert with NAT antibiotics as the selection marker was ordered. Additionally, the pRS42H-gRNA-trp-HYB plasmid (Addgene number, 64331) with the gRNA sequence targeting the TRP1 gene of *S.boulardii* was obtained. The Cas-9 enzyme is under the control of a constitutive yeast promotor TEF1, and CYC1 terminator. The production of the TRP1-gRNA block is controlled by the SNR52 promoter and SUP4 terminator. The mechanism of action for CRISPR Cas-9 mediated knock-out of the TRP1 gene is illustrated in Figure 3.

The transformation of the Cas-9 plasmid to wild-type *S.boulardii* was achieved via electroporation. However, the first attempts to perform the electroporation protocol failed. Initially, the required overnight cell amount could not be reached due to insufficient initial cell amount. The cell amount issue was solved by significantly increasing the initial cell amount taken from freshly prepared YPD agar plates. Then, the electroporation method failed again, although the cell number problem had been solved. Therefore, different protocol conditions were tried to optimize the protocol, including changing the DNA amount transformed, increasing or decreasing the inoculation duration after electroporation, and doubling the wash step. In the end, the protocol was finalized as described above. The colonies that grew on YPD agar plate

containing NAT antibiotics were picked up and screened via colony PCR using Pfu DNA polymerase with the primer pair p22seq(DNA)/ORI-F(DNA) given in Table B.1

The pRS42H-gRNA-trp-HYB plasmid and the donor DNA were co-transformed to *S.boulardii* cells harboring Cas-9 plasmid via electroporation. The pRS42H-gRNA-trp-HYB plasmid carries the TRP1 disruption gene cassette and produces the gRNA that mediates the CRISPR Cas-9 enzyme to the exact location where the enzyme will cut the DNA strands. The primer pair ONK-5/ONK-6, giving the donor DNA, has a 30 bp of an overlapping region which also contains the "TAAAAT" sequence, aiming to insert the stop codon to both sense and antisense strands of the DNA. The donor DNA has two homology arms flanking the stop codon and overlapping with the TRP1 gene of *S.boulardii*. The donor DNA was created via overlap extension PCR using Q5 DNA polymerase, as shown in Figure 3.B. The placement of the donor DNA to the TRP1 was achieved via Cas-9 induced homology-directed repair mechanism (HDR), as shown in Figure 3.A The gRNA is responsible for navigating the Cas-9 enzyme to its target and also keeping it stable while binding to DNA.

A.



B.

Figure 4. The Illustration of the Insertion of the Donor DNA to the TRP1 Gene by Cas-9-Induced HDR Mechanism. A.

The schematic presentation of the construction of the donor DNA using primers via overlap extension PCR. B.

Following the co-transformation of gRNA plasmid and the donor DNA to the *S.boulardii* cells with Cas-9 plasmid, 10 colonies were picked from the YPD plate supplied with NAT and HyB antibiotics and inoculated simultaneously on either a YPD plate with two antibiotics (NAT and HyB) or a synthetic drop-out agar without tryptophan, and grown at 30 ºC for 3 days. Then, 4 of 10 colonies were found to survive on YPD plate with two antibiotics but could not on SDO agar w/o trp. The TRP1 gene sequences of those 4 colonies were amplified with Q5 DNA polymerase using the ONK-7/ONK-8 primer pair and sent to NGS for sequence analysis. It was found that those four colonies were successfully knocked out by means of the insertion of the stop codon into the TRP1 gene. The NGS results showing that the stop codon is inserted into the TRP1 gene were provided in Figure 4., and given in Table D 1., in FASTA format.

A.

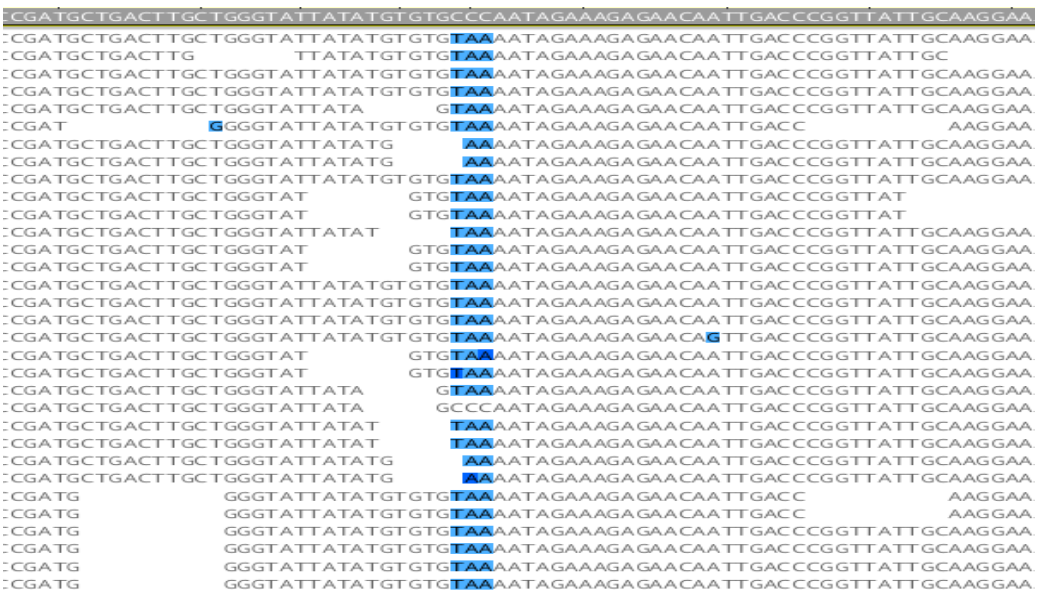


Figure 5. The NGS Data Aligned with the Reference Map of TRP1 Given in Table A.

The result shows that the stop codon TAA is inserted into the targeted location.

3.2. Removal of the Plasmids Carrying Antibiotic Resistance

To remove the plasmids carrying antibiotic resistance genes, colonies verified to harbor the desired stop codon insertion into the TRP1 gene and thus being knocked out were inoculated on YPD media with NAT and YPD media with HyB antibiotics separately at the same time and grown at 30 ºC for 2 days until single colonies became visible. Then, single colonies were picked up from YPD with NAT plate and inoculated on YPD with NAT plate. Same, single colonies from YPD with HyB were selected and inoculated on YPD with HyB. This inoculation step was repeated at the end of the 2nd day, resulting in 3 fresh generations of each colony in total. At the end of the 3rd inoculation, each colony was inoculated on a YPD agar plate without antibiotics at 30 ºC for 2 days. This process is repeated three times, aiming to remove selective pressure from the yeast cells' environment and thus make the cells release the plasmids.

In order to check whether the colonies were cured from the plasmids with the antibiotic resistance genes, each colony was inoculated on YPD plates with NAT+HYB, only NAT, and only Hyb, respectively. It was found that none of the colonies chosen could grow on YPD agar plates with NAT and/or HyB, meaning that they did not carry the relevant plasmids.

3.3. Construction and Cloning of pETcon(-)-TEF1p-Alpha-His-GS-6His/GIF-CYC1t-TRP Plasmid

Initially, the construction and cloning of pETcon-TEF1p-Alpha-His-GS-6His/GIF-CYC1t-TRP plasmid were planned to be completed as follows: The pETcon α-synuclein-sfGFP plasmid (sequence and map are not provided), another plasmid constructed in our laboratory for another project, was digested with KpnI-HF (NEB), and BsrgI restriction enzymes sequentially. According to the manufacturer's instructions, digestion using the KpnI-HF and BsrgI can be performed in two ways. First, the sample is digested with KpnI-HF, run on agarose gel, and the corresponding fragment is cleaned up from the agarose gel. Then, the fragment digested with KpnI-HF is digested with the BsrgI enzyme. Second, the sample is digested using the KpnI-HF enzyme in its suggested reaction buffer. Then the salt concentration of the reaction mix is adjusted to the salt concentration of the BsrgI enzyme's reaction buffer.

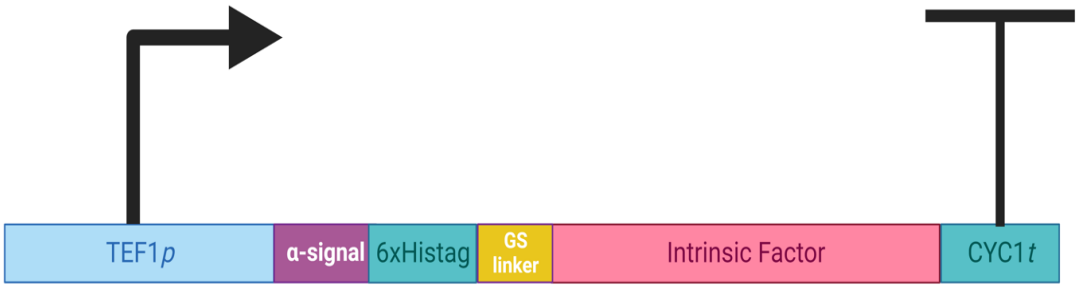


Figure 6. Schematic Representation of the Gene Insert, Carrying the IF.

The expression of IF is controlled by a constitutive TEF1 promoter and CYC1 terminator. The extracellular secretion of the protein is achieved by the Alpha secretion signal. The 6xHistag is fused to the protein for immunochemical

visualization assays. The GS linker is added between the tag and the protein to provide flexibility and keep the Histag and the IF separated.

Meanwhile, the TEF1 promoter was amplified from the CAS-9 plasmid using the ONK-25/ONK-32 primer pair via Q5 DNA polymerase. The ONK-25 primer contains the KpnI cut site within its overhang and has a 30 bp overlap with the pETcon(-) vector digested with KpnI and BsrG-I enzymes. The ONK-32 primer has a 30 bp overlapping region with the Alpha secretion signal. The Alpha secretion signal was amplified from the pGAPZ A plasmid (Thermo Fisher Scientific, Catalog NoV20520)) using the ONK-29/ONK30 primer pair via Q5 DNA polymerase. The ONK-30 primer has a 6xHis-Tag sequence plus GS linker sequence that overlaps with the GIF sequence given in Table A.1. The Intrinsic Factor was amplified from the construct synthesized by Genewiz using the ONK-24/ONK-31 primer pair via Q5 DNA polymerase. The ONK-31 primer has a 6xHis-Tag sequence plus GS linker sequence that overlaps with the Alpha secretion signal sequence. The CYC1 terminator was amplified from the Cas-9 plasmid with the ONK-27/ONK-28 primer pair using Q5 DNA polymerase. The ONK-27 primer has a 31 bp overhang, 6 of which adds two stod codons (TAATAA) at the end of the GIF sequence, 25 of which overlap with the GIF sequence. The ONK-28 primer contains the BsrgI cut site sequence and has a 30 bp overlap with the pETcon(-) vector digested with BsrgI and KpNI-HF enzymes. After amplifying the insert fragments with proper overlaps, Gibson assembly was performed to fuse the pieces with the order of TEF1 promoter, Alpha signal, GIF protein, and CYC1 terminator into the pETcon(-) vector digested with KpnI-HF and BsrgI restriction enzymes. The Gibson reaction products were transformed into *E. coli* DH5α cells. Although some colonies were able to grow on the LB agar plates with the relevant antibiotics, none of the colonies were found

positive for the insert. The presence of the insert in the cells was checked via colony PCR using the ONK-25/ONK-28 primer pair via Pfu DNA polymerase. Then, different ratios of the insert to insert, and insert to vector, were tried to perform Gibson assembly. However, all the attempts to fuse the parts with Gibson assembly failed. There are some possible reasons the Gibson assembly experiments failed: It is possible that using restriction enzymes with different reaction buffers caused insufficient digestion of the vector. It is also possible that the appropriate molar ratios among the inserts or between the inserts and the vector were not applied. Alternatively, using five different DNA samples that were cleaned from the agarose might have failed the experiment due to the organic residuals coming from the agarose gel and/or due to the high salt concentrations in the DNA samples coming from the agarose gel clean-up buffers. As a result, the Gibson assembly experiment to fuse the fragments into the pETcon(-) digested with KpnI-HF, and BsrgI enzymes failed. Then, a new experimental design was planned.

The empty vector, pETcon(-) (Addgene number, 41522) (Figure C.2), was employed in the final design. First, the pETcon(-) plasmid enzyme was digested with KpnI-HF and BamHI-HF restriction enzymes following the protocol provided by the NEB's online tool. Then, the TEF1 promoter was amplified from the Cas-9 plasmid via Q5 DNA polymerase using the ONK-25/ONK-32 primer pair. The ONK-25 primer contains the KpnI enzyme cut site used in the T4 ligation experiment and has a 30 bp overlapping region with the pETcon(-) enzyme used in the Gibson assembly experiment. The ONK-32 primer has a 30 bp overlap to be used for fusing with the Alpha secretion signal via the overlap extension PCR. The Alpha secretion signal was amplified from the pGAPZ A plasmid (Thermo Fisher Scientific, Catalog NoV20520)) with the ONK-29/ONK30 primer pair using Q5 DNA polymerase. The

ONK-30 primer has an overhang, 33 bp in length, which includes a GS linker sequence to provide flexibility, and 6His-Tag. The GIF protein sequence is amplified from gene stock synthesized by Genewiz using the ONK-31/ONK-34 primer pair. The ONK-31 primer has a 33 bp sequence overlapping with the Alpha signal. The CYC1 terminator was amplified from the Cas-9 plasmid using the ONK-27/ONK-34 primer pair. The 30 bp of the overhang of the ONK-27 overlaps with the GIF sequence, and the ONK-34 primer contains the BamHI cut site in addition to the 30 bp overlap with the pETcon(-) vector digested with KpnI and BamHI enzymes. PCR products of the inserts are shown in Figure 5.

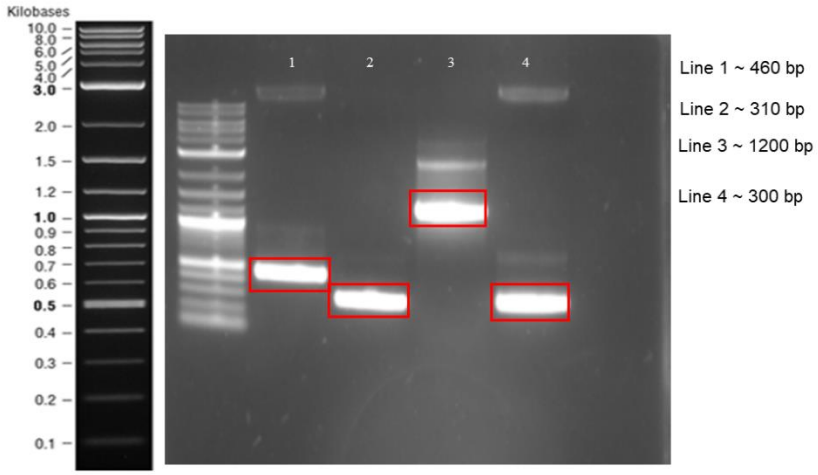


Figure 7. Agarose Gel Image of the Insert Fragments.

Lines show the TEF1 promoter (Line 1) amplified with ONK-25/ONK-32 primer pair, the CYC1 terminator (Line 2) amplified with ONK-27/ONK-34, the GIF sequence amplified with ONK-24/ONK-31, and the Alpha secretion signal (Line 4) amplified with ONK-29/ONK-30 with their expected sizes.

All the insert fragments amplified with their corresponding primer pairs were run on agarose gel and cleaned up from the gel. Then, overlap extension PCR via Q5 DNA polymerase was employed to fuse the parts as TEF1 plus Alpha signal and GIF plus

CYC1 as follows: PCR products of TEF1 and the Alpha signal were mixed in PCR master mix and amplified using the ONK-25, the forward primer of the TEF1, and the ONK-30, the reverse primer of the Alpha signal. Then, the PCR products of the GIF protein and CYC1 terminator were mixed in the PCR master mix and amplified with the ONK-31, the forward primer of the GIF, and the ONK-34, the reverse primer of the CYC1 terminator. Next, the corresponding bands of TEFp plus Alpha signal, and GIF plus CYC1t were run on agarose gel (Figure 6.) and cleaned up from the gel. Following clean-up, the proper amounts of inserts (TEF1p +Alpha, GIF + CYC1t) were mixed in the PCR master mix and amplified via overlap extension PCR using the forward primer of the TEF1 promoter (ONK-25), and the reverse primer of the CYC1 terminator (ONK-34). The final product giving the final gene insert cassette was run on agarose gel(Figure 6.) and cleaned up to be used in the T4 ligation assay.

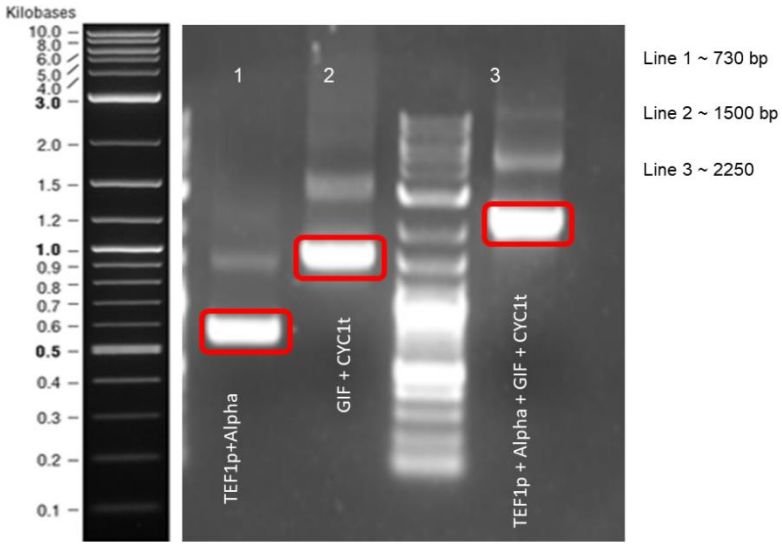


Figure 8. Overlap Extension PCR Products of the Insert Parts.

Line 1 shows the PCR product of TEF1 plus Alpha signal. Line 2 shows the PCR product of the GIF protein sequence and CYC1 terminator. Line 3 shows the PCR product of the "TEF1 plus Alpha signal" plus "GIF plus CYC1".

After the parts of the final gene insert were assembled through overlap extension PCR, the gene insert was digested with KpnI-HF and BamHI-HF to create the appropriate restriction enzyme sites through which the insert will be ligated with the pETcon(-) vector having KpnI and BamHI cut sites. The insert digested with KpnI and BamHI were run on agarose gel and cleaned up from the gel. Then, T4 ligation reaction was set up to fuse the pETcon(-) vector with the insert made up of the TEF1 promoter, Alpha signal, GIF sequence, and CYC1 terminator (details of the reaction are given in Table E.). The whole T4 reaction mix was transformed into *E. coli* DH5α cells and transformed cells were inoculated on an LB agar plate supplied with proper antibiotics at 37 °C overnight. Next day, eleven colonies were selected from the transformation plate, and colony PCR was performed using the ONK-25/ONK-34 primer pair to check the presence of the insert. (Figure 7 A.) However, the amplified regions resulted in weak bants on the agarose gel. Therefore, colony PCR was repeated using the ONK-24/ONK-29 primer pair, and 5 of 11 colonies were clearly positive for the insert (Colonies 2, 3, 4, 6, 9, respectively, Figure 7 B.). Positive colonies were inoculated overnight for plasmid isolation. Then, isolated plasmids were sent for NGS to be sequenced.

A. B.

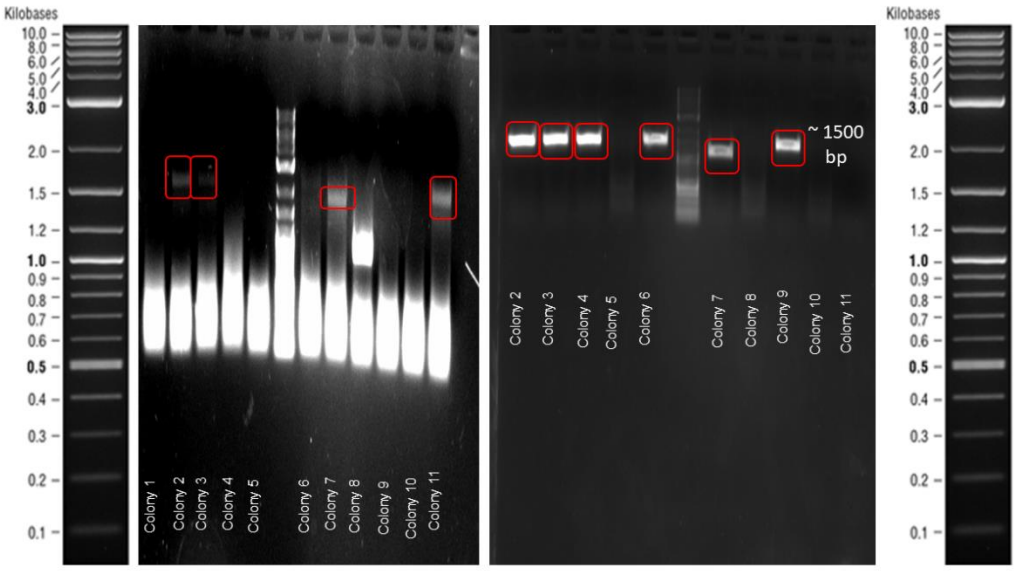


Figure 9. Colony PCR Results of the 11 Colonies Chosen with an Expected Size of 2250 bp.

PCR was done using a primer pair specific for the gene insert. Forward primer belongs to the TEF1 promoter, and reverse primer belongs to the CYC1 terminator. A. Second colony PCR results following the failed colony PCR. The primers used to check the presence of the insert were specific for the Alpha sequence and GIF protein sequence, with an expected size of 1500 bp. B

The NGS results showed that the gene cassette made up of TEF1 promoter, Alpha secretion signal, 6xHistag, GS linker, GIF protein, and CYC1 terminator was successfully fused with the pETcon(-) vector in all five colonies. NGS result showing the insertion of the insert into the vector is given in Table D 2. Then, colony number two was chosen, and experiments proceeded using the plasmid isolated from colony 2.

3.4. Expression Assay of Recombinant Intrinsic Factor

To produce Intrinsic Factor protein using *S.boulardii*, pETcon-TEF1p-Alpha-His-GS-6His/GIF-CYC1t-TRP plasmid, verified via NGS data, was transformed into TRP1 auxotroph *S.boulardii* cells through chemical transformation method described previously. Then, transformed cells were grown at 30 °C for 60 hours. At the 60th hour, 1 mL culture was pelleted, and the supernatant was transferred into a 15 mL falcon tube. The remaining cell pellet was kept at -20 °C until the SDS-Page assay was established. Next, 4 X volume of the supernatant (4 mL) cold acetone (-20 °C) was mixed with the supernatant sample. The mixture was incubated at -20 °C for 16 hours. The next day, the liquid phase of the mix was decanted without resuspending the pellet at the bottom. Then, the pellet was evaporated at room temperature for 30 minutes. The pellet obtained from acetone precipitation and the cell pellet kept at -20°C were dissolved in 1 X SDS loading dye, and the gel was run at 100V in 1X SDS Running Buffer for 45 minutes and then at 140V until the end of the run. According to the SDS-Page assay data, it was not observed any relevant protein band on the gel, neither from the supernatant nor from the cell lysate. At the same time, it was proceeded to Western Blot assay by considering that there was not enough amount of protein gathered from the samples. Surprisingly, no his-tagged protein bands were seen on the gel image (Figure 10.). These results are unexpected because all the constitutional elements of the expression vector, which are the promoter, secretion signal, and terminator, were verified via NGS analysis, meaning that it should have been produced theoretically. It was assumed that this promoter and terminator would work well together since the Cas9 enzyme used to knock out TRP1 in this study was expressed within these elements.

Additionally, it would have been expected to see the protein production intracellularly if the broken part of the system was the secretion signal. On the other hand, the secretion signal precedes the Intrinsic Factor protein within the gene cassette. Thus, it is possible not to see protein expression if the problem was related to the translation of the Alpha secretion signal. Also, there is no rare codon for *S.boulardii* within the recombinant protein sequence that would interfere with the expression of the protein. Another possibility is that the recombinant protein production and auxotrophic environment created a metabolic burden failing the production. However, this is less likely since this organism has already been used to produce more complex substances in the industry. It is also possible that we could not ensure the proper environment for the organism to express the recombinant protein. Thus, it might be necessary to try different media and incubation conditions, such as changing the carbon source or amount, incubation time, and overall media composition.

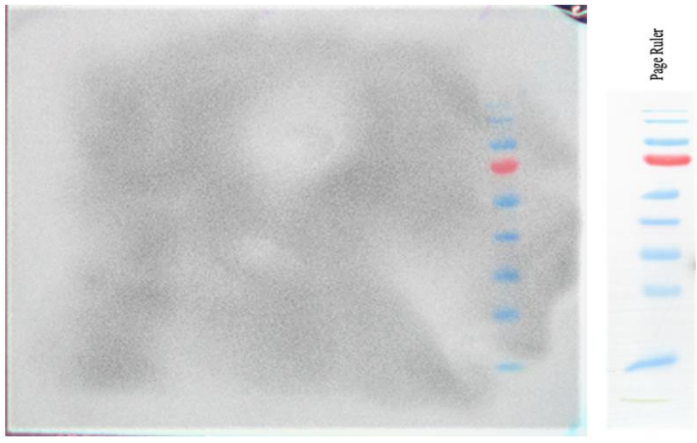


Figure 10. Western Blot analysis of Recombinant His-Tagged Intrinsic Factor Expression in *S.boulardii.*

No his-tagged protein bands were observed.

As a result, it is clear that there is a non-functional element, or more than one element, within the system that failed the production of the Intrinsic Factor.

CONCLUSION

To conclude, a TRP1Δ auxotroph *Saccharomyces boulardii* strain to be used as a chassis organism for expressing therapeutic proteins in the gut was created using the CRISPR CAS-9 system in this study and confirmed via Next Generation Sequencing analysis. Throughout the work, a great number of optimizations have been made to fix the experiment-related issues, such as reviving the cells, finding the right conditions for the electroporation, and employing the proper cloning method to construct the final design. In addition to creating an auxotrophic *S.boulardii* strain to be used as a therapeutic delivery system, it was also targeted to combat pernicious anemia caused by Intrinsic Factor-related cobalamin deficiency. For this purpose, we modified a yeast expression plasmid with a high copy number by fusing a strong, constitutive promoter (TEF1), a well-studied secretion signal (alpha signal), the protein sequence coding for Intrinsic Factor, and a well-working terminator (CYC1). Also, a Histag sequence was fused to the protein sequence to be used in characterization and purification steps. However, the planned recombinant protein expression could not be established even though all the vital parts of the expression vector were successfully fused and verified. These results require further investigation regarding the reason the expression system failed.

As mentioned above, some possible reasons that would cause the protein was not expressed include the grown conditions of the organism, the metabolic burden created by the auxotrophy and the recombinant protein, or a structural issue caused by the fact that some parts of the gene cassette could not work together. For the future of this work, it has been planned to analyze those possibilities and optimize our engineered *S.boulardii* to be used as a living therapeutic device for various diseases.

BIBLIOGRAPHY

[1] A. Cubillos-Ruiz et al., “Engineering living therapeutics with synthetic biology,” doi: 10.1038/s41573-021-00285-3.

[2] V. W. Kelly, B. K. Liang, and S. J. Sirk, “Living Therapeutics: The Next Frontier of Precision Medicine,” Cite This ACS Synth. Biol, vol. 2020, pp. 3184–3201, 2020, doi: 10.1021/acssynbio.0c00444.

[3] D. B. Pedrolli et al., “Engineering Microbial Living Therapeutics: The Synthetic Biology Toolbox,” Trends in Biotechnology, vol. 37, no. 1. 2019, doi: 10.1016/j.tibtech.2018.09.005.

[4] Z. Z. R. Hamady et al., “Xylan-regulated delivery of human keratinocyte growth factor-2 to the inflamed colon by the human anaerobic commensal bacterium Bacteroides ovatus,” Gut, vol. 59, no. 4, pp. 461–469, Apr. 2010, doi: 10.1136/GUT.2008.176131.

[5] C. Grangette, H. Müller-Alouf, D. Goudercourt, M. C. Geoffroy, M. Turneer, and A. Mercenier, “Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant Lactobacillus plantarum,” Infect. Immun., vol. 69, no. 3, pp. 1547–1553, 2001, doi: 10.1128/IAI.69.3.1547-1553.2001.

[6] S. Caluwaerts et al., “AG013, a mouth rinse formulation of Lactococcus lactis secreting human Trefoil Factor 1, provides a safe and efficacious therapeutic tool for treating oral mucositis,” Oral Oncol., vol. 46, no. 7, pp. 564–570, Jul. 2010, doi: 10.1016/J.ORALONCOLOGY.2010.04.008.

[7] M. R. Charbonneau, V. M. Isabella, N. Li, and C. B. Kurtz, “Developing a new class of engineered live bacterial therapeutics to treat human diseases,” doi: 10.1038/s41467-020-15508-1.

[8] D. Liu, “CAR-T ‘the living drugs’, immune checkpoint inhibitors, and precision medicine: a new era of cancer therapy,” J. Hematol. Oncol., vol. 12, no. 1, Nov. 2019, doi: 10.1186/S13045-019-0819-1.

[9] G. Reid, A. A. Gadir, and R. Dhir, “Probiotics: Reiterating What They Are and What They Are Not,” Front. Microbiol., vol. 10, no. MAR, 2019, doi: 10.3389/FMICB.2019.00424.

[10] J. Plaza-Diaz, F. J. Ruiz-Ojeda, M. Gil-Campos, and A. Gil, “Mechanisms of Action of Probiotics,” Adv. Nutr., vol. 10, no. Suppl 1, p. S49, Jan. 2019, doi: 10.1093/ADVANCES/NMY063.

[11] Z. Zhou et al., “Engineering probiotics as living diagnostics and therapeutics for improving human health,” Microbial Cell Factories, vol. 19, no. 1. 2020, doi: 10.1186/s12934-020-01318-z.

[12] N. Saeidi et al., “Engineering microbes to sense and eradicate Pseudomonas aeruginosa, a human pathogen,” Mol. Syst. Biol., vol. 7, no. 1, p. 521, Jan. 2011, doi: 10.1038/MSB.2011.55.

[13] C. N. Sarkissian et al., “A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase,” 1999. [Online]. Available: www.pnas.org.

[14] L. Wang et al., “An engineered probiotic secreting Sj16 ameliorates colitis via Ruminococcaceae/butyrate/retinoic acid axis,” Bioeng. Transl. Med., vol. 6, no. 3, Sep. 2021, doi: 10.1002/btm2.10219.

[15] C. Huang et al., “Hypoxic Tumor Radiosensitization Using Engineered Probiotics,” Adv. Healthc. Mater., vol. 10, no. 10, May 2021, doi: 10.1002/ADHM.202002207.

[16] T. Danino et al., “Programmable probiotics for detection of cancer in urine,” Sci. Transl. Med., vol. 7, no. 289, p. 289ra84, May 2015, doi: 10.1126/SCITRANSLMED.AAA3519.
[17] I. Khatri, R. Tomar, K. Ganesan, G. S. Prasad, and S. Subramanian, “Complete genome sequence and comparative genomics of the probiotic yeast Saccharomyces boulardii,” Sci. Rep., vol. 7, no. 1, Dec. 2017, doi: 10.1038/S41598-017-00414-2.
[18] K. Kaźmierczak-Siedlecka, · Jakub Ruszkowski, M. Fic, M. Folwarski, and W. Makarewicz, “Saccharomyces boulardii CNCM I-745: A Non-bacterial Microorganism Used as Probiotic Agent in Supporting Treatment of Selected Diseases,” vol. 77, pp. 1987–1996, 2053, doi: 10.1007/s00284-020-02053-9.
[19] “Genotypic and Physiological Characterization of Saccharomyces boulardii, the Probiotic Strain of Saccharomyces cerevisiae.” https://journals.asm.org/doi/epdf/10.1128/AEM.02201-06?src=getftr (accessed Aug. 31, 2022).
[20] J. L. R. Fietto et al., “Molecular and physiological comparisons between Saccharomyces cerevisiae and Saccharomyces boulardii,” Can. J. Microbiol., vol. 50, no. 8, 2004, doi: 10.1139/w04-050.
[21] C. Cordonnier et al., “Dynamic In Vitro Models of the Human Gastrointestinal Tract as Relevant Tools to Assess the Survival of Probiotic Strains and Their Interactions with Gut Microbiota,” Microorganisms, vol. 3, no. 4, p. 725, Dec. 2015, doi: 10.3390/MICROORGANISMS3040725.
[22] D. Czerucka and P. Rampal, “Diversity of Saccharomyces boulardii CNCM I-745 mechanisms of action against intestinal infections,” World J. Gastroenterol., vol. 25, no. 18, 2019, doi: 10.3748/wjg.v25.i18.2188.
[23] D. Czerucka, T. Piche, and P. Rampal, “Review article: Yeast as probiotics - Saccharomyces boulardii,” Alimentary Pharmacology and Therapeutics, vol. 26, no. 6. pp. 767–778, Sep. 2007, doi: 10.1111/j.1365-2036.2007.03442.x.
[24] M. I. Moré and A. Swidsinski, “Saccharomyces boulardii CNCM I-745 supports regeneration of the intestinal microbiota after diarrheic dysbiosis - a review,” Clin. Exp. Gastroenterol., vol. 8, pp. 237–255, Aug. 2015, doi: 10.2147/CEG.S85574.
[25] G. Bagherpour et al., “Oral Administration of Recombinant Saccharomyces boulardii Expressing Ovalbumin-CPE Fusion Protein Induces Antibody Response in Mice,” Front. Microbiol., vol. 9, no. APR, Apr. 2018, doi: 10.3389/FMICB.2018.00723.
[26] S. Kwak, B. Mahmud, and G. Dantas, “A Tunable and Expandable Transactivation System in Probiotic Yeast Saccharomyces boulardii,” ACS Synth. Biol., vol. 11, no. 1, 2022, doi: 10.1021/acssynbio.1c00384.
[27] A. L. Demain and P. Vaishnav, “Production of recombinant proteins by microbes and higher organisms,” Biotechnol. Adv., vol. 27, no. 3, pp. 297–306, May 2009, doi: 10.1016/J.BIOTECHADV.2009.01.008.
[28] L. E. Hudson et al., “Functional heterologous protein expression by genetically engineered probiotic yeast Saccharomyces boulardii,” PLoS One, vol. 9, no. 11, 2014, doi: 10.1371/journal.pone.0112660.
[29] L. Raida, “Pernicious anemia,” Prakticky Lekar, vol. 88, no. 7. 2008, doi: 10.2307/3412441.
[30] “Open Peer Review on Pernicious Anemia,” doi: 10.32388/6PA1CL.
[31] A. Ankar and S. S. Bhimji, “Vitamin B12 Deficiency,” in StatPearls, StatPearls Publishing, 2022.

[32] T. W. Htut, K. Z. Thein, and T. H. Oo, “Pernicious anemia: Pathophysiology and diagnostic difficulties,” Journal of Evidence-Based Medicine, vol. 14, no. 2. 2021, doi: 10.1111/jebm.12435.
[33] E. Morawiec-Szymonik, W. Foltyn, B. Marek, J. Głogowska-Szeląg, B. Kos-Kudła, and D. Kajdaniuk, “Antibodies involved in the development of pernicious anemia and other autoimmune diseases,” Polish Arch. Intern. Med., vol. 130, no. 1, pp. 31–37, 2020, doi: 10.20452/PAMW.15094.
[34] E. Andres and K. Serraj, “Optimal management of pernicious anemia,” J. Blood Med., vol. 3, p. 97, Sep. 2012, doi: 10.2147/JBM.S25620.
[35] N. Bizzaro and A. Antico, “Diagnosis and classification of pernicious anemia,” Autoimmun. Rev., vol. 13, no. 4–5, pp. 565–568, Apr. 2014, doi: 10.1016/J.AUTREV.2014.01.042.
[36] C. Q. H. Chan, L. L. Low, and K. H. Lee, “Oral Vitamin B12 replacement for the treatment of Pernicious Anemia,” Front. Med., vol. 3, no. AUG, pp. 1–6, 2016, doi: 10.3389/fmed.2016.00038.
[37] G. Radau, “Vitamin B12 and B12-Proteins Edited by B. Kräutler, D. Arigoni, B. T. Golding, WILEY-VCH Weinheim, 1998, 542 S., 174 Abb., DM 238,-, ISBN 3-527-29480-5,” Pharm. Unserer Zeit, vol. 28, no. 1, pp. 47–47, Jan. 1999, doi: 10.1002/PAUZ.19990280114.
[38] S. N. Fedosov, “Physiological and Molecular Aspects of Cobalamin Transport,” doi: 10.1007/978-94-007-2199-9_18.
[39] R. Kozyraki and O. Cases, “Vitamin B12 absorption: Mammalian physiology and acquired and inherited disorders,” Biochimie, vol. 95, no. 5, pp. 1002–1007, May 2013, doi: 10.1016/J.BIOCHI.2012.11.004.

APPENDIX A

DNA SEQUENCES OF CONSTRUCTS

Table A 1. Sequences of Plasmids and The DNA Fragments Used in This Study

Name	Sequence
pRS414-TEF1p-Cas9-CYC1t plasmid (Addgene number, 64329)	GACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTA ATGTCATGATAATAATGGTTTCTTAGACGGATCGCTTGC CTGTAACTTACACGCGCCTCGTATCTTTTAATGATGGAAT AATTTGGGAATTTACTCTGTGTTTATTTATTTTTATGTTTT GTATTTGGATTTTAGAAAGTAAATAAAGAAGGTAGAAG AGTTACGGAATGAAGAAAAAAAAATAAACAAAGGTTTA AAAAATTTCAACAAAAAGCGTACTTTACATATATATTTA TTAGACAAGAAAAGCAGATTAAATAGATATACATTCGAT TAACGATAAGTAAAATGTAAAATCACAGGATTTTCGTGT GTGGTCTTCTACACAGACAAGATGAAACAATTCGGCATT AATACCTGAGAGCAGGAAGAGCAAGATAAAAGGTAGTA TTTGTTGGCGATCCCCCTAGAGTCTTTTACATCTTCGGAA AACAAAAACTATTTTTTCTTTAATTTCTTTTTTTACTTTCT ATTTTTAATTTATATATTTATATTAAAAAATTTAAATTAT AATTATTTTTATAGCACGTGATGAAAAGGACCCAGGTGG CACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTT ATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGA CAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGG AAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTC CCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCA

	GAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTT GGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAG CGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTT TCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGC GGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGG TCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTA CTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGAC AGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGA TAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGG ACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGA TCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAA TGAAGCCATACCAAACGACGAGCGTGACACCACGATGC CTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTG GCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAG ACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGC GCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATC TGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGC ACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTAT CTACACGACGGGGAGTCAGGCAACTATGGATGAACGAA ATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGC ATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTA GATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAG GTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTT AACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAG AAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCG

	CGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACC AGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCT TTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACC AAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCA CTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCT GCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAA GTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACC GGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGT GCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCAC GCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAA GCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTT CCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGG TTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCT CGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAAC GCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTG CTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGA TAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCG CCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCG AGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTC CCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGAC AGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGC AATTAATGTGAGTTACCTCACTCATTAGGCACCCCAGGC TTTACACTTTATGCTTCCGGCTCCTATGTTGTGTGGAATT GTGAGCGGATAACAATTTCACACAGGAAACAGCTATGA

	CCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAG GGAACAAAAGCTGGAGCTCATAGCTTCAAAATGTTTCTA CTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCA TCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTA AATTTCCCCTCTTTCTTCCTCTAGGGTGTCGTTAATTACC CGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCTC GTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTA TCACGTTTCTTTTTCTTGAAAATTTTTTTTTTGATTTTTTT CTCTTTCGATGACCTCCCATTGATATTTAAGTTAATAAAC GGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTC TATTACAACTTTTTTTACTTCTTGCTCATTAGAAAGAAAG CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGATC CCCCGGGAAAAATGGACAAGAAGTACTCCATTGGGCTC GATATCGGCACAAACAGCGTCGGTTGGGCCGTCATTACG GACGAGTACAAGGTGCCGAGCAAAAAATTCAAAGTTCT GGGCAATACCGATCGCCACAGCATAAAGAAGAACCTCA TTGGCGCCCTCCTGTTCGACTCCGGGGAGACGGCCGAAG CCACGCGGCTCAAAAGAACAGCACGGCGCAGATATACC CGCAGAAAGAATCGGATCTGCTACCTGCAGGAGATCTTT AGTAATGAGATGGCTAAGGTGGATGACTCTTTCTTCCAT AGGCTGGAGGAGTCCTTTTTGGTGGAGGAGGATAAAAA GCACGAGCGCCACCCAATCTTTGGCAATATCGTGGACGA GGTGGCGTACCATGAAAAGTACCCAACCATATATCATCT GAGGAAGAAGCTTGTAGACAGTACTGATAAGGCTGACT TGCGGTTGATCTATCTCGCGCTGGCGCATATGATCAAAT

	TTCGGGGACACTTCCTCATCGAGGGGGACCTGAACCCAG ACAACAGCGATGTCGACAAACTCTTTATCCAACTGGTTC AGACTTACAATCAGCTTTTCGAAGAGAACCCGATCAACG CATCCGGAGTTGACGCCAAAGCAATCCTGAGCGCTAGGC TGTCCAAATCCCGGCGGCTCGAAAACCTCATCGCACAGC TCCCTGGGGAGAAGAAGAACGGCCTGTTTGGTAATCTTA TCGCCCTGTCACTCGGGCTGACCCCCAACTTTAAATCTA ACTTCGACCTGGCCGAAGATGCCAAGCTTCAACTGAGCA AAGACACCTACGATGATGATCTCGACAATCTGCTGGCCC AGATCGGCGACCAGTACGCAGACCTTTTTTTGGCGGCAA AGAACCTGTCAGACGCCATTCTGCTGAGTGATATTCTGC GAGTGAACACGGAGATCACCAAAGCTCCGCTGAGCGCT AGTATGATCAAGCGCTATGATGAGCACCACCAAGACTTG ACTTTGCTGAAGGCCCTTGTCAGACAGCAACTGCCTGAG AAGTACAAGGAAATTTTCTTCGATCAGTCTAAAAATGGC TACGCCGGATACATTGACGGCGGAGCAAGCCAGGAGGA ATTTTACAAATTTATTAAGCCCATCTTGGAAAAAATGGA CGGCACCGAGGAGCTGCTGGTAAAGCTTAACAGAGAAG ATCTGTTGCGCAAACAGCGCACTTTCGACAATGGAAGCA TCCCCCACCAGATTCACCTGGGCGAACTGCACGCTATCC TCAGGCGGCAAGAGGATTTCTACCCCTTTTTGAAAGATA ACAGGGAAAAGATTGAGAAAATCCTCACATTTCGGATA CCCTACTATGTAGGCCCCCTCGCCCGGGGAAATTCCAGA TTCGCGTGGATGACTCGCAAATCAGAAGAGACCATCACT CCCTGGAACTTCGAGGAAGTCGTGGATAAGGGGGCCTCT

	GCCCAGTCCTTCATCGAAAGGATGACTAACTTTGATAAA AATCTGCCTAACGAAAAGGTGCTTCCTAAACACTCTCTG CTGTACGAGTACTTCACAGTTTATAACGAGCTCACCAAG GTCAAATACGTCACAGAAGGGATGAGAAAGCCAGCATT CCTGTCTGGAGAGCAGAAGAAAGCTATCGTGGACCTCCT CTTCAAGACGAACCGGAAAGTTACCGTGAAACAGCTCA AAGAAGACTATTTCAAAAAGATTGAATGTTTCGACTCTG TTGAAATCAGCGGAGTGGAGGATCGCTTCAACGCATCCC TGGGAACGTATCACGATCTCCTGAAAATCATTAAAGACA AGGACTTCCTGGACAATGAGGAGAACGAGGACATTCTT GAGGACATTGTCCTCACCCTTACGTTGTTTGAAGATAGG GAGATGATTGAAGAACGCTTGAAAACTTACGCTCATCTC TTCGACGACAAAGTCATGAAACAGCTCAAGAGGCGCCG ATATACAGGATGGGGGCGGCTGTCAAGAAAACTGATCA ATGGGATCCGAGACAAGCAGAGTGGAAAGACAATCCTG GATTTTCTTAAGTCCGATGGATTTGCCAACCGGAACTTC ATGCAGTTGATCCATGATGACTCTCTCACCTTTAAGGAG GACATCCAGAAAGCACAAGTTTCTGGCCAGGGGGACAG TCTTCACGAGCACATCGCTAATCTTGCAGGTAGCCCAGC TATCAAAAAGGGAATACTGCAGACCGTTAAGGTCGTGG ATGAACTCGTCAAAGTAATGGGAAGGCATAAGCCCGAG AATATCGTTATCGAGATGGCCCGAGAGAACCAAACTACC CAGAAGGGACAGAAGAACAGTAGGGAAAGGATGAAGA GGATTGAAGAGGGTATAAAAGAACTGGGGTCCCAAATC CTTAAGGAACACCCAGTTGAAAACACCCAGCTTCAGAAT

	GAGAAGCTCTACCTGTACTACCTGCAGAACGGCAGGGA CATGTACGTGGATCAGGAACTGGACATCAATCGGCTCTC CGACTACGACGTGGATCATATCGTGCCCCAGTCTTTTCTC AAAGATGATTCTATTGATAATAAAGTGTTGACAAGATCC GATAAAAATAGAGGGAAGAGTGATAACGTCCCCTCAGA AGAAGTTGTCAAGAAAATGAAAAATTATTGGCGGCAGC TGCTGAACGCCAAACTGATCACACAACGGAAGTTCGATA ATCTGACTAAGGCTGAACGAGGTGGCCTGTCTGAGTTGG ATAAAGCCGGCTTCATCAAAAGGCAGCTTGTTGAGACAC GCCAGATCACCAAGCACGTGGCCCAAATTCTCGATTCAC GCATGAACACCAAGTACGATGAAAATGACAAACTGATT CGAGAGGTGAAAGTTATTACTCTGAAGTCTAAGCTGGTC TCAGATTTCAGAAAGGACTTTCAGTTTTATAAGGTGAGA GAGATCAACAATTACCACCATGCGCATGATGCCTACCTG AATGCAGTGGTAGGCACTGCACTTATCAAAAAATATCCC AAGCTTGAATCTGAATTTGTTTACGGAGACTATAAAGTG TACGATGTTAGGAAAATGATCGCAAAGTCTGAGCAGGA AATAGGCAAGGCCACCGCTAAGTACTTCTTTTACAGCAA TATTATGAATTTTTTCAAGACCGAGATTACACTGGCCAA TGGAGAGATTCGGAAGCGACCACTTATCGAAACAAACG GAGAAACAGGAGAAATCGTGTGGGACAAGGGTAGGGAT TTCGCGACAGTCCGGAAGGTCCTGTCCATGCCGCAGGTG AACATCGTTAAAAAGACCGAAGTACAGACCGGAGGCTT CTCCAAGGAAAGTATCCTCCCGAAAAGGAACAGCGACA AGCTGATCGCACGCAAAAAAGATTGGGACCCCAAGAAA

	TACGGCGGATTCGATTCTCCTACAGTCGCTTACAGTGTA CTGGTTGTGGCCAAAGTGGAGAAAGGGAAGTCTAAAAA ACTCAAAAGCGTCAAGGAACTGCTGGGCATCACAATCAT GGAGCGATCAAGCTTCGAAAAAAACCCCATCGACTTTCT CGAGGCGAAAGGATATAAAGAGGTCAAAAAAGACCTCA TCATTAAGCTTCCCAAGTACTCTCTCTTTGAGCTTGAAAA CGGCCGGAAACGAATGCTCGCTAGTGCGGGCGAGCTGC AGAAAGGTAACGAGCTGGCACTGCCCTCTAAATACGTTA ATTTCTTGTATCTGGCCAGCCACTATGAAAAGCTCAAAG GGTCTCCCGAAGATAATGAGCAGAAGCAGCTGTTCGTGG AACAACACAAACACTACCTTGATGAGATCATCGAGCAA ATAAGCGAATTCTCCAAAAGAGTGATCCTCGCCGACGCT AACCTCGATAAGGTGCTTTCTGCTTACAATAAGCACAGG GATAAGCCCATCAGGGAGCAGGCAGAAAACATTATCCA CTTGTTTACTCTGACCAACTTGGGCGCGCCTGCAGCCTTC AAGTACTTCGACACCACCATAGACAGAAAGCGGTACAC CTCTACAAAGGAGGTCCTGGACGCCACACTGATTCATCA GTCAATTACGGGGCTCTATGAAACAAGAATCGACCTCTC TCAGCTCGGTGGAGACAGCAGGGCTGACCCCAAGAAGA AGAGGAAGGTGTGATCTCTTCTCGAGTCATGTAATTAGT TATGTCACGCTTACATTCACGCCCTCCCCCCACATCCGCT CTAACCGAAAAGGAAGGAGTTAGACAACCTGAAGTCTA GGTCCCTATTTATTTTTTTATAGTTATGTTAGTATTAAGA ACGTTATTTATATTTCAAATTTTTCTTTTTTTTCTGTACAG ACGCGTGTACGCATGTAACATTATACTGAAAACCTTGCT

	TGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCGG CCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACGCG CGCTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAA AACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACAT CCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGC ACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGC GAATGGCGCGACGCGCCCTGTAGCGGCGCATTAAGCGC GGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACT TGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCT TCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTC TAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTT ACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGG TTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCG CCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTC TTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTC TATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCT ATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACG CGAATTTTAACAAAATATTAACGTTTACAATTTCCTGAT GCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACAC CGCATAGGCAAGTGCACAAACAATACTTAAATAAATACT ACTCAGTAATAACCTATTTCTTAGCATTTTTGACGAAATT TGCTATTTTGTTAGAGTCTTTTACACCATTTGTCTCCACA CCTCCGCTTACATCAACACCAATAACGCCATTTAATCTA AGCGCATCACCAACATTTTCTGGCGTCAGTCCACCAGCT AACATAAAATGTAAGCTTTCGGGGCTCTCTTGCCTTCCA

	ACCCAGTCAGAAATCGAGTTCCAATCCAAAAGTTCACCT GTCCCACCTGCTTCTGAATCAAACAAGGGAATAAACGAA TGAGGTTTCTGTGAAGCTGCACTGAGTAGTATGTTGCAG TCTTTTGGAAATACGAGTCTTTTAATAACTGGCAAACCG AGGAACTCTTGGTATTCTTGCCACGACTCATCTCCATGC AGTTGGACGATATCAATGCCGTAATCATTGACCAGAGCC AAAACATCCTCCTTAGGTTGATTACGAAACACGCCAACC AAGTATTTCGGAGTGCCTGAACTATTTTTATATGCTTTTA CAAGACTTGAAATTTTCCTTGCAATAACCGGGTCAATTG CGTTTTCGACACTGGATGGCGGCGTTAGTATCGAATCGA CAGCAGTATAGCGACCAGCATTCACATACGATTGACGCA TGATATTACTTTCTGCGCACTTAACTTCGCATCTGGGCAG ATGATGTCGAGGCGAAAAAAAATATAAATCACGCTAAC ATTTGATTAAAATAGAACAACTACAATATAAAAAAACTA TACAAATGACAAGTTCTTGAAAACAAGAATCTTTTTATT GTCAGTACTGATTAGGGGCAGGGCATGCTCATGTAGAGC GCCTGCTCGCCGTCCGAGGCGGTGCCGTCGTACAGGGCG GTGTCCAGGCCGCAGAGGGTGAACCCCATCCGCCGGTAC GCGTGGATCGCCGGTGCGTTGACGTTGGTGACCTCCAGC CAGAGGTGCCCGGCGCCCCGCTCGCGGGCGAACTCCGTC GCGAGCCCCATCAACGCGCGCCCGACCCCGTGCCCCCGG TGCTCCGGGGCGACCTCGATGTCCTCGACGGTCAGCCGG CGGTTCCAGCCGGAGTACGAGACGACCACGAAGCCCGC CAGGTCGCCGTCGTCCCCGTACGCGACGAACGTCCGGGA GTCCGGGTCGCCGTCCTCCCCGTCGTCCGATTCGTCGTCC

	GATTCGTCGTCGGGGAACACCTTGGTCAGGGGCGGGTCC ACCGGCACCTCCCGCAGGGTGAAGCCGTCCCCGGTGGCG GTGACGCGGAAGACGGTGTCGGTGGTGAAGGACCCATC CAGTGCCTCGATGGCCTCGGCGTCCCCCGGGACACTGGT GCGGTACCGGTAAGCCGTGTCGTCAAGAGTGGTACCCAT GGTTGTTTATGTTCGGATGTGATGTGAGAACTGTATCCT AGCAAGATTTTAAAAGGAAGTATATGAAAGAAGAACCT CAGTGGCAAATCCTAACCTTTTATATTTCTCTACAGGGG CGCGGCGTGGGGACAATTCAACGCGTCTGTGAGGGGAG CGTTTCCCTGCTCGCAGGTCTGCAGCGAGGAGCCGTAAT TTTTGCTTCGCGCCGTGCGGCCATCAAAATGTATGGATG CAAATGATTATACATGGGGATGTATGGGCTAAATGTACG GGCGACAGTCACATCATGCCCCTGAGCTGCGCACGTCAA GACTGTCAAGGAGGGTATTCTGGGCCTCCATGTCGCTGG CCGGGTGACCCGGCGGGGACAAGGCAAGCTAAACAGAT CTACGTAAGGTGACAAGCTATTTTTCAATAAAGAATATC TTCCACTACTGCCATCTGGCGTCATAACTGCAAAGTACA CATATATTACGATGCTGTTCTATTAAATGCTTCCTATATT ATATATATAGTAATGTCGTTTATGGTGCACTCTCAGTAC AATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACA CCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCT GCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTC CGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACC GAAACGCGCGA

S.cerevisiae TEF1 Promoter	CATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCA GATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAA ACACCCAAGCACAGCATACTAAATTTCCCCTCTTTCTTCC TCTAGGGTGTCGTTAATTACCCGTACTAAAGGTTTGGAA AAGAAAAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGA AAAAGGCAATAAAAATTTTTATCACGTTTCTTTTTCTTGA AAATTTTTTTTTTGATTTTTTTCTCTTTCGATGACCTCCCA TTGATATTTAAGTTAATAAACGGTCTTCAATTTCTCAAGT TTCAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACT TCTTGCTCATTAGAAAGAAAGCATAGCAATCTAATCTAA G
TRP1-disrupting gRNA	TGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG TCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG
Donor DNA	TCCGATGCTGACTTGCTGGGTATTATATGTGTGTAAAAT AGAAAGAGAACAATTGACCCGGTTATTGCAAGGAAAAT TTCAAGTCTTGTA
Reference Sequence Containing the Complete-Wild-type TRP1 gene	CACCCGCCCGTCTGGACGCGCCGCTCACCCGCACGGCAG AGACCAATCAGTAAAAATCAACGGTTAACGACATTACTA TATATATAATATAGGAAGCATTTAATAGAACAGCATCGC AATATATGTGTACTTTGCAGTTATGACGCCAGATGGCAG TGGTGGAAGATATTCTTTATTGAAAAATAGCTTGTCACC TTACGTACAATCTTGATCCGGAGCTTTTCTTTTTTTGCCG ATTAAGAATTCGGTCGAAAAAAGAAAAGGAGAGGGCCA AGAGGGAGGGCATTGGTGACTATTGAGCACGTGAGTAT

	ACGTGATTAAGCACACAAAGGCAGCTTGGAGTATGTCTG TTATTAATTTCACAGGTAGTTCTGGTCCATTGGTGAAAGT TTGCGGCTTGCAGAGCACAGAGGCCGCAGAATGTGCTCT AGATTCCGATGCTGACTTGCTGGGTATTATATGTGTGCC CAATAGAAAGAGAACAATTGACCCGGTTATTGCAAGGA AAATTTCAAGTCTTGTAAAAGCATATAAAAATAGTTCAG GCACTCCGAAATACTTGGTTGGCGTGTTTCGTAATCAAC CTAAGGAGGATGTTCTGGCTCTGGTCAATGATTACGGCA TTGATATCGTCCAACTGCATGGAGATGAGTCGTGGCAAG AATACCAAGAGTTCCTCGGTTTGCCAGTTATTAAAAGAC TCGTATTTCCAAAAGACTGCAACATACTACTCAGTGCAG CTTCACAGAAACCTCATTCGTTTATTCCCTTGTTTGATTC AGAAGCAGGTGGGACAGGTGAACTTTTGGATTGGAACT CGATTTCTGACTGGGTTGGAAGGCAAGAGAGCCCCGAA AGCTTACATTTTATGTTAGCTGGTGGACTGACGCCAGAA AATGTTGGTGATGCGCTTAGATTAAATGGCGTTATTGGT GTTGATGTAAGCGGAGGTGTGGAGACAAATGGTGTAAA AGACTTTAACAAAATAGCAAATTTCGTCAAAAATGCTAA GAAATAGGTTATTACTGAGTAGTATTTATTTAAGTATTGT TTGTGCACTTGCCTGCAGGCCTTTTGAAAAGCAAGCATA AAAGATCTAAACATAAAATCTGTAAAATAACAAGATGT AAAGATAATGCTAAATCATTTGGCTTTTTGACTGATTGT ACAGGAAAATATACATCGCAGGGGTTGACTTTTACCATT TCACCGCAATGGAATCAAACTTGTTGAAGAGAATGTTCA CAGGCGCATACGCTACAATGACCCGATTCTTGCTAGCCT

	TTTCTCGGTCTTGCAAACAACCGCCGGCAGCTTAGTATA TAAATACACATGTACATACCTCTCTCCGTATCCTCGTAAT CATTTTCTTGTATTTATCGTCTTTTCGCTGTAAAAACTTT ATCACACTTATCTCAAATACACTTATTAACCGCTTTTACT ATTATCTTCTACGCTGACAGTAATATCAAACAGTGACAC ATATTAAACACAGTGGTTTCTTTGCATAAACACCATCAG CCTCAAGTCGTCAAGTAAAGATTTCGTGTTCATGC
S. cerevisiae Alpha Secretion Signal gene	ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAG CATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAG AAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATC GGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTT TTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTT ATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAA GGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCT
Intrinsic Factor with GS Linker and His-Tag	CACCACCACCACCACCACGGTTCTGGTGGTTCTTCTACC CAGACCCAGTCTTCTTGCTCTGTTCCGTCTGCGCAGGAA CCGCTGGTTAACGGTATCCAGGTTCTGATGGAAAACTCT GTTACCTCTTCTGCGTACCCGAACCCGTCTATCCTGATCG CGATGAACCTGGCGGGTGCGTACAACCTGAAAGCGCAG AAACTGCTGACCTACCAGCTGATGTCTTCTGACAACAAC GACCTGACCATCGGTCAGCTGGGTCTGACCATCATGGCG CTGACCTCTTCTTGCCGTGACCCGGGTGACAAAGTTTCT ATCCTGCAGCGTCAGATGGAAAACTGGGCGCCGTCTTCT CCGAACGCGGAAGCGTCTGCGTTCTACGGTCCGTCTCTG GCGATCCTGGCGCTGTGCCAGAAAAACTCTGAAGCGACC

	CTGCCGATCGCGGTTCGTTTCGCGAAAACCCTGCTGGCG AACTCTTCTCCGTTCAACGTTGACACCGGTGCGATGGCG ACCCTGGCGCTGACCTGCATGTACAACAAAATCCCGGTT GGTTCTGAAGAAGGTTACCGTTCTCTGTTCGGTCAGGTT CTGAAAGACATCGTTGAAAAAATCTCTATGAAAATCAAA GACAACGGTATCATCGGTGACATCTACTCTACCGGTCTG GCGATGCAGGCGCTGTCTGTTACCCCGGAACCGTCTAAA AAAGAATGGAACTGCAAAAAAACCACCGACATGATCCT GAACGAAATCAAACAGGGTAAATTCCACAACCCGATGT CTATCGCGCAGATCCTGCCGTCTCTGAAAGGTAAAACCT ACCTGGACGTTCCGCAGGTTACCTGCTCTCCGGACCACG AAGTTCAGCCGACCCTGCCGTCTAACCCGGGTCCGGGTC CGACCTCTGCGTCTAACATCACCGTTATCTACACCATCA ACAACCAGCTGCGTGGTGTTGAACTGCTGTTCAACGAAA CCATCAACGTTTCTGTTAAATCTGGTTCTGTTCTGCTGGT TGTTCTGGAAGAAGCGCAGCGTAAAAACCCGATGTTCAA ATTCGAAACCACCATGACCTCTTGGGGTCTGGTTGTTTCT TCTATCAACAACATCGCGGAAAACGTTAACCACAAAACc TACTGGCAGTTCCTGTCTGGTGTTACCCCGCTGAACGAA GGTGTTGCGGACTACATCCCGTTCAACCACGAACACATC ACCGCGAACTTCACCCAGTAC
S. cerevisiae CYC1 Terminator	TCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTCC CCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAGAC AACCTGAAGTCTAGGTCCCTATTTATTTTTTTATAGTTAT GTTAGTATTAAGAACGTTATTTATATTTCAAATTTTTCTT

	TTTTTTCTGTACAGACGCGTGTACGCATGTAACATTATAC TGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGG CTTTAATTTGC
GS Linker	GGTTCTGGTGGTTCT
His-Tag	CACCACCACCACCACCAC
pETcon- TEF1p-Alpha- His-GS- 6His/GIF- CYC1t-TRP	TTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCG CGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCG GTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGT ACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGA CAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTG ATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAG GACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGG ATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGA ATGAAGCCATACCAAACGACGAGCGTGACACCACGATG CCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACT GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATA GACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCT GCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAA ATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGC AGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGT TATCTACACGACGGGGAGTCAGGCAACTATGGATGAAC GAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTA AGCATTGGTAACTGTCAGACCAAGTTTACTCATATATAC

	TTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGAT CTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAAT CCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCC CGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTT TCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACC GCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACC AACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCA GATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGG CCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCT CGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGG CGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATA GTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGG GTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACA CCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGC GCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCC GGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGG AGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTG TCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTG ATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCA GCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCC TTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTG TGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCG CTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTG AGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCC TCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCA

	CGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCA ACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCC AGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGG AATTGTGAGCGGATAACAATTTCACACAGGAAACAGCT ATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACT AAAGGGAACAAAAGCTGGTACCCATAGCTTCAAAATGT TTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCG CGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCAT ACTAAATTTCCCCTCTTTCTTCCTCTAGGGTGTCGTTAAT TACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCG CCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATT TTTATCACGTTTCTTTTTCTTGAAAATTTTTTTTTTGATTT TTTTCTCTTTCGATGACCTCCCATTGATATTTAAGTTAAT AAACGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCT TGTTCTATTACAACTTTTTTTACTTCTTGCTCATTAGAAA GAAAGCATAGCAATCTAATCTAAGATGAGATTTCCTTCA ATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAG CTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCA CAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTA GAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACA GCACAAATAACGGGTTATTGTTTATAAATACTACTATTG CCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGA AAAGAGAGGCTGAAGCTCACCACCACCACCACCACGGT TCTGGTGGTTCTTCTACCCAGACCCAGTCTTCTTGCTCTG TTCCGTCTGCGCAGGAACCGCTGGTTAACGGTATCCAGG

	TTCTGATGGAAAACTCTGTTACCTCTTCTGCGTACCCGAA CCCGTCTATCCTGATCGCGATGAACCTGGCGGGTGCGTA CAACCTGAAAGCGCAGAAACTGCTGACCTACCAGCTGAT GTCTTCTGACAACAACGACCTGACCATCGGTCAGCTGGG TCTGACCATCATGGCGCTGACCTCTTCTTGCCGTGACCCG GGTGACAAAGTTTCTATCCTGCAGCGTCAGATGGAAAAC TGGGCGCCGTCTTCTCCGAACGCGGAAGCGTCTGCGTTC TACGGTCCGTCTCTGGCGATCCTGGCGCTGTGCCAGAAA AACTCTGAAGCGACCCTGCCGATCGCGGTTCGTTTCGCG AAAACCCTGCTGGCGAACTCTTCTCCGTTCAACGTTGAC ACCGGTGCGATGGCGACCCTGGCGCTGACCTGCATGTAC AACAAAATCCCGGTTGGTTCTGAAGAAGGTTACCGTTCT CTGTTCGGTCAGGTTCTGAAAGACATCGTTGAAAAAATC TCTATGAAAATCAAAGACAACGGTATCATCGGTGACATC TACTCTACCGGTCTGGCGATGCAGGCGCTGTCTGTTACC CCGGAACCGTCTAAAAAAGAATGGAACTGCAAAAAAAC CACCGACATGATCCTGAACGAAATCAAACAGGGTAAAT TCCACAACCCGATGTCTATCGCGCAGATCCTGCCGTCTC TGAAAGGTAAAACCTACCTGGACGTTCCGCAGGTTACCT GCTCTCCGGACCACGAAGTTCAGCCGACCCTGCCGTCTA ACCCGGGTCCGGGTCCGACCTCTGCGTCTAACATCACCG TTATCTACACCATCAACAACCAGCTGCGTGGTGTTGAAC TGCTGTTCAACGAAACCATCAACGTTTCTGTTAAATCTG GTTCTGTTCTGCTGGTTGTTCTGGAAGAAGCGCAGCGTA AAAACCCGATGTTCAAATTCGAAACCACCATGACCTCTT

	GGGGTCTGGTTGTTTCTTCTATCAACAACATCGCGGAAA ACGTTAACCACAAAACCTACTGGCAGTTCCTGTCTGGTG TTACCCCGCTGAACGAAGGTGTTGCGGACTACATCCCGT TCAACCACGAACACATCACCGCGAACTTCACCCAGTACT AATAATCATGTAATTAGTTATGTCACGCTTACATTCACG CCCTCCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGT TAGACAACCTGAAGTCTAGGTCCCTATTTATTTTTTTATA GTTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTT TTCTTTTTTTTCTGTACAGACGCGTGTACGCATGTAACAT TATACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTC GAAGGCTTTAATTTGCTGTACAAAGAACAAAAGCTTATT TCTGAAGAGGACTTGTAATAGAGATCTGATAACAACAGT GTAGATGTAACAAAATCGACTTTGTTCCCACTGTACTTTT AGCTCGTACAAAATACAATATACTTTTCATTTCTCCGTAA ACAACATGTTTTCCCATGTAATATCCTTTTCTATTTTTCG TTCCGTTACCAACTTTACACATACTTTATATAGCTATTCA CTTCTATACACTAAAAAACTAAGACAATTTTAATTTTGCT GCCTGCCATATTTCAATTTGTTATAAATTCCTATAATTTA TCCTATTAGTAGCTAAAAAAAGATGAATGTGAATCGAAT CCTAAGAGAATTGAGCTCCAATTCGCCCTATAGTGAGTC GTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGA CTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGC AGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGA GGCCCGCACCGATCGCCTTTCCCAACAGTTGCGCAGCCT GAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAG

	CGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTAC ACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTC CCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAG CTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTG CTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTG ATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTT TTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGG ACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTC GGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCG GCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTT AACGCGAATTTTAACAAAATATTAACGCTTACAATTTCC TGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTC ACACCGCATAGATCGGCAAGTGCACAAACAATACTTAA ATAAATACTACTCAGTAATAACCTATTTCTTAGCATTTTT GACGAAATTTGCTATTTTGTTAGAGTCTTTTACACCATTT GTCTCCACACCTCCGCTTACATCAACACCAATAACGCCA TTTAATCTAAGCGCATCACCAACATTTTCTGGCGTCAGTC CACCAGCTAACATAAAATGTAAGCTTTCGGGGCTCTCTT GCCTTCCAACCCAGTCAGAAATCGAGTTCCAATCCAAAA GTTCACCTGTCCCACCTGCTTCTGAATCAAACAAGGGAA TAAACGAATGAGGTTTCTGTGAAGCTGCACTGAGTAGTA TGTTGCAGTCTTTTGGAAATACGAGTCTTTTAATAACTGG CAAACCGAGGAACTCTTGGTATTCTTGCCACGACTCATC TCCATGCAGTTGGACGATATCAATGCCGTAATCATTGAC CAGAGCCAAAACATCCTCCTTAGGTTGATTACGAAACAC

	GCCAACCAAGTATTTCGGAGTGCCTGAACTATTTTTATA TGCTTTTACAAGACTTGAAATTTTCCTTGCAATAACCGG GTCAATTGTTCTCTTTCTATTGGGCACACATATAATACCC AGCAAGTCAGCATCGGAATCTAGAGCACATTCTGCGGCC TCTGTGCTCTGCAAGCCGCAAACTTTCACCAATGGACCA GAACTACCTGTGAAATTAATAACAGACATACTCCAAGCT GCCTTTGTGTGCTTAATCACGTATACTCACGTGCTCAATA GTCACCAATGCCCTCCCTCTTGGCCCTCTCCTTTTCTTTTT TCGACCGAATTAATTCTTAATCGGCAAAAAAAGAAAAG CTCCGGATCAAGATTGTACGTAAGGTGACAAGCTATTTT TCAATAAAGAATATCTTCCACTACTGCCATCTGGCGTCA TAACTGCAAAGTACACATATATTACGATGCTGTTCTATT AAATGCTTCCTATATTATATATATAGTAATGTCGTGATCT ATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATA GTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGC GCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAG ACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAG GTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGG GCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGAT AATAATGGTTTCTTAGACGGATCGCTTGCCTGTAACTTA CACGCGCCTCGTATCTTTTAATGATGGAATAATTTGGGA ATTTACTCTGTGTTTATTTATTTTTATGTTTTGTATTTGGA TTTTAGAAAGTAAATAAAGAAGGTAGAAGAGTTACGGA ATGAAGAAAAAAAAATAAACAAAGGTTTAAAAAATTTC AACAAAAAGCGTACTTTACATATATATTTATTAGACAAG

	AAAAGCAGATTAAATAGATATACATTCGATTAACGATAA GTAAAATGTAAAATCACAGGATTTTCGTGTGTGGTCTTC TACACAGACAAGATGAAACAATTCGGCATTAATACCTGA GAGCAGGAAGAGCAAGATAAAAGGTAGTATTTGTTGGC GATCCCCCTAGAGTCTTTTACATCTTCGGAAAACAAAAA CTATTTTTTCTTTAATTTCTTTTTTTACTTTCTATTTTTAAT TTATATATTTATATTAAAAAATTTAAATTATAATTATTTT TATAGCACGTGATGAAAAGGACCCAGGTGGCACTTTTCG GGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAA ATACATTCAAATATGTATCCGCTCATGAGACAATAACCC TGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATG AGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTG CGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCT GGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCAC GAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAG ATCCTTGAGAGTTTTCGCCCCGAAGAACGTT
pETcon(-)	ATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTAT GACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAA AGGGAACAAAAGCTGGTACCAATTCCTTGAATTTTCAAA AATTCTTACTTTTTTTTTGGATGGACGCAAAGAAGTTTAA TAATCATATTACATGGCATTACCACCATATACATATCCA TATCTAATCTTACTTATATGTTGTGGAAATGTAAAGAGC CCCATTATCTTAGCCTAAAAAAACCTTCTCTTTGGAACTT TCAGTAATACGCTTAACTGCTCATTGCTATATTGAAGTA CGGATTAGAAGCCGCCGAGCGGGTGACAGCCCTCCGAA

	GGAAGACTCTCCTCCGTGCGTCCTCGTCTTCACCGGTCG CGTTCCTGAAACGCAGATGTGCCTCGCGCCGCACTGCTC CGAACAATAAAGATTCTACAATACTAGCTTTTATGGTTA TGAAGAGGAAAAATTGGCAGTAACCTGGCCCCACAAAC CTTCAAATGAACGAATCAAATTAACAACCATAGGATGAT AATGCGATTAGTTTTTTAGCCTTATTTCTGGGGTAATTAA TCAGCGAAGCGATGATTTTTGATCTATTAACAGATATAT AAATGCAAAAACTGCATAACCACTTTAACTAATACTTTC AACATTTTCGGTTTGTATTACTTCTTATTCAAATGTAATA AAAGTATCAACAAAAAATTGTTAATATACCTCTATACTT TAACGTCAAGGAGAAAAAACCCCGGATCGAATTCCCTA CTTCATACATTTTCAATTAAGATGCAGTTACTTCGCTGTT TTTCAATATTTTCTGTTATTGCTTCAGTTTTAGCACAGGA ACTGACAACTATATGCGAGCAAATCCCCTCACCAACTTT AGAATCGACGCCGTACTCTTTGTCAACGACTACTATTTT GGCCAACGGGAAGGCAATGCAAGGAGTTTTTGAATATT ACAAATCAGTAACGTTTGTCAGTAATTGCGGTTCTCACC CCTCAACAACTAGCAAAGGCAGCCCCATAAACACACAG TATGTTTTTAAGGACAATAGCTCGACGATTGAAGGTAGA TACCCATACGACGTTCCAGACTACGCTCTGCAGGCTAGT GGTGGAGGAGGCTCTGGTGGAGGCGGTAGCGGAGGCGG AGGGTCGGCTAGCCATATGTGAAGCATGCTTAATTAAGG CGCCTTATCTCGAGGGGGGCGGATCCGAACAAAAGCTTA TTTCTGAAGAGGACTTGTAATAGAGATCTGATAACAACA GTGTAGATGTAACAAAATCGACTTTGTTCCCACTGTACT

	TTTAGCTCGTACAAAATACAATATACTTTTCATTTCTCCG TAAACAACATGTTTTCCCATGTAATATCCTTTTCTATTTT TCGTTCCGTTACCAACTTTACACATACTTTATATAGCTAT TCACTTCTATACACTAAAAAACTAAGACAATTTTAATTTT GCTGCCTGCCATATTTCAATTTGTTATAAATTCCTATAAT TTATCCTATTAGTAGCTAAAAAAAGATGAATGTGAATCG AATCCTAAGAGAATTGAGCTCCAATTCGCCCTATAGTGA GTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGT GACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTT GCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAA GAGGCCCGCACCGATCGCCTTTCCCAACAGTTGCGCAGC CTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAA GCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTA CACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTT CCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAA GCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGT GCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGT GATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTT TTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTG GACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCT CGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTC GGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATT TAACGCGAATTTTAACAAAATATTAACGCTTACAATTTC CTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTT CACACCGCATAGATCGGCAAGTGCACAAACAATACTTA

	AATAAATACTACTCAGTAATAACCTATTTCTTAGCATTTT TGACGAAATTTGCTATTTTGTTAGAGTCTTTTACACCATT TGTCTCCACACCTCCGCTTACATCAACACCAATAACGCC ATTTAATCTAAGCGCATCACCAACATTTTCTGGCGTCAG TCCACCAGCTAACATAAAATGTAAGCTTTCGGGGCTCTC TTGCCTTCCAACCCAGTCAGAAATCGAGTTCCAATCCAA AAGTTCACCTGTCCCACCTGCTTCTGAATCAAACAAGGG AATAAACGAATGAGGTTTCTGTGAAGCTGCACTGAGTAG TATGTTGCAGTCTTTTGGAAATACGAGTCTTTTAATAACT GGCAAACCGAGGAACTCTTGGTATTCTTGCCACGACTCA TCTCCATGCAGTTGGACGATATCAATGCCGTAATCATTG ACCAGAGCCAAAACATCCTCCTTAGGTTGATTACGAAAC ACGCCAACCAAGTATTTCGGAGTGCCTGAACTATTTTTA TATGCTTTTACAAGACTTGAAATTTTCCTTGCAATAACCG GGTCAATTGTTCTCTTTCTATTGGGCACACATATAATACC CAGCAAGTCAGCATCGGAATCTAGAGCACATTCTGCGGC CTCTGTGCTCTGCAAGCCGCAAACTTTCACCAATGGACC AGAACTACCTGTGAAATTAATAACAGACATACTCCAAGC TGCCTTTGTGTGCTTAATCACGTATACTCACGTGCTCAAT AGTCACCAATGCCCTCCCTCTTGGCCCTCTCCTTTTCTTT TTTCGACCGAATTAATTCTTAATCGGCAAAAAAAGAAAA GCTCCGGATCAAGATTGTACGTAAGGTGACAAGCTATTT TTCAATAAAGAATATCTTCCACTACTGCCATCTGGCGTC ATAACTGCAAAGTACACATATATTACGATGCTGTTCTAT TAAATGCTTCCTATATTATATATATAGTAATGTCGTGATC

	TATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCAT AGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACG CGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACA GACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGA GGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAG GGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGA TAATAATGGTTTCTTAGACGGATCGCTTGCCTGTAACTTA CACGCGCCTCGTATCTTTTAATGATGGAATAATTTGGGA ATTTACTCTGTGTTTATTTATTTTTATGTTTTGTATTTGGA TTTTAGAAAGTAAATAAAGAAGGTAGAAGAGTTACGGA ATGAAGAAAAAAAAAATAAACAAAGGTTTAAAAAATTTC AACAAAAAGCGTACTTTACATATATATTTATTAGACAAG AAAAGCAGATTAAATAGATATACATTCGATTAACGATAA GTAAAATGTAAAATCACAGGATTTTCGTGTGTGGTCTTC TACACAGACAAGATGAAACAATTCGGCATTAATACCTGA GAGCAGGAAGAGCAAGATAAAAGGTAGTATTTGTTGGC GATCCCCCTAGAGTCTTTTACATCTTCGGAAAACAAAAA CTATTTTTTCTTTAATTTCTTTTTTTACTTTCTATTTTTAAT TTATATATTTATATTAAAAAATTTAAATTATAATTATTTT TATAGCACGTGATGAAAAGGACCCAGGTGGCACTTTTCG GGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAA ATACATTCAAATATGTATCCGCTCATGAGACAATAACCC TGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATG AGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTG CGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCT

	GGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCAC GAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAG ATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATG ATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTA TCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGC ATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCA GTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG AGAATTATGCAGTGCTGCCATAACCATGAGTGATAACAC TGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAA GGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGT AACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGC CATACCAAACGACGAGCGTGACACCACGATGCCTGTAG CAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAAC TACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGA TGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGG CCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAG CCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGG GGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACA CGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGA CAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGG TAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTG ATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGA AGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAAC GTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAA AGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGT

	AATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGC GGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTT CCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAA TACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTC AAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTA ATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCG TGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGAT AAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCAC ACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGA GATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTC CCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGC AGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGG GGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCG CCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCA GGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGC CTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCAC ATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACC GTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCA GCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAA GCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGC GCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTT TCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTA ATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTAC ACTTTATGCTTCCGGCTCGTATGTTGTGTGGA

APPENDIX B

LIST OF PRIMERS

Table B 1. Primer Sequences Used in Experiments

Primer Name	**Sequence (5' – 3')**
ONK-5	TCCGATGCTGACTTGCTGGGTATTATATGTGTGTAAA ATAGAAAGAGAACAATTGACCCG
ONK-6	TACAAGACTTGAAATTTTCCTTGCAATAACCGGGTCA ATTGTTCTCTTTCTATTTTACAC
ONK-7	CATTGGTGACTATTGAGCAC
ONK-8	CAAAAGGCCTGCAGGCAAGT
ONK-24	GTACTGGGTGAAGTTCGCG
ONK-25	CCCTCACTAAAGGGAACAAAAGCTGGTACCCATAGC TTCAAAATGTTTCTACTCC
ONK-27	CATCACCGCGAACTTCACCCAGTACTAATAATCATGT AATTAGTTATGTCACGC
ONK-28	CTTCAGAAATAAGCTTTTGTTCTTTGTACAGCAAATT AAAGCCTTCGAGC
ONK-29	ATGAGATTTCCTTCAATTTTTACTGC
ONK-30	ACCACCAGAACCGTGGTGGTGGTGGTGGTGAGCTTC AGCCTCTCTTTTCTC
ONK-31	CACCACCACCACCACCACGGTTCTGGTGGTTCTTCTA CCCAGACCCAGTCTTC

ONK-32	AACAGCAGTAAAAATTGAAGGAAATCTCATCTTAGATTAGATTGCTATGCTTTC
ONK-34	CTTCAGAAATAAGCTTTTGTTCTTTGTACAGCAAATTAAAGCCTTCGAGC
P22seqDNA	GCCGCTACAGGGCGCGT
ORI-F(DNA)	CTCAAGACGATAGTTACCGG

APPENDIX C

PLASMID MAPS

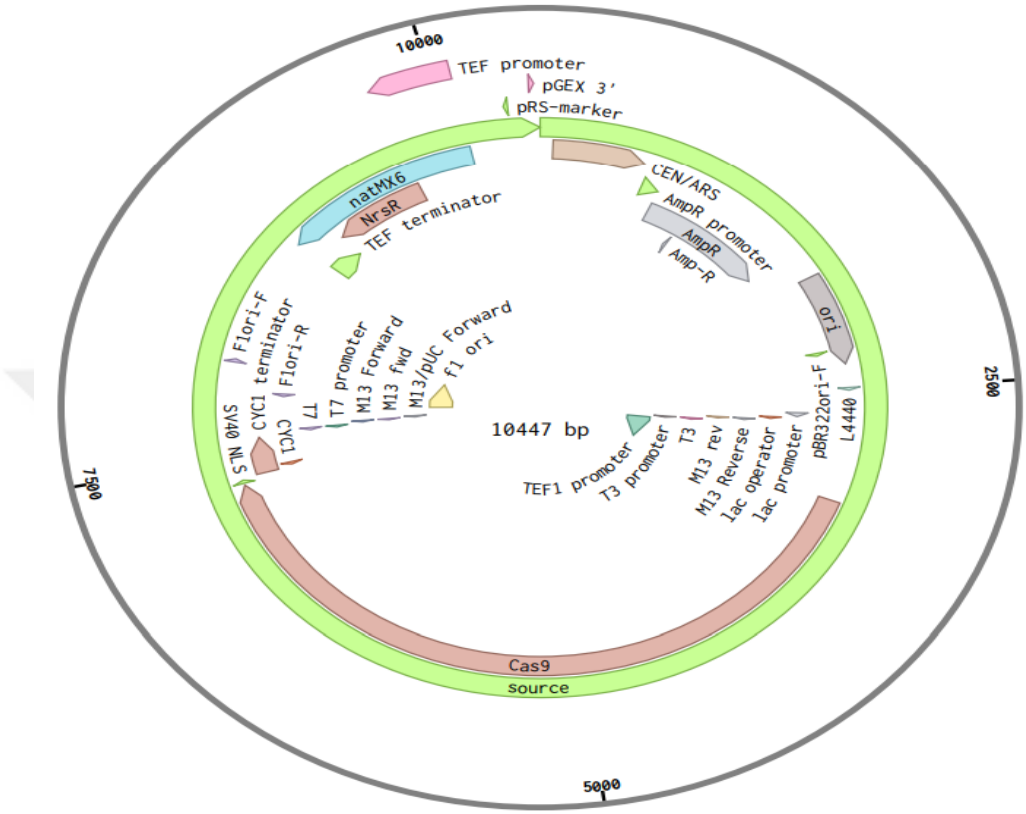


Figure C. 1. Schematic representation of pRS414-TEF1p-Cas9-CYC1t plasmid

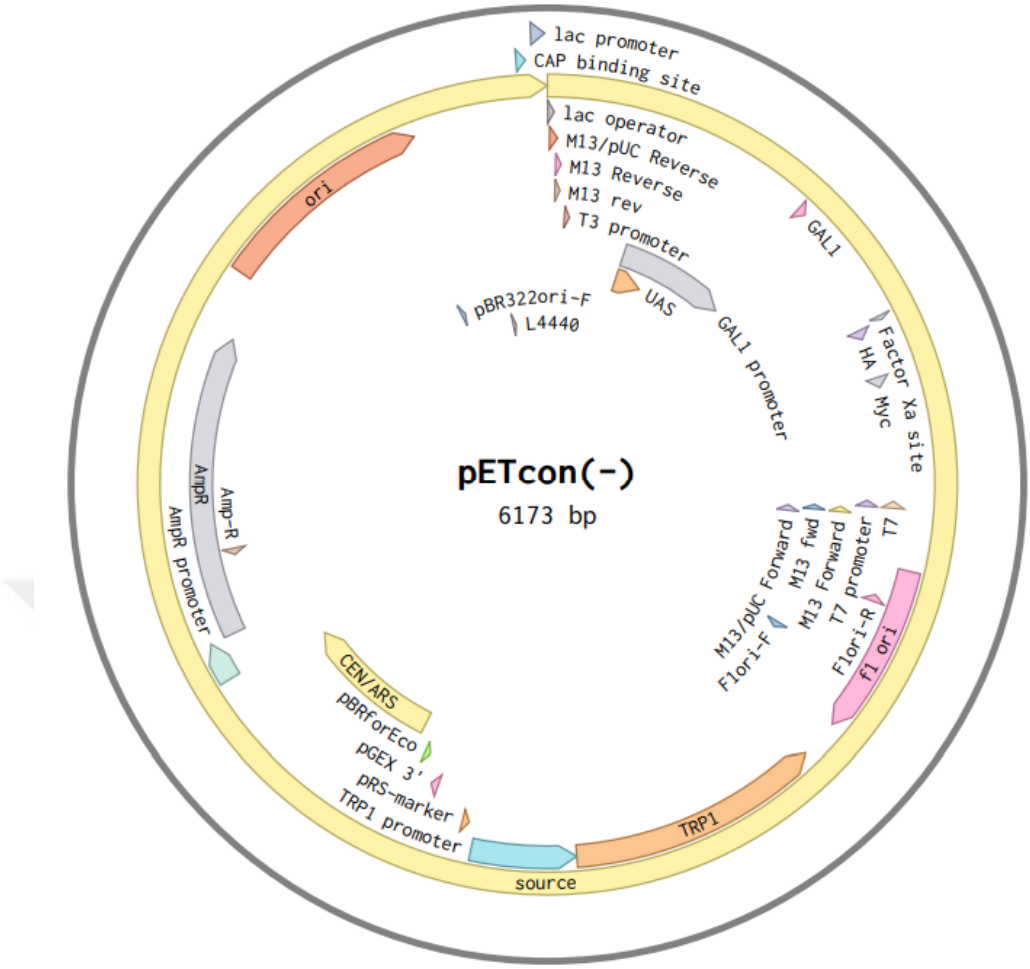


Figure C. 2. Schematic representation of pETcon(-) plasmid

Figure C. 3. Schematic representation of pETcon-TEF1p-Alpha-His-GS-6His/GIF-CYC1t-TRP plasmid

APPENDIX D

SEQUENCING RESULTS

Figure D. 1. Sequence alignment of the NGS results for colony number 1. The result shows that the TRP1 gene of the *S.boulardii* colony 1 is knocked out by the insertion of the stop codon, TAA, to the targeted site in the gene. Alignment was performed in Geneious software

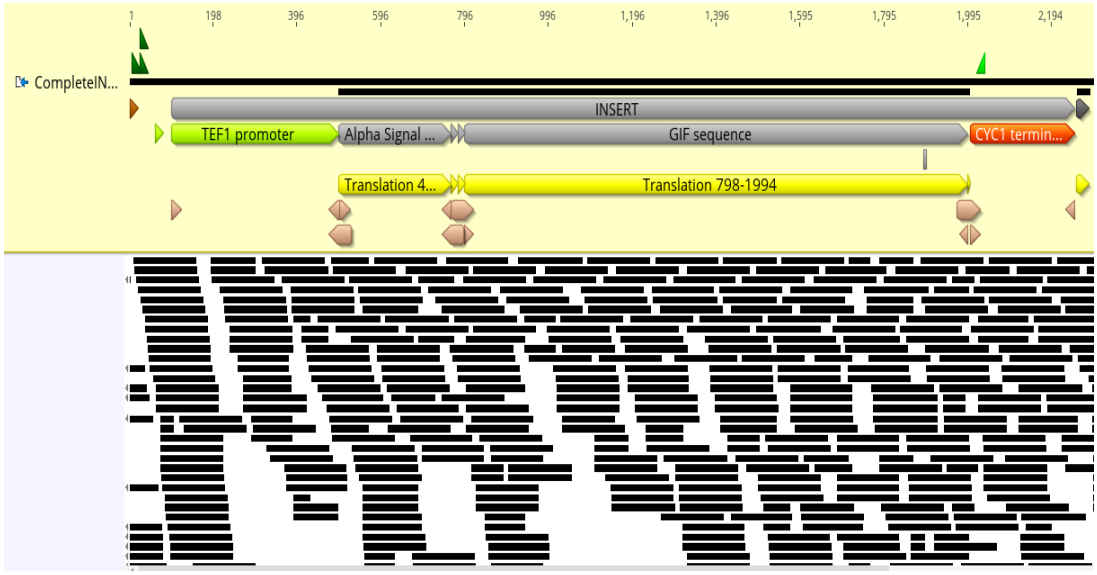


Figure D. 2. Sequence alignment of the NGS results for colony number 2. Result shows the successful insertion of the gene cassette carrying TEF1 promoter, Alpha signal sequence, GIF sequence, and CYC1 terminator. Alignment was performed in Geneious software

APPENDIX E

ADDITIONAL REACTION RECIPES AND CONDITIONS

Table E. 1.Lysogeny Broth (LB) Growth Medium, LB Agar

Component	**Amount**
Tryptone	10 g
Sodium Chloride (NaCl)	10 g
Yeast Extract	5 g
Agar (only for LB Agar)	15 g
H_2O	1000 ml

Table E. 2. YPD (Yeast Extract, Peptone/Tryptone, Dextrose) Medium

Component	**Amount**
Yeast Extract	10 g
Tryptone	20 g
Dextrose	20 g
Agar (only for LB Agar)	15 g
H_2O	1000 ml

Table E 3. 1X YPAD (Yeast Extract, Peptone/Tryptone, Adenine Hemisulfate, Dextrose) Medium

Component	**Amount**
Yeast Extract	10 g
Tryptone	20 g
Dextrose	20 g
Agar (only for LB Agar)	15 g
Adenine Hemisulfate	80 mg
H_2O	1000 ml

Table E 4. 2X YPAD (Yeast Extract, Peptone/Tryptone, Adenine Hemisulfate, Dextrose) Medium

Component	**Amount**
Yeast Extract	20 g
Tryptone	40 g
Dextrose	40 g
Agar (only for LB Agar)	15 g
Adenine Hemisulfate	80 mg
H_2O	1000 ml

Table E 5. Synthetic Drop-Out Medium without Tryptophan

Component	**Amount**
Drop-out mix	1.92 g
Yeast Nitrogen Base w/o amino acids	6.7 g
Dextrose	20 g
Agar (only for LB Agar)	15 g
H_2O	1000 ml

Table E 6. Double Digestion Reaction Set Up with High Fidelity Restriction Enzymes

10X Cut Smart Buffer	2 µl
DNA Sample	1 µg
Restriction Enzyme 1	1 µl
Restriction Enzyme 2	1 µl
Nuclease Free Water	Up to 20 µl

Table E 7. PCR Set up with Q5 DNA Polymerase

Components	**Final Concentration**	**25 µL Reaction Volume**
5X Q5 Reaction Buffer	1 X	5 µl
10 mM dNTP	200 µM	0.5 µl
10 µM Forward Primer	0.5 µM	1.25 µl
10 µM Reverse Primer	0.5 µM	1.25 µl
Template DNA	< 1 µg	< 1 µg
Q5 DNA Polymerase	0.02 U/µl	0.5 µl
5X Q5 GC Enhancer (Optional)	1 X	5 µl
Nuclease Free Water	Up to total reaction volume	Up to 25 µl

Table E 8. PCR Conditions of Q5 DNA Polymerase

Step	**Temperature**	**Time**
Initial Denaturation	98 ºC	30 seconds
Denaturation	98 ºC	5 - 10 seconds
Annealing	50 - 72 ºC	10 - 30 seconds
Extension (25 – 35 cycles)	72 ºC	20 - 30 seconds/kb
Final Extension	72 ºC	2 minutes
Hold	+4 ºC	∞

Table E 9. PCR Set up with Pfu DNA Polymerase

Components	**Final Concentration**	**For 25 µl Reaction Volume**
10X Pfu Reaction Buffer	1 X	2.5 µl
10 mM dNTP	200 µM	0.5 µl
10 µM Forward Primer	0.5 µM	1.25 µl
10 µM Reverse Primer	0.5 µM	1.25 µl
Template DNA	< 1 µg	< 1 µg
Pfu DNA Polymerase	0.02 U/µl	0.5 µl
Nuclease Free Water	Up to the total reaction volume	Up to 25 µl

Table E 10. PCR Conditions of Pfu DNA Polymerase

Step	**Temperature**	**Time**
Initial Denaturation	98 ºC	3 minutes
Denaturation	98 ºC	30 seconds
Annealing	50 - 72 ºC	30 seconds
Extension (25 – 35 cycles)	72 ºC	2 minutes/kb
Final Extension	72 ºC	5 minutes
Hold	+4 ºC	∞

Table E 11. Gibson Assembly Mix (1.33X)

Component	**Amount**
Taq Ligase (40 U/µl)	50 µl
5X Isothermal Buffer	100 µl
T5 Exonuclease (1 U/µl)	2 µl
Phusion Polymerase (2 U/µl)	6.25 µl
Nuclease Free Water	216.75 µl

Table E 12. T4 Ligation Reaction Recipe

Component	**Amount**
10X T4 DNA Ligase Buffer	2 µl
Vector DNA	50 ng
Insert DNA	3 x molar to vector DNA
T4 DNA Ligase	1 µl
10 mM ATP	1 µl
Nuclease Free Water	Up to 20 µl

Table E 13. Overlap Extension PCR Conditions and Components for Two Fragments

Step	**Temperature**	**Time**
PART 1 Initial Denaturation Note: Primers are not mixed. Only fragments to be fused are added to the mixture as 15 ng of each	98 ºC	3 minutes
Denaturation	98 ºC	30 seconds
Annealing (Tm of the overlapping region)	50 - 72 ºC	30 seconds
Extension (5- cycles)	72 ºC	2 minutes/kb
PART 2 Initial Denaturation Note: PCR reaction is stopped, and primers are added to the mixture	98 ºC	3 minutes
Denaturation	98 ºC	30 seconds
Annealing (Tm of the primers)	50 - 72 ºC	30 seconds
Extension (25-35 cycles)	72 ºC	2 minutes/kb
Final Extension	72 ºC	5 minutes
Hold	+4 ºC	∞