UNIVERSITY OF CALIFORNIA,
IRVINE

Protein Yield Testing for Adhesion Molecule Plasmids for Targeting Therapeutic Nanoparticles

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE
in Biomedical Engineering

by

Kevin Lee

Thesis Committee:
Associate Professor Jered Haun, Chair
Associate Professor Michelle Digman
Associate Professor James Brody

DEDICATION

To

my family and friends

in recognition of their love and support

Table of Contents

LIST OF FIGURES

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Abstract of the Thesis

Protein Yield Testing for Adhesion Molecule Plasmids for Targeting Therapeutic Nanoparticles

By

Kevin Lee

Master of Biomedical Engineering

University of California, Irvine, 2020

Professor Jered B. Haun, Chair

Many studies have been done on different approaches for cancer therapy and one of the most promising fields is using nanoparticles as a drug delivery cancer therapeutic agent. The unique optical properties and high surface volume ratio of nanoparticles can improve the drug delivery system for not only cancer but other diseases. Moreover, nanoparticles can be attached to small proteins which provide the ability to target specific areas or inflamed tissues to minimize the death of healthy cells and tissues. Fusing the protein with fluorescent proteins also enables us to know the localization while targeting cells. Engineering a platform design for nanoparticles with adherent small proteins together with adding specific fluorescent proteins of interest on nanoparticles remains challenging. To achieve the goals, two different expression systems: E. coli and yeast protein expression system are being tested in this thesis. The E. coli expression system is known to have higher protein yield but with protein folding problems while the yeast expression system has lower yields but no protein folding problems which is easier for downstream applications. Therefore, the first part of this thesis is aiming to fuse different fluorescent proteins to an existing recombinant sequence which is construct in our lab and optimize the protein expression via E. coli and yeast expression system. The second part of the thesis is performing the protein yield testing of plasmids contains enzymatic tags. To perform the enzymatic test of our plasmid, we need to be sure that the enzymatic tags fused to our plasmid does not interact with the protein of interest which causes the protein yield to drop. Once we make sure that the enzymatic tags are not interfering the protein

yield, enzymatic tests can be performed to know more about the enzyme activity our protein of interest and enzymes. Three different enzyme/tag systems are planned to be tested, Sfp synthase/S6 tag, and Sortase A/LPETG tag, and Lipoic Acid Ligase (LplA)/LAP2 tag. Different colors of fluorescent protein in this construct will create a library which enable us to have more options to choose while doing imaging and targeting in the future. It also creates a possibility to image multiple fluorescence simultaneously.

Introduction

Cancer is now the second leading cause of death in the United States according to the data of the Centers of Disease Control and Prevention website [(1)]. Therefore, research is widely been done in cancer field to help slowing down or flattening the curve of cancer deaths each year. Traditional therapy such as chemotherapy is one of the methods to treat cancer nowadays but this treatment contains high toxicity which can cause harm to human body and cells. To minimize the side effects causes by the treatment, nanomedicine and nanobiotechnology are being brought into picture for cancer therapies [(2)].

One of the main concerns of traditional drug admission route such as oral doses and injections is the low efficiency of the drug delivery to the inflammatory tissue. Oral doses and injections often have higher concentration of drug since the concentration of drug will gradually decrease while it is circulating through the whole body to reach the disease site. The higher concentration of drug often leads to higher toxicity which will also kill healthy cells and eventually leads to unpleasant side effect. Targeted drug delivery is one of the best options nowadays to overcome these disadvantages with only increasing drug concentration at the disease site. Nanotechnology has been introduced not only to research field but also medical field nowadays (Figure 1). Incorporating nanotechnology to drug delivery can also help prolong the degradation of drug while circulating in human body.

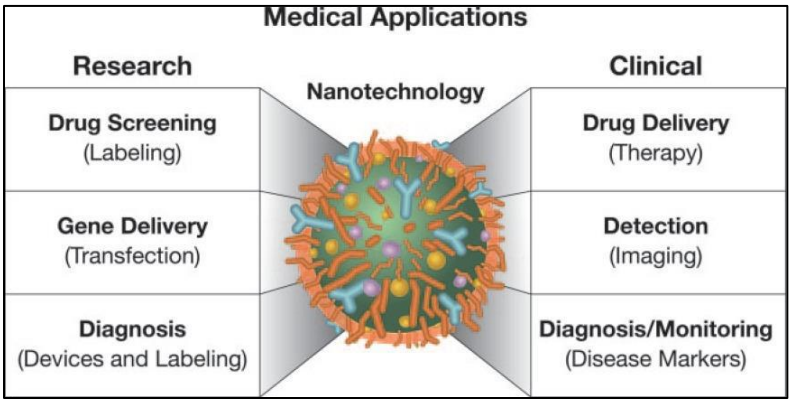


Figure 1. Applications of Nanotechnology [(3)]

A successful anticancer drug needs to have the ability to penetrate barriers inside human body with minimal loss of the drug concentration. After the drug reaches the cancer site, it needs to only target the cancer cells and release the drug gradually and continuously within a limited time span. Without affecting the normal cells, the complexity of adverse side effect can be reduced therefore higher up the quality of life of the patient. Also, by increasing the intracellular drug concentration and lowering the toxicity simultaneously can also increase the survival rate of the patient.

Nano size molecules such as peptides, proteins and nucleic acid can also be one of the tools for targeted drug delivery. These designed small molecules can target cancer cells through binding to surface receptors on the cancer cells. There are two categories for targeted drug delivery: the passive and active targeting (Figure 2a). The passive targeting brings the nanocarriers into disease site through the leakage of the blood vessels. To be more specific in cancer therapeutics, the passive targeting relies on the tumor microvasculature and other factors such as the size, shape and the charges of the nanocarrier. To target cancer cells in a more efficient way, researchers designed small molecules and nanocarriers which can bind on the surface receptor which is normally overexpressed in tumor cells (Figure 2b). This route is known as the active targeting and has a higher specificity in targeting tumor cells when compared to passive targeting.

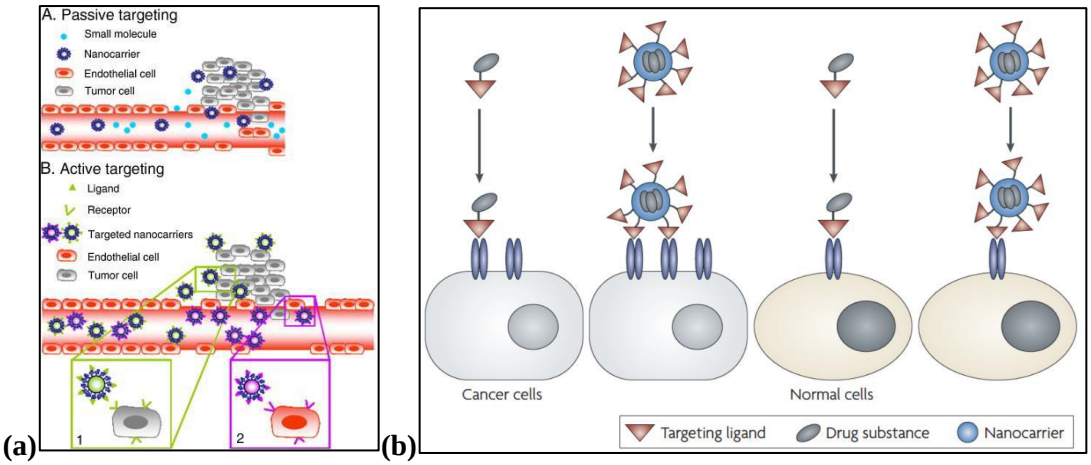


Figure 2. (a) Passive and Active Targeting [4]. (b) Nanoparticle with multiple targeting ligands can bind to overexpressed surface receptors on tumor cells [5].

Previous studies in our lab has constructed a platform which includes 4420 scFv fused fluorescent protein (mCherry), biotin acceptor protein (Avitag), six-histidine tag (6His) and c-myc epitope in expression vector. The recombinant single chain antibody is biotinylated and functionalized to avidin-coated nanoparticles. The binding reaction were measured in flow chamber under fluid flow [6]. (Figure 3.)

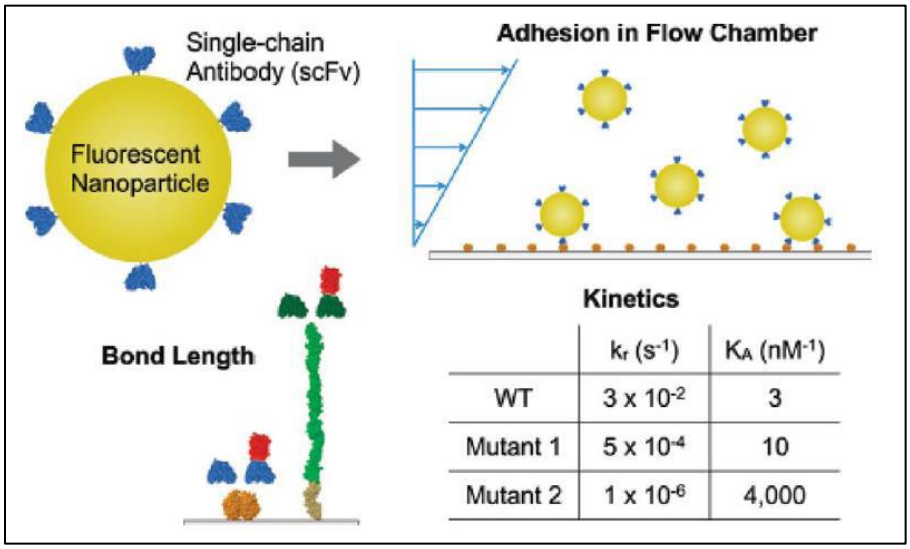


Figure 3. Model of targeted drug delivery system [6]

In this thesis, mRFP and moxGFP fluorescent protein have been introduced into the construct mentioned above and the biotin acceptor protein is replaced by S6 tag (Figure 4). The whole construct has been done in pRS314 vector for yeast expression system and pGEM-3Z vector for E. coli expression system. The LAP2 and LPETG tags are then cloned onto the construct for the enzymatic test.

Figure 4. Construct of scFv with peptide linkers in pRS expression system

This construct includes a GAL1-10 promotor, a synthetic prepro leader as sequence to direct the protein, pRS314 or pGEM-3Z vector (depends on yeast or E.coli system), fluorophore (mRFP or

moxGFP), S6 tag as the orthogonal binding site for the nanoparticle to attach via enzymatic reaction, 6His protein purification tag and c-myc epitope tag (Figure 5).

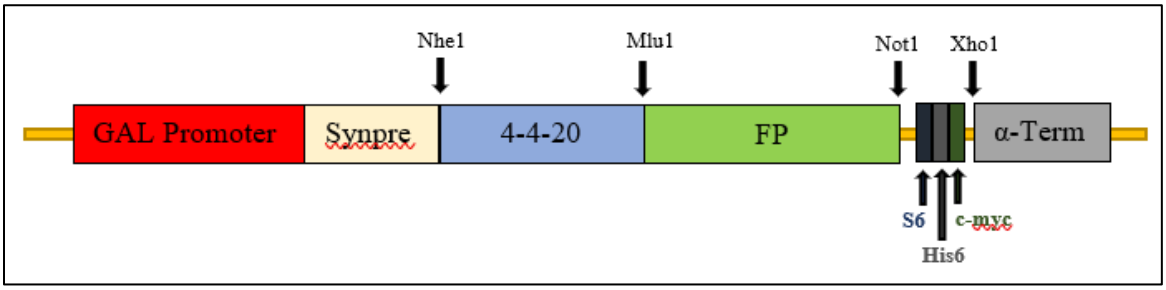


Figure 5. Detailed view of the construct with restriction enzyme sites.

Chapter 1: Construction of recombinant protein with moxGFP and mRFP1 in pGEM-3Z vector

1.1 Background

Fluorescent proteins are good markers when it comes to understanding the protein-protein interaction within the cell. Since mCherry has been expressed successfully in the previous lab work, we are trying to express different colors of fluorescent proteins inside the plasmid to make a library of different colors of fluoresceins and possibly combine different color markers in the future. In our research, green fluorescent protein (moxGFP) and red fluorescent protein (mRFP1) are inserted onto two different pGEM-3Z plasmids, respectively. Due to the higher quantum yield of mRFP1, we swap the mCherry fluorescein to mRFP1. Our lab has designed a unique sequence which fused the fluorescent proteins with different tag proteins which enabled us to track the localization of the protein of interest.

The green fluorescent protein (GFP) (Figure 6) derived from jellyfish *Aequorea Victoria* is widely used in the field of biology research due to its non-toxicity to cells. GFP is also a stable protein which allows researchers to insert its DNA sequence to the gene of interest easily therefore GFP becomes one of the most popular fluorescent proteins for imaging. There are different versions of GFPs in the market, our lab decided to work on moxGFP which is derived from oxGFP, a superfolder GFP, with several mutations in the sequence. The moxGFP is a monomer which matures faster for fluorescence detection. [7] [8]

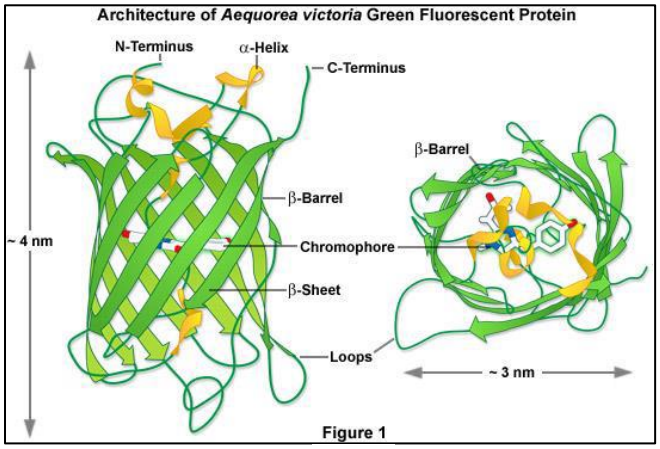


Figure 6. Structure of green fluorescent protein

The red fluorescent protein (RFP) also has different versions in the market. The one which we picked for this specific project is the mRFP1. Though the most popular RFP series in the market is the mfruit series such as mCherry (Figure 7), mOrange, mStrawberry and so on due to their high photostability, we decided on using mRFP1 as our RFP choice since mRFP1 has a higher quantum yield if compared to mCherry. One of the future goals of our experiment is to fuse different fluorescent proteins onto our plasmids to create a protein which can do multiple labeling in cells. Therefore, quantum yield is relatively more important in our future experiment. To sum up, though the mfruits series have higher photostability and faster maturation, mRFP1 has slightly worse photostability and slower maturation but higher quantum yield which balanced the cons and make mRFP1 the better choice for our experiment.

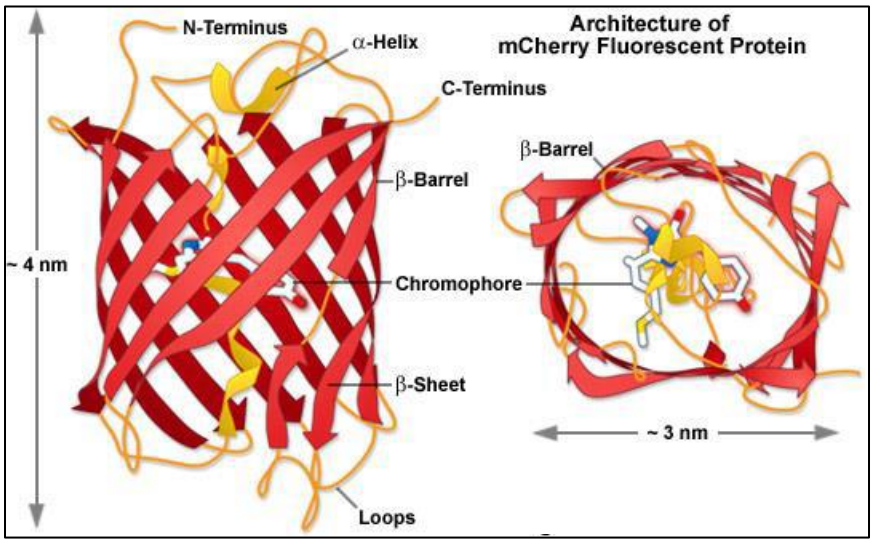


Figure 7. Structure of mCherry

1.2 Materials and Methods

1.2.1 Reagents, Materials and Kits

See appendix for all reagents, materials and kits

1.2.2 Strains and Media

Escherichia coli strain BL21(DE3) cells are from New England Biolab. This strain is used for recombinant plasmids gene cloning. Luria-Bertani medium is used to culture BL21(DE3) cells.

1.2.3 Plasmid Design

Two fluorescence proteins' DNA sequences are as follow:

moxGFP sequence (770 base pairs):

5'-

TCAGTGCTAAGACGCGTGGTGGCGGTCGTACGATGGTGTCAAAGGGTGAAGAATTGTTTACT
GGTGTTGTTCCTATTTTGGTTGAATTGGATGGTGACGTTAATGGACATAAGTTCTCTGTTAGA
GGGGAAGGTGAGGGAGATGCTACTAATGGTAAATTGACCTTGAAATTCATTTCTACTACTGG
GAAATTGCCAGTTCCATGGCCAACCTTGGTTACTACTTTGACTTATGGTGTTCAATCTTTTTC
CAGATATCCAGATCACATGAAAAGACATGATTTTTTCAAATCTGCTATGCCAGAGGGTTATG
TTCAAGAAAGAACTATTAGTTTCAAAGATGATGGCACCTATAAAACAAGAGCTGAGGTTAA
ATTCGAAGGAGACACTTTGGTTAACAGAATTGAACTAAAAGGTATTGATTTTAAGGAAGATG
GTAATATTCTGGGTCATAAATTGGAATACAACTTCAACTCCCACAACGTCTACATTACTGCT
GATAAGCAAAAAAACGGTATTAAGGCTAACTTCAAAATTAGACATAACGTTGAAGACGGCA
GTGTTCAGTTGGCAGATCATTATCAACAAAATACTCCAATCGGTGATGGTCCAGTCTTGTTG
CCAGATAATCACTATTTGTCTACTCAATCTAAGCTTTCCAAAGATCCAAATGAGAAAAGAGA
TCATATGGTTTTGCTTGAATTTGTTACTGCAGCCGGTATCACTCATGGTATGGATGAACTATA
TAAGGCGGCCGCTACATGCTACTAC - 3'

mRFP1 sequence (678 base pairs):

5' -

ATGGCCTCCTCCGAGGACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTC
CGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACC
CAGACCGCCAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTC
CCCTCAGTTCCAGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACT
TGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGG

CGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGC
TGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGA
GGCCTCCACCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGATGAGG
CTGAAGCTGAAGGACGGCGGCCACTACGACGCCGAGGTCAAGACCACCTACATGGCCAAGA
AGCCCGTGCAGCTGCCCGGCGCCTACAAGACCGACATCAAGCTGGACATCACCTCCCACAA
CGAGGACTACACCATCGTGGAACAGTACGAGCGCGCCGAGGGCCGCCACTCCACCGGCGCC
TAA – 3’

The designed plasmid is constructed by a previous graduate student. This plasmid is stored in -20 degree Celsius freezer and the partial sequence is shown below (Figure 8). The plasmid has a mCherry sequence and the goal is to swap the mCherry to moxGFP or mRFP1. Two restriction sites which are Mlu1 and Not1 are marked in red in the figure below respectively. Mlu1 restriction sites are at the beginning of the mCherry sequence and Not1 is at the end.

The pRS vector is not suitable for the BL21(DE3) cells. Therefore, we do a second digestion after successfully ligate the moxGFP or mRFP1 on to the pRS vector backbone. For the second digestion, we used restriction enzyme Nhe1 and Xho1 to move the whole 4420-moxGFP/mRFP1-S6-His6-cmyc sequence onto a pGEM backbone. See Figure 9 below.

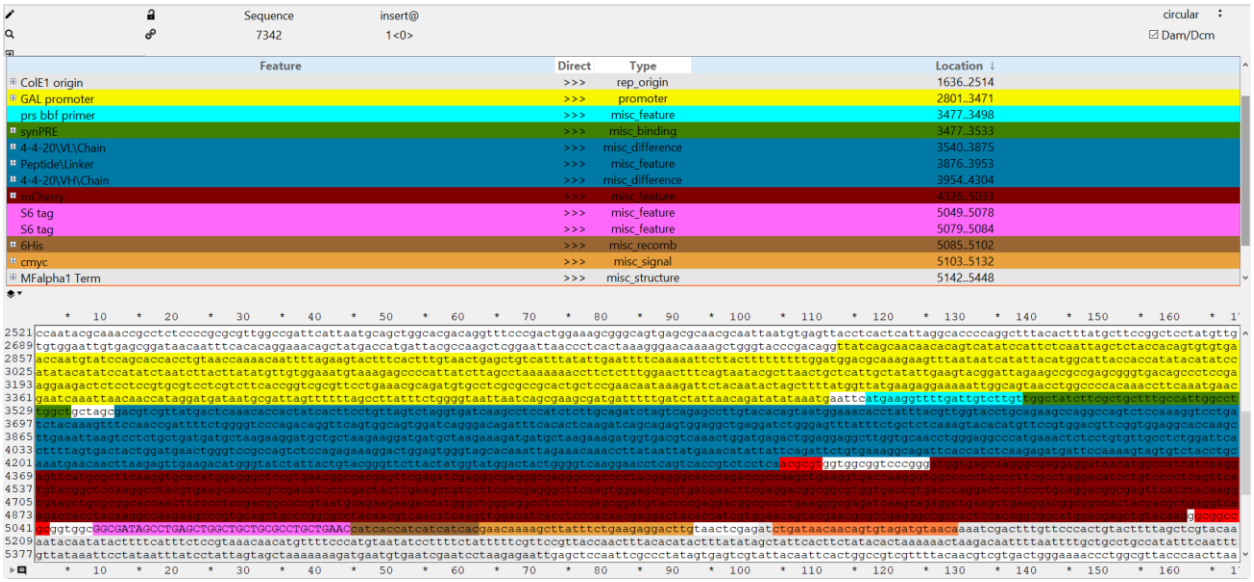


Figure 8. pRS-4420-mCherry-S6-His6 sequence. 4420 sequence is highlighted in blue, mCherry is highlighted in dark red, S6 sequence in pink, His6 sequence in brown and cmyc sequence in light orange. The two bright red regions highlighted on both sides of the mCherry sequence is the two restriction sites which are Mlu1 (acgcgt) at the beginning of the mCherry sequence and Not1 (gcggccgc) at the end of the mCherry sequence. We use these two restriction enzymes to swap mCherry to other fluorescent protein sequences

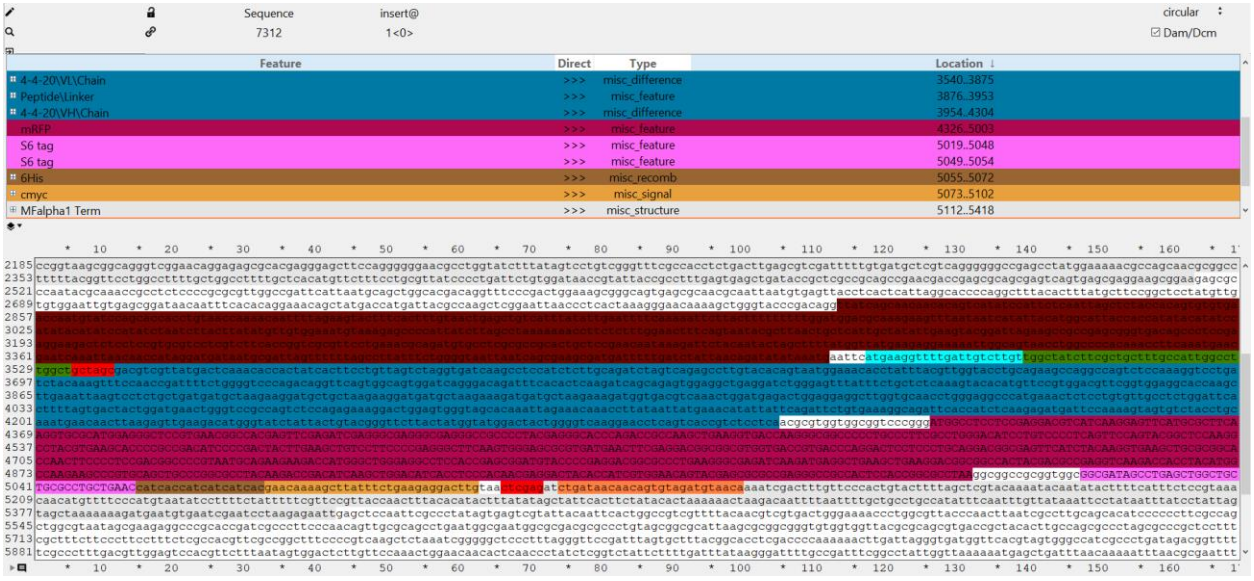


Figure 9. pRS-4420-mRFP1-S6-His6-cmyc sequence. This plasmid is the product after swapping out the mCherry sequence into mRFP1. In order to swap out the pRS vector to a pGEM vector, a second digestion is needed. Two bright red restriction sites are marked in the figure. The restriction site in the front of the 4-4-20 sequence is the Nhe1 restriction site (gctagc) and the one which is at the end of the cmyc sequence is the Xho1 restriction site (ctcgag).

1.2.4 Digestion and Ligation

MoxGFP and mRFP1 inserts are amplified by thermo-cycler in Professor Chang Liu's lab. The settings for the polymerase chain reaction (PCR) in appendix. The designed primers are used to create an insert with restriction sites on both ends of the fluorescent protein sequence after the PCR. (Mlu1 at the beginning of our forward primer and a Xho1 site at the end of the reverse primer for both moxGFP and mRFP1 primers.)

After the PCR, double digestions are carried out on both insert and plasmid. Both insert and plasmid are digested with Mlu1 and Not1 restriction enzymes. The digestion mix are put in the 37-degree Celsius water bath for 1 hour and gel electrophoresis (Figure 10, 11 and 12) is done to check if the insert and plasmid have been digested completely and successfully.

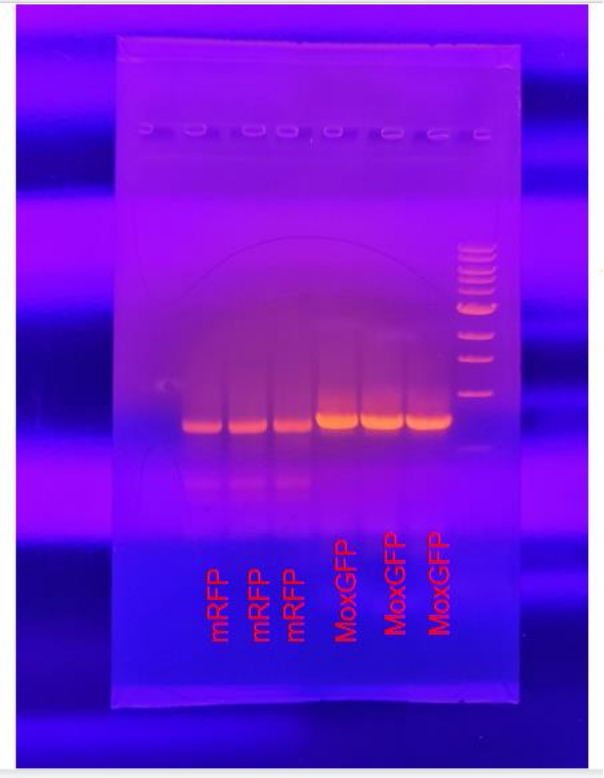


Figure 10. Gel Electrophoresis for double digested moxGFP and mRFP1 using Mlu1 and Not1 restriction enzymes. The far-right lane is the 1k DNA ladder. The mRFP1 bands are slightly lower than the moxGFP bands. The total basepairs for mRFP1 and moxGFP sequence are 700 and 770 basepairs respectively.

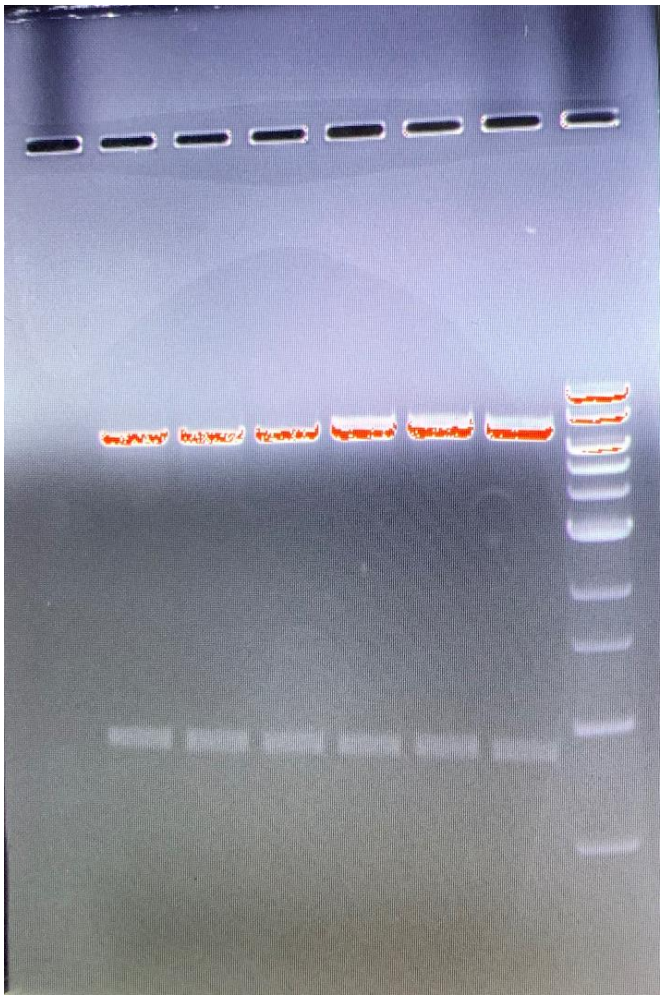


Figure 11. Gel Electrophoresis for double digested pRS-4420-mCherry-S6-His6-cmyc using Mlu1 and Not1 restriction enzymes. The far-right lane is the 1k DNA ladder. The length of the whole pRS-4420-mCherry-S6-His6-cmyc is 7342 base pairs and the length of the mCherry is 708 base pairs. After the double digestion, the lower bands show that the mCherry has been cut out of the whole plasmid which means that the upper bands are the product for the ligation step.

The similar procedure is used for the second digestion and ligation. Instead of using the Mlu1 and Not1 restriction enzymes, Nhe1 and Xho1 restriction enzymes are used to cut the whole sequence (4420-moxGFP/mRFP1-S6-His6-cmyc) from the pRS vector then ligate on to the pGEM-3Z vector for further transformation. The electrophoresis result after the double digestion is shown below. Note that the pGEM-3Z plasmid for the double digestion is the pGEM-4420 plasmid.

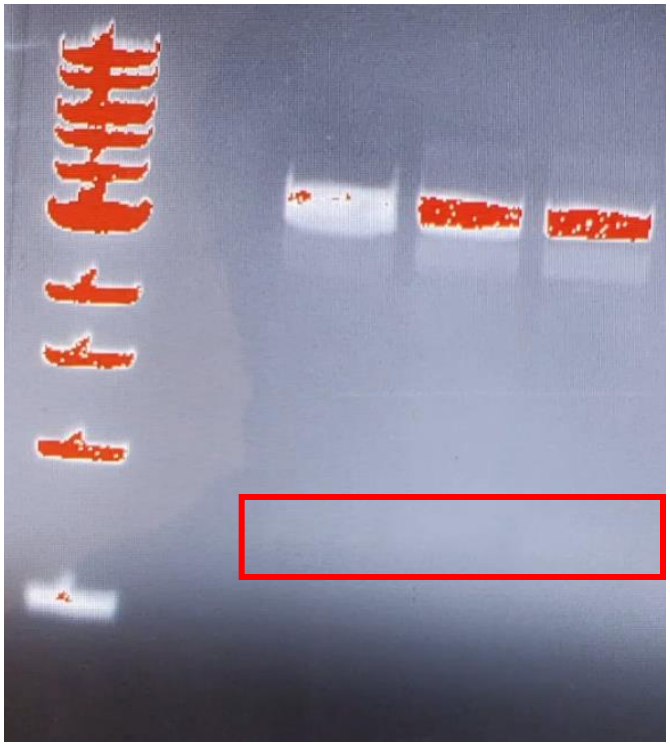


Figure 12. Gel Electrophoresis for double digested pGEM-4420 using Nhe1 and Xho1 restriction enzymes. The far-left lane is the 1k DNA ladder. The length of the whole pGEM-4420 is 3508 base pairs and the length of the 4420 is 765 base pairs. After the double digestion, the faint lower bands show that the 4420 has been cut out of the whole plasmid which means that the upper bands are the product that we want for the second ligation step.

1.2.5 Transformation

We carefully transformed the ligated pGEM-4420-moxGFP/mRFP1-S6-His6-cmyc plasmid to the BL21(DE3) cells after the ligation. For the transformation step, all the temperatures and timings need to be accurate and precise in order to transform successfully. The BL21 cells are thawed on ice for 10 mins until the crystals disappear before the whole procedure starts. Pipette the BL21 cell mixture gently then aliquot 50 uL to a new Eppendorf tube. Add 5 uL of the pGEM plasmid to the cell mixture, flick 4-5 times (note: do not vortex) and let it stay on ice for 30 minutes. Heat shock the cell mixture in 42-degree Celsius water bath for exactly 10 seconds then immediately place the Eppendorf tube back on ice for another 5 minutes. Add 950 uL of room temperature SOC media to the cell mixture and invert the tube 4-5 times to mix the cells thoroughly. Incubate the cell mixture in an ambient 37 degree Celsius incubator for an hour and with 250 rpm shaking. Prewarm the LB-Ampicillin plates to 37 degree Celsius. Pipette

100 uL to each selection plate and spread the liquid with a cell inoculator. Incubate the plates in a 37 degree Celsius ambient incubator overnight to let the BL21(DE3) cells grow. Figure 13 below shows the transformed BL21(DE3) cells.

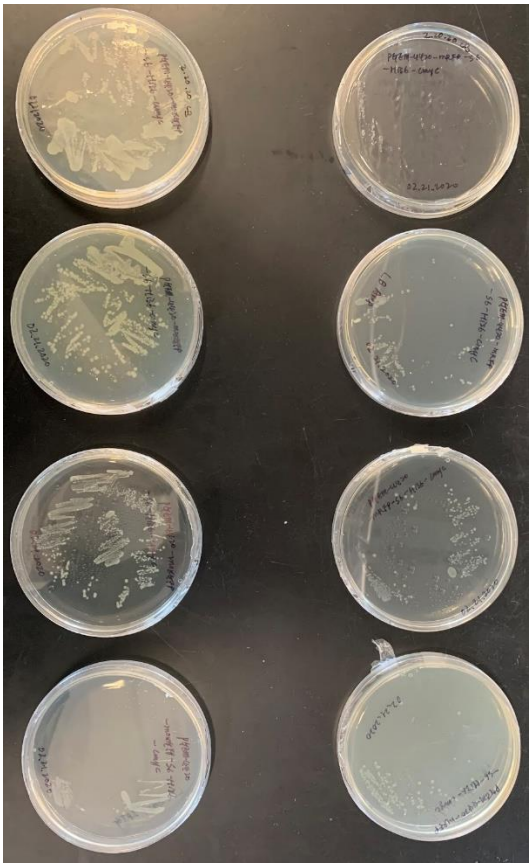


Figure 13. Transformed BL21(DE3) cells. The 4 plates on the left are the BL21(DE3) cells with pGEM-4420-moxGFP-S6-His6-cmyc plasmid and the 4 on the right are the BL21(DE3) cells with pGEM-4420-mRFP1-S6-His6-cmyc plasmid.

The colony PCR is done after the BL21(DE3) cells grow on the selection plates. A colony on the selection plate is picked and dipped the into 50 uL of ultrapure water. The reagents are the same as doing the normal PCR. The only two differences are that the volume of each reagents are half of the normal PCR and the primers which we select for colony PCR are 4420 forward primer and fluorescent protein (moxGFP or mRFP1) reverse primer. The figure 14 below verified that the pGEM-4420-moxGFP/mRFP1 plasmid has been successfully cloned into the BL21(DE3) cells.

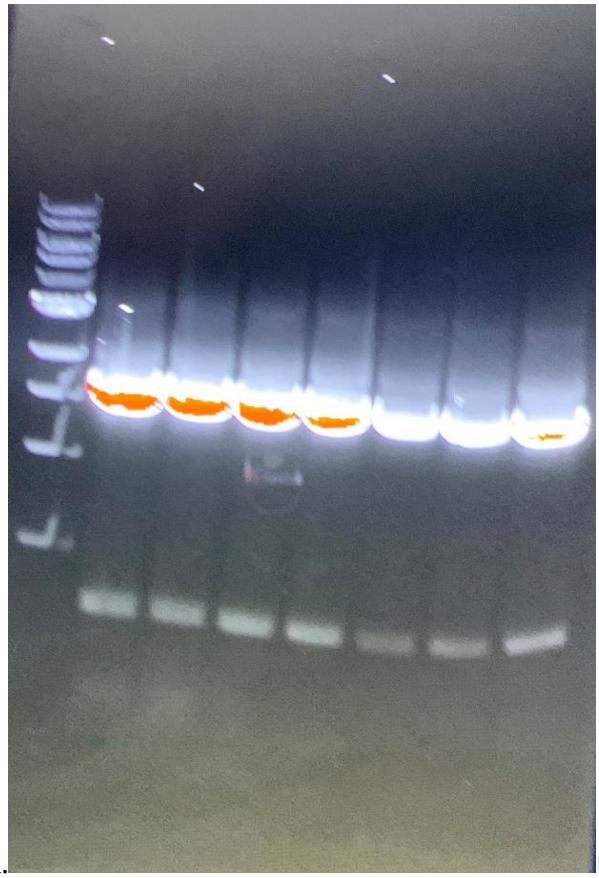
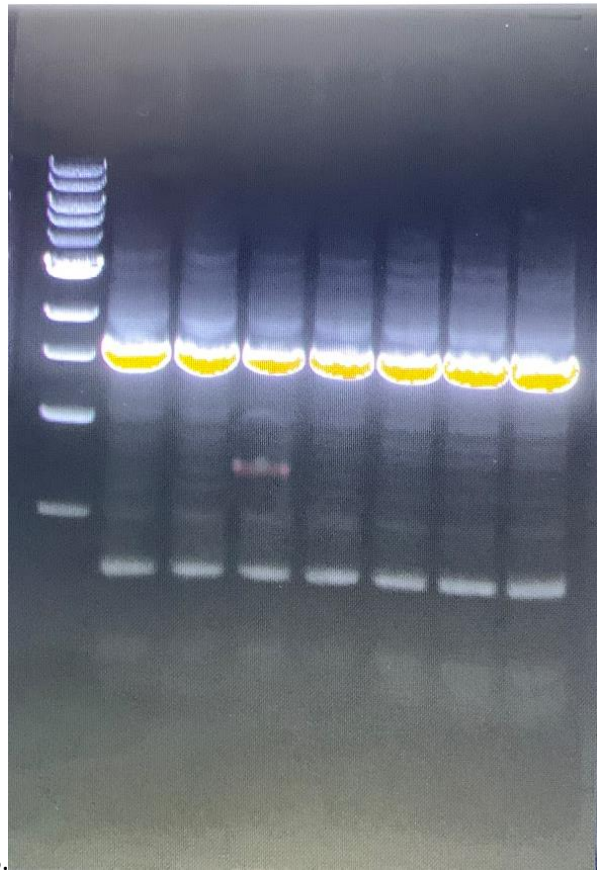
A.  B. 

Figure 14. Gel Electrophoresis for colony PCR. (A.) colony PCR using 4420 forward primer and moxGFP reverse primer of pGEM-4420-moxGFP-S6-His6-cmyc BL21(DE3) cells and (B.) colony PCR using 4420 forward primer and mRFP1 reverse primer of pGEM-4420-mRFP1-S6-His6-cmyc BL21(DE3) cells. The amplifying region from 4420 to the fluorescent protein (moxGFP or mRFP1) is around 1500 base pairs which is the upper band on both picture A and B. The lower bands are the primer bands.

1.2.6 Sequencing Results

The last step to double check that the BL21(DE3) cells which has been transformed successfully is to send it out to an off-campus company to sequence our samples. A monocolony is picked from each selection plate and liquid culture is performed overnight in a 37-degree Celsius ambient incubator with 250 rpm shaking. After the incubation, the samples have been miniprepped and are ready to be sent out for sequencing. The chosen primers are the T7 promotor forward primer and the sp6 promotor reverse primer. Both T7 and sp6 promotor can be found on the pGEM plasmid not the pRS plasmid. If we are able to get a sequencing result using these two primers, we are sure that our designed sequence has been

successfully transferred from the pRS vector to pGEM vector. The figures below show the sequencing result from the off-campus company, Genewiz. (Figure 15-18)

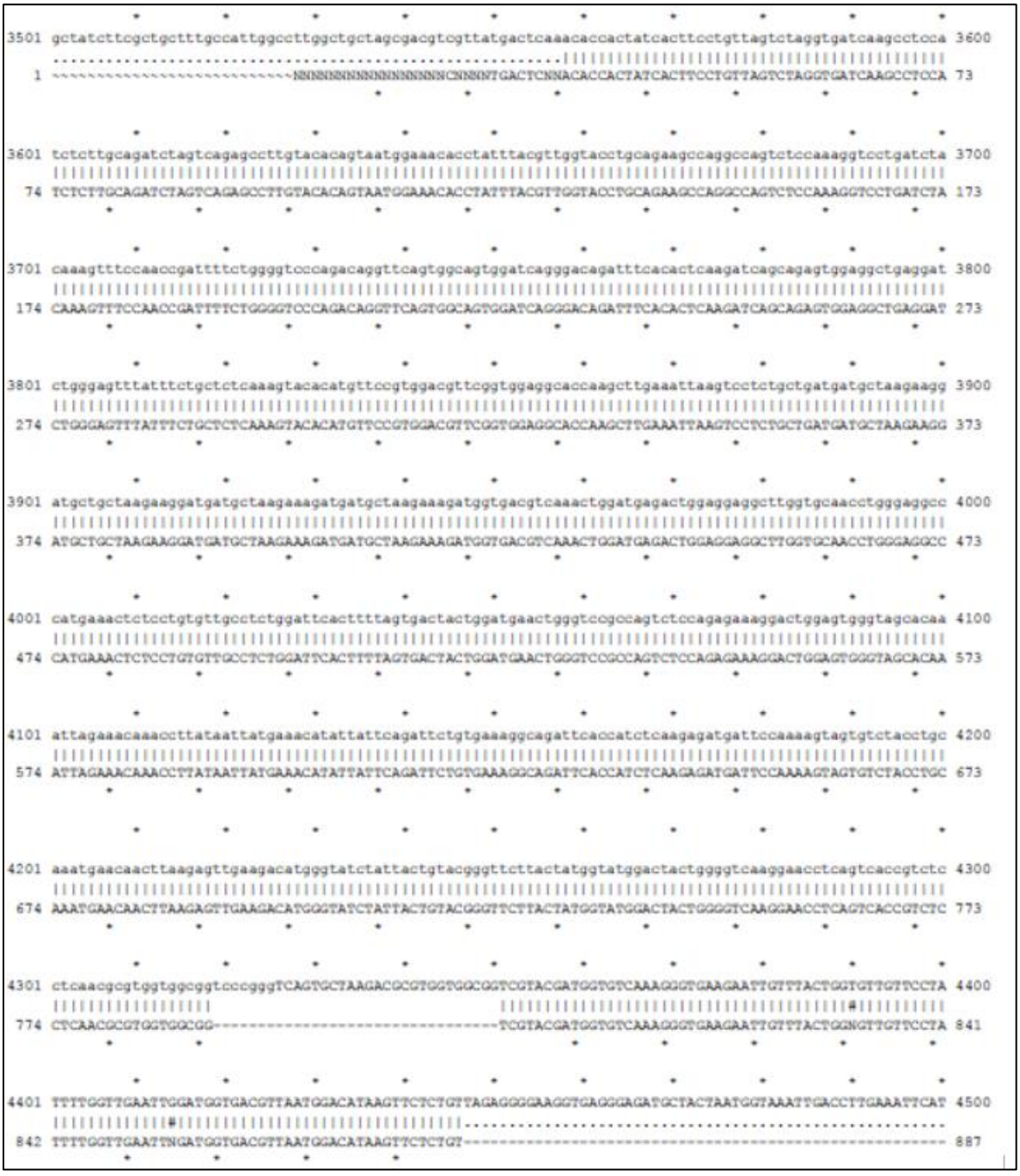


Figure 15. Align pGEM-4420-moxGFP-S6-His6-cmyc sequencing result using T7 forward primer to pRS-4420-moxGFP-S6-His6-cmyc. (from 4420 to moxGFP)

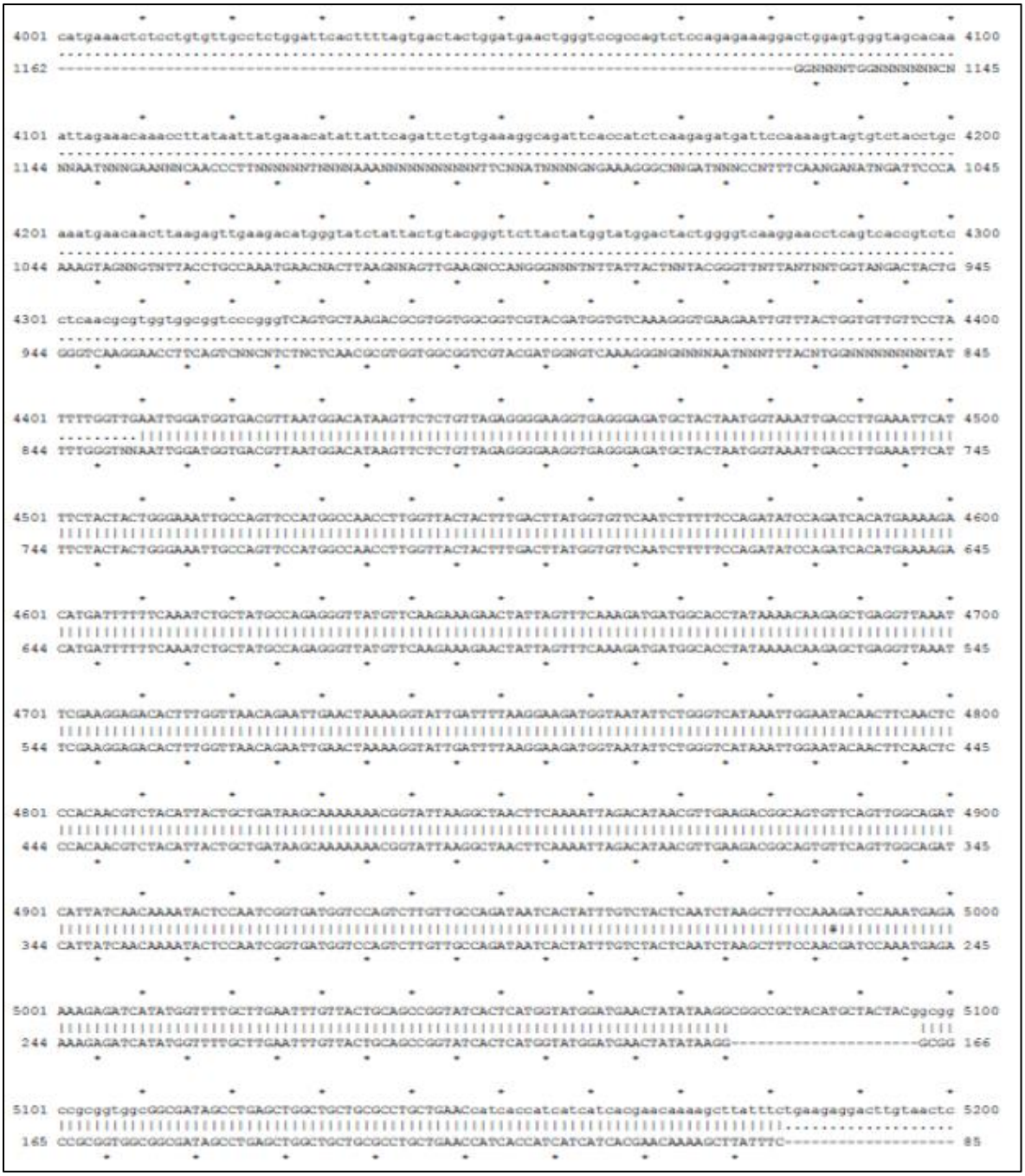


Figure 16. Align pGEM-4420-moxGFP-S6-His6-cmyc sequencing result using sp6 reverse primer to pRS-4420-moxGFP-S6-His6-cmyc. (from GFP to cmyc)

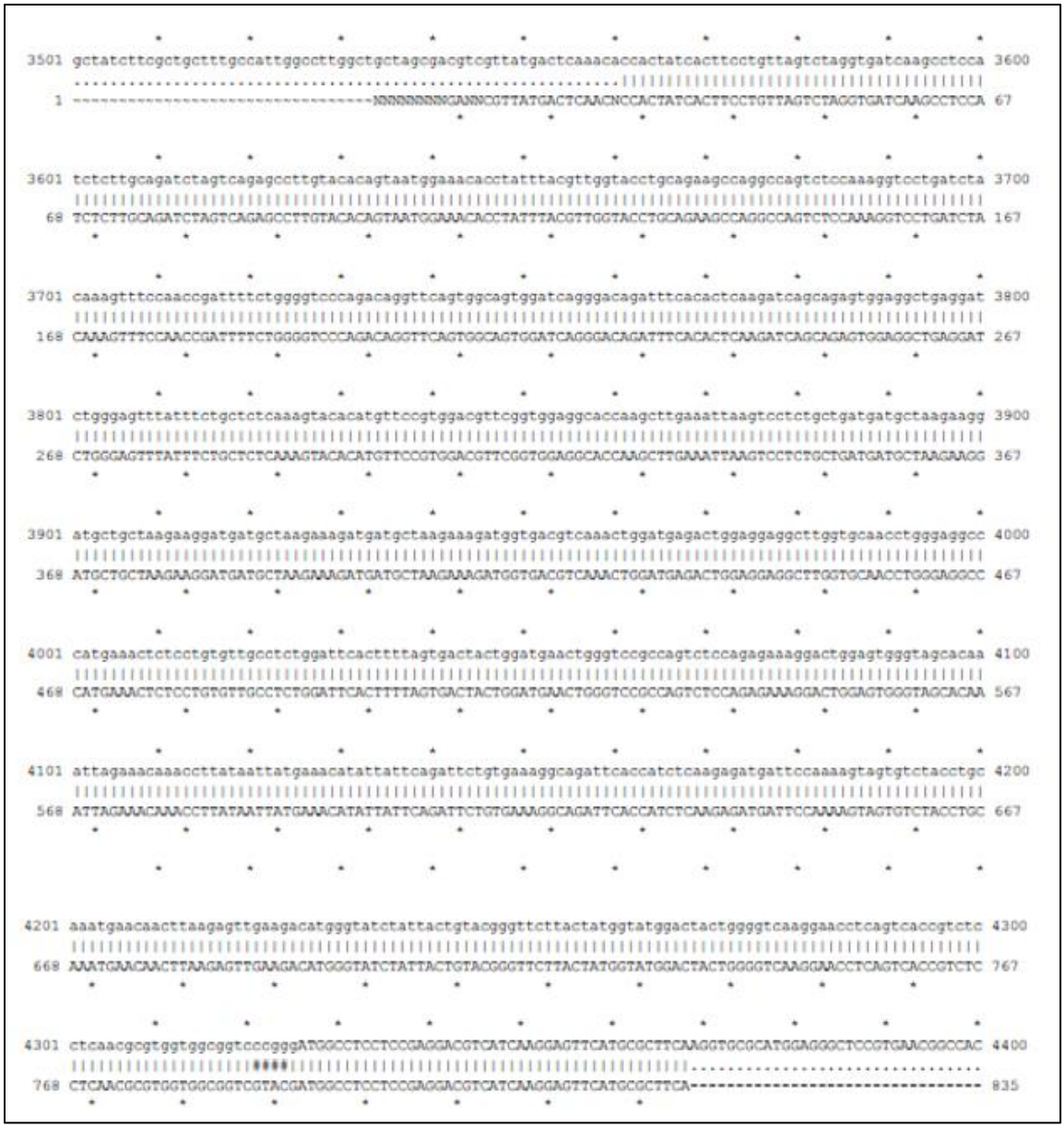


Figure 17. Align pGEM-4420-mRFP1-S6-His6-cmyc sequencing result using T7 forward primer to pRS-4420-mRFP1-S6-His6-cmyc. (from 4420 to mRFP1)

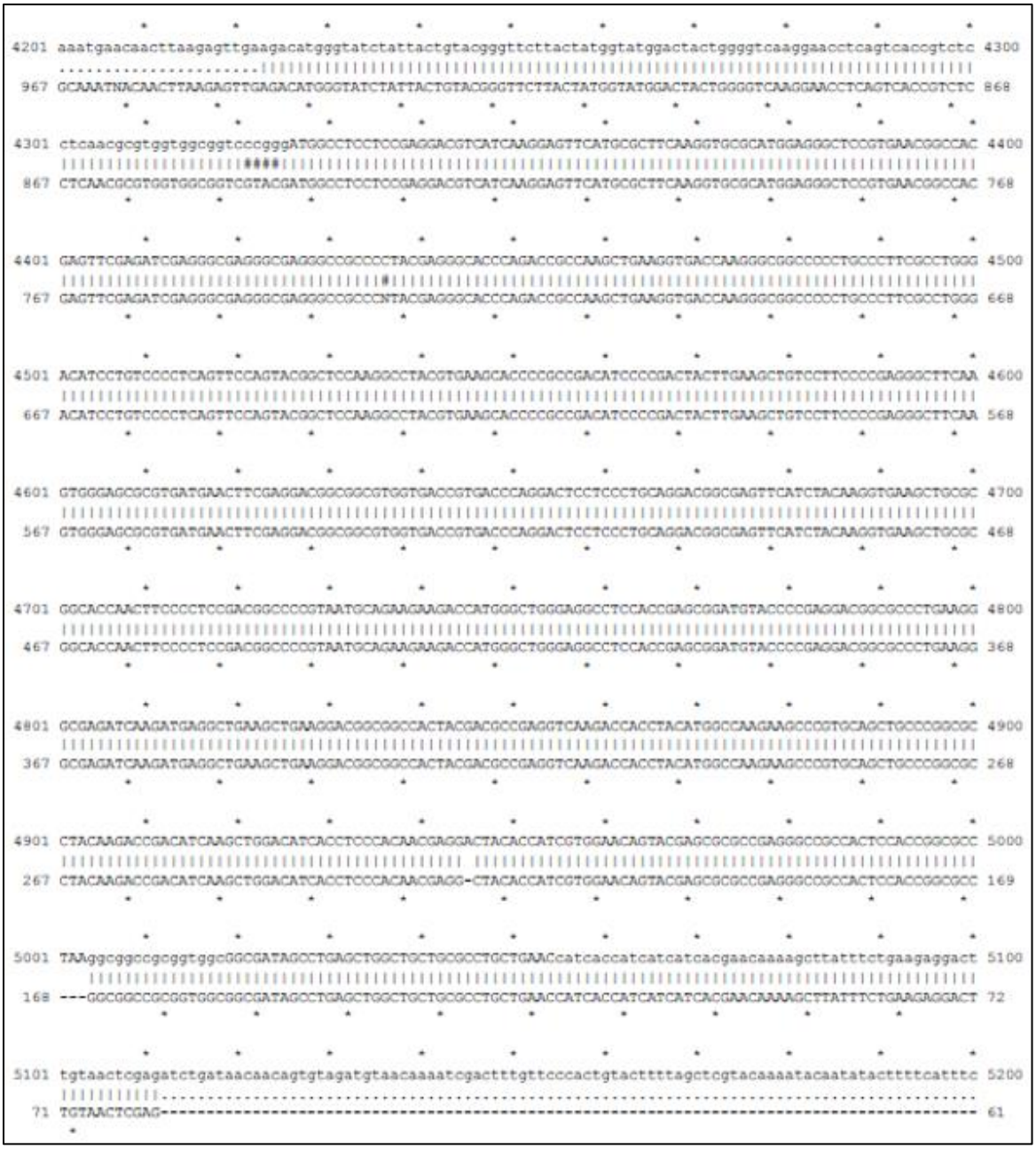


Figure 18. Align pGEM-4420-mRFP1-S6-His6-cmyc sequencing result using sp6 reverse primer to pRS-4420-mRFP1-S6-His6-cmyc. (from end of 4420 to cmyc)

Chapter 2: pGEM-4420-mRFP1/moxGFP-S6-His6-cmyc Protein Expression

2.1 Background

The main reason of ligating the fluorescent protein sequence to our designed sequence is because the fluorescent proteins will act as an indicator after the protein expression and it will help us understand the cellular pathways and the protein-protein interaction within the cells. Our designed sequence has included different tag systems for further applications. The short peptide tag, S6 tag, is a substrate which can be catalyzed by the Sfp PPTases enables site-specific protein labeling [(9)]. The His6 tags are used for protein purification and the cmyc tag is used for affinity chromatography, the Western Blot. These different tags help us localize where our proteins are and therefore, they enable us to have a better understanding of the protein-protein interaction.

The mesoporous silica nanoparticle is being widely research as a drug delivery material for cancer therapy and other treatments because of its advantages such as biocompatibility, biodegradability and non-toxicity etc. The surface properties of the mesoporous silica nanoparticles can be complicated and hard to understand once after the PEGylation process [(10)]. In order to integrate our samples with the mesoporous silica nanoparticle to achieve the goal for drug delivery, knowing more about the protein-protein interaction beforehand is especially important and necessary.

2.2 Materials and Methods

2.2.1 Chemical Reagents and Kits

See appendix for all reagents, materials and kits

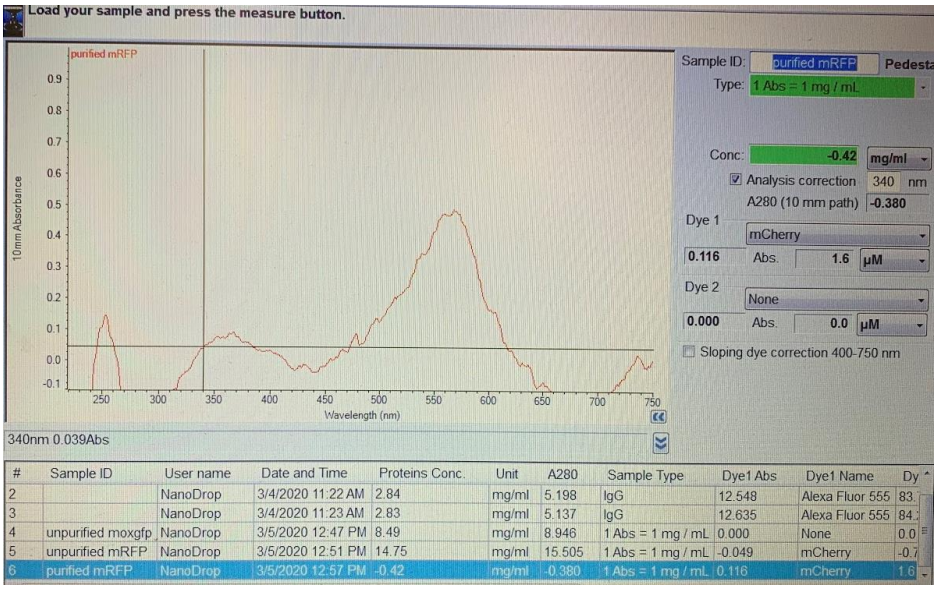
2.2.2 Strains and Media

Escherichia coli strain BL21(DE3) cells are from New England Biolab. Luria-Bertani medium (LB broth) is used to culture BL21(DE3) cells. Isopropyl β-d-1-thiogalactopyranoside (IPTG) is used as an induce agent for protein induction.

2.2.3 Protein Expression, Purification and Characterization

After the transformed BL21(DE3) cells grow on the LB-Amp plates, a single colony is picked by using a pipette tip or inoculator for 10 mL liquid culture in LB-Amp media. Incubate the cell mixture in an ambient incubator (37 degree Celsius with 200 rpm shaking). Monitor the OD600 value of the liquid culture cell mixture until the OD600 value reaches 0.4-0.8 (normally it takes around 3 to 5 hours). Spin the cells down with 4000g for 5 mins and resuspend the cells with LB-Amp media with 0.1 mM IPTG for protein induction (induce for 3-5 hours, potentially 24 hours in 37-degree Celsius ambient incubator with 200 rpm shaking). After the protein induction, spin the cells down then collect the supernatant and save the cell pellets.

As for the purification step, run the soluble and insoluble protein through the Amicon Ultra 15 mL centrifugal filters and collect the proteins (read Amicon Ultra centrifugal filter protocol). Run the filtered proteins through Ni-NTA columns from Qiagen to purify the His-tag proteins (read Ni-NTA column protocol). The purified proteins are then checked by NanoDrop, Cary eclipse fluorescence meter and verified by running a Coomassie stained protein gel. The result of our product is shown below (Figure 19 and 20). Since the Coomassie gel staining can only verify that there are proteins being expressed, Western blot is done to make sure that the expressed protein has the cmyc tag by using primary cmyc antibody. The western blot is shown below and the bands of the 4420-mRFP/moxGFP-S6-His6-cmyc is around 70 kDa which are shown in the figure below (Figure 21).



#	Sample ID	User name	Date and Time	Proteins Conc.	Unit	A280	Sample Type	Dye1 Abs	Dye1 Name	Dy
2		NanoDrop	3/4/2020 11:22 AM	2.84	mg/ml	5.198	IgG	12.548	Alexa Fluor 555	83.
3		NanoDrop	3/4/2020 11:23 AM	2.83	mg/ml	5.137	IgG	12.635	Alexa Fluor 555	84.
4	unpurified moxgfp	NanoDrop	3/5/2020 12:47 PM	8.49	mg/ml	8.946	1 Abs = 1 mg / mL	0.000	None	0.0
5	unpurified mRFP	NanoDrop	3/5/2020 12:51 PM	14.75	mg/ml	15.505	1 Abs = 1 mg / mL	-0.049	mCherry	-0.7
6	purified mRFP	NanoDrop	3/5/2020 12:57 PM	-0.42	mg/ml	-0.380	1 Abs = 1 mg / mL	0.116	mCherry	1.6

Figure 19. NanoDrop results for 4420-mRFP1-S6-His6-cmyc protein. According to literature [(11)], the absorption peak of mRFP1 is around 550 nm and our sample has a peak around 550 nm which shows that it is highly possible that we successfully have protein expressed with mRFP1. Based on Beer's Law and the mRFP molar absorptivity extinction coefficient of 44,000 mol^{-1} cm^{-1}, the concentration of the mRFP contained protein is around 0.11 mM.

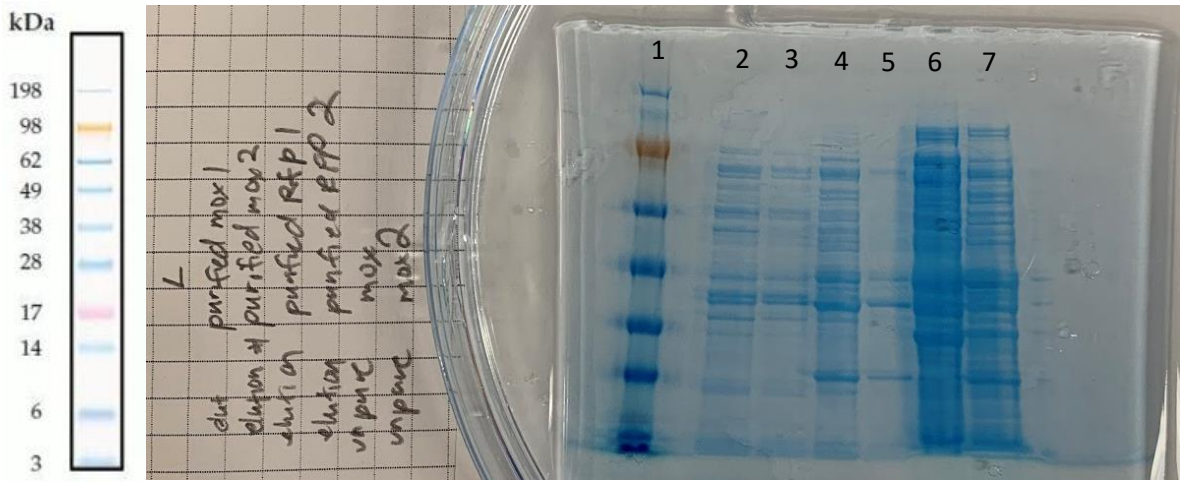


Figure 20. Coomassie staining for purified protein.

Columns from left to right: 1. See Blue Ladder. 2 and 3. Purified 4420-moxGFP-S6-His6-cmyc from Ni-NTA column. 4 and 5. Purified 4420-mRFP-S6-His6-cmyc from Ni-NTA column. 6 and 7. Unpurified 4420-moxGFP-S6-His6-cmyc from Ni-NTA column. From the Coomassie staining result above, it shows that there are a lot of different sizes of protein in our samples. To know find out which proteins are c-myc positive, running a western blot with a primary anti-cmyc antibody is necessary therefore we can be sure which band is the protein of interest.

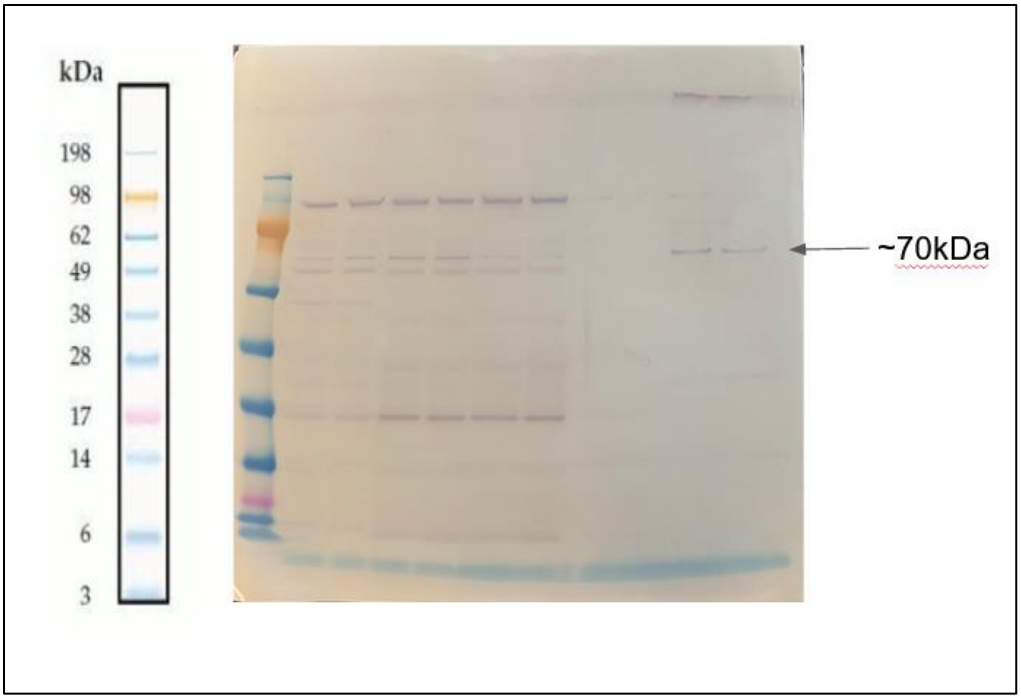


Figure 21. Western Blot for purified protein 4420-mRFP-S6-His6-cmyc. The bands shown on the blot is the c-myc specific protein. The protein size of 4-4-20 is ~30kDa, mRFP is ~25kDa and the S6-His6-cmyc tags are around 10kDa in total. To sum up, the total size of our protein of interest is around 70kDa.

Chapter 3: Construction of recombinant protein with eGFP and mRFP1 in pRS314 vector

3.1 Background

Since protein misfolding issue and proteins stuck at the insoluble fraction of E. coli while expressing. Yeast expression system is an alternative way to express protein since it is known to avoid protein misfolding problem. To test the yeast protein expression system, moxGFP or mRFP1 is also replaced with eGFP. We chose eGFP not moxGFP is because to have an optimize protein expression of moxGFP in the yeast expression system, another step which is the codon optimization need to be done with the moxGFP sequence. It would be more straightforward to test the plasmid with eGFP than doing an additional step to manipulate the moxGFP sequence since we are adding an uncertain factor to our plasmid. Codon optimized moxGFP will be cloned into the plasmids once the yeast expression system is a better expression system compared to E. coli expression system.

3.2 Materials and Methods

3.2.1 Reagents, Materials and Kits

See appendix for all reagents, materials and kits

3.2.2 Strains and Media

5-alpha competent E. coli cells are from New England Biolab. This strain is used for recombinant plasmids gene cloning. Luria-Bertani medium is used to culture the 5-alpha cells.

3.3.3 Plasmid Design: Digestion, Ligation and Transformation

The design concept is the same as previous chapter. In our previous work, pRS-4420-mCherry-S6-His6-cmyc has been constructed. To swap the fluorescent protein mCherry to mRFP or eGFP, a plasmid double digestion is needed using the restriction enzymes, Mlu1 and Not,1 as mentioned above in the previous chapter. mRFP and eGFP is also being amplified by using forward primer with Mlu1 restriction site and reverse primer with Not1 site then the double digestion using Mlu1 and Not1

restriction enzymes are done after PCR. Ligate both double digested plasmid and inserted then transform to NEB 5-alpha cells (Figure 22). (See all protocols and methods in appendix, same method as previous chapter) The sequencing results are shown below in figure 23 and 24.

Figure 22. pRS-4420-mRFP tags transformed to 5-alpha cells.

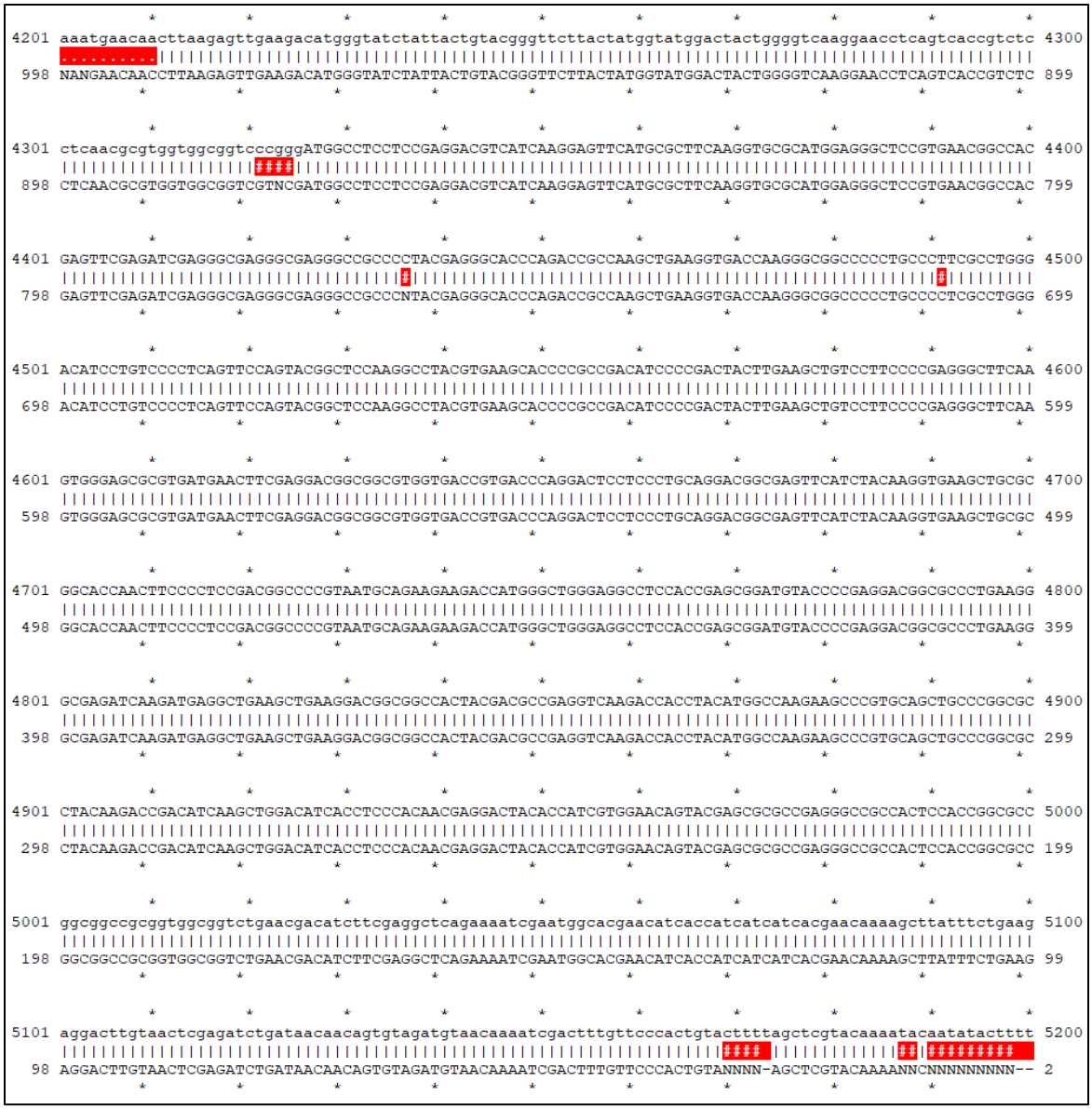


Figure 23. Align our sample (pRS-4420-mRFP-tags) to expected pRS-4420-mRFP-tags. Sequences aligned to each other.

Figure 24. Align our sample (pRS-4420-eGFP-tags) to expected pRS-4420-eGFP-tags. Sequences aligned to each other.

Chapter 4: pRS-4420-mRFP1/eGFP-S6-His6-cmyc protein expression test

4.1 Background

Though E. coli expression may have a higher yield in protein expression, there are also some disadvantages of E. coli expression system. From the result shown in the previous chapters, the size of the 4420-mRFP/moxGFP-S6-His6-cmyc has been verified via Coomassie gel staining and western blot but the cell pellets and the soluble fraction of the protein did not show fluorescence or shown only a very small amount of fluorescent through NanoDrop. Based on previous experience and literatures, the E. coli expression system will often encounter protein misfolding problems after the expression which will need an extra step for the proteins to refold into the correct structures. Also, another common problem for the E. coli expression system is that the expressed protein could be trapped inside the insoluble fraction which can be extracted carefully using lysis buffers.

To minimize the possibility of encountering the potential problems listed above, yeast protein expression system is a good alternative since the expressed protein will only be soluble and can easily been collected from the supernatant after expression. In this chapter, we will examine if the soluble fraction we get from yeast expression system has a higher amount of fluorescent via measuring from NanoDrop and Cary Eclipse Fluorimeter.

4.2 Material and Methods

4.2.1 Reagents, Materials and Kits

See appendix for all reagents, materials and kits

4.2.2 Strains and Media

The BJ5464 yeast cells are from New England Biolab. For soluble protein expression, SDCAA+Uracil media is used for yeast culture and SGCAA+Uracil+0.1% BSA is used for protein expression. See appendix for media recipe.

4.2.3 Protein Expression, Purification and Characterization

After getting the positive results from sequencing, the designed plasmids need to be transformed into yeast cells via the lithium acetate method for the yeast protein expression system. According to Gietz RD and RA Woods transformation protocol, the salmon sperm single stranded DNA needed to be boiled in water bath for 5 mins, vortexed then added to the transformation mix which includes PEG 3500, lithium acetate, plasmid DNA. The transformation mix is then added to the BJ5464 yeast cells and incubated in 42-degree Celsius water bath for 1 hour. The transformed cells are streaked on the SDCAA + uracil plate and grew for 3 days.

A monocolony is picked from the cultured plate and cultured in the SDCAA + uracil media for another day for protein expression. The yeast cells will be centrifuged down then resuspended in SGCAA + uracil + BSA and will be ready for western blot after a 3-day culture in 30-degree Celsius incubator.

The western blot result for pRS-4420-mRFP-S6-His6-cmyc and pRS-4420-eGFP-S6-His6-cmyc is shown below. (Figure 25.)

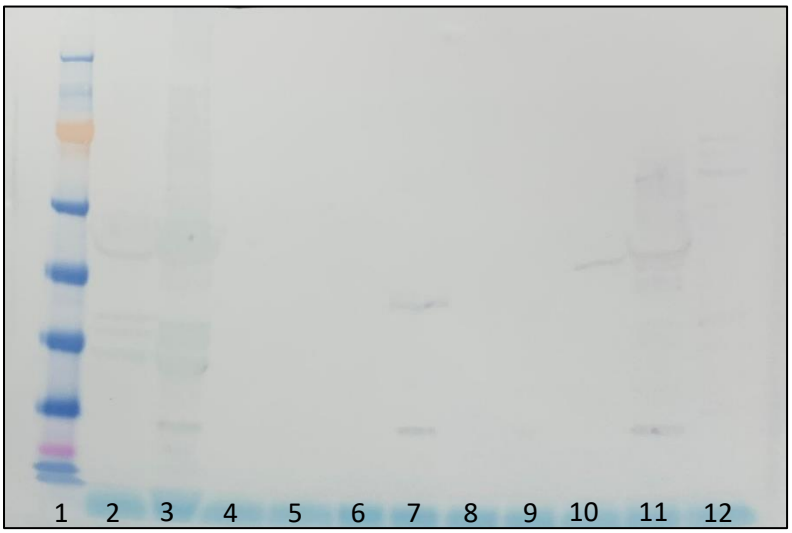


Figure 25. The western blot result for pRS-4420-mRFP-S6-His6-cmyc and pRS-4420-eGFP-S6-His6-cmyc. Columns: 1. See blue plus ladder 2. pRS-4420-eGFP-S6-His6-cmyc yeast cells suspension. 3. pRS-4420-mRFP-S6-His6-cmyc suspension. 4. 4420-eGFP-S6-His6-cmyc running through Amicon filter. 5. 4420-mRFP-S6-His6-cmyc running through Amicon filter. 6. Protein fraction of 4420-eGFP-S6-His6-cmyc running through Amicon filter. 7. Protein fraction of 4420-mRFP-S6-His6-cmyc running through Amicon filter. 8. 4420-eGFP-S6-His6-cmyc first time running through Ni-NTA column. 9. 4420-mRFP-S6-His6-cmyc first time running through Ni-NTA column. 10. His6-tag Purified 4420-eGFP-S6-His6-cmyc protein. 11. His6-tag Purified 4420-mRFP-S6-His6-cmyc protein. 12. E. coli expression of 4420-moxGFP-S6-His6-cmyc as positive control.

The 4420-mRFP-S6-His6-cmyc protein after purification band (column 11 in figure 25) shows that the size of the protein is around 70kDa which also matches the size of the protein using E. coli expression system. Though both E. coli and yeast expression system show the correct band of the protein of interest, yeast expression system should be better than the E. coli expression system since there are no concerns about protein misfolding and proteins get stuck in the insoluble fraction. To sum up, the yeast expression system will be used in the enzymatic tests in the future.

Chapter 5: E. coli and Yeast Expression System Protein Yield Testing with Plasmids contain Enzymatic Tags

5.1 Background

Researchers in molecular biology field have been focusing on developing methods for chemo-selective ligation and site-specific labeling. The discovery will help understanding the cellular pathway and molecular mechanisms. Fluorescent proteins which fused to targeting therapeutic agents can act as a good marker to help localize of the binding of therapeutic agent and cells.

One of the most popular enzymatic tests is the Sortase A ligation test. Sortase A, also known as surface protein sorting A, is a transpeptidase which is crucial in cell wall biosynthesis and protein attachment to cell walls and. A calcium dependent Sortase A is used to examine the site-specific binding in living cells. Sortase A catalyzes the cleavage of the LPXTG (Leu-Pro-XXX-Thr-Gly, where XXX is any amino acid) motif between the threonine and glycine and release an epitope tag as a result [(12)]. The LPETG motif is usually designed to append to fluorophores, biotins, lipid etc. In our experiment, the motif is appended to fluorophores which can act as a marker after the sortase tagging (Figure 26).

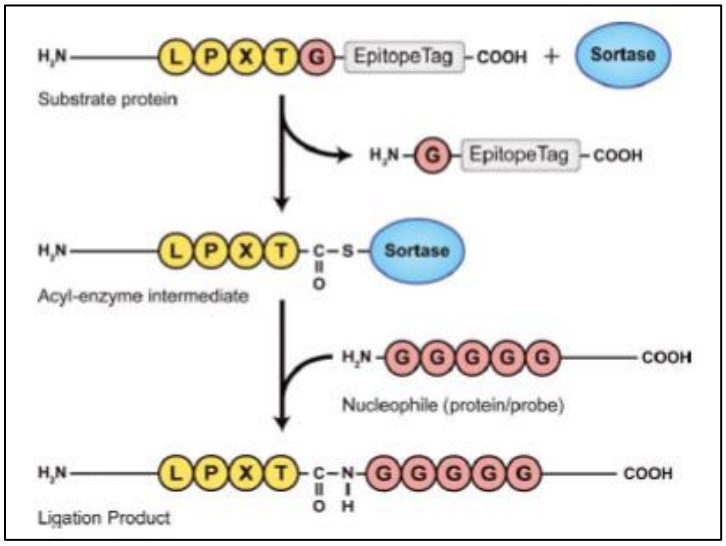


Figure 26 [(12)]. Schematic representation of sortase-mediated protein ligation. A substrate protein containing an LPXTG motif near the C-terminus followed by an epitope tag is cleaved between the threonine and the glycine by sortase, thereby releasing the epitope tag (top). The formed acylenzyme intermediate can be resolved by an (oligo) glycine-based nucleophile (middle), resulting in the release of sortase and the formation of a substrate-nucleophile ligation product (bottom).

Sortase A ligation method has been widely used in different areas since it can provide a good selectivity for N-terminal labeling with a high yield and purity of the labeled protein product. The sortase A ligation method can also be used in the field of protein-drug conjugation, therapeutic nanoparticle design and so on [11].

The sequencing result of our plasmid contains LPETG tag is shown below (Figure 27). In this chapter, we will find out whether enzymatic tags will affect the protein yield. In the future, cmyc staining and sortase A catalyzation can be tested by flow cytometry once we are sure that the protein yield of the plasmid is not being affected by the fusion of enzymatic tags. We will use GGG-FL (GK (FITC)-4) {sequence GGGK (FITC)} as the nucleophile for LPETG recombinant peptide. Fluorophore FITC is attached at the C terminus of GGGK, the N terminus us glycine free to conjugate with C terminus of the acyl-enzyme intermediate.

Researches have been done on lipoic acid and the results show that the use of lipoic acid for therapeutic purposes is possible [13]. Therefore, the LAP2 tag will also be fused into the protein to check if this enzymatic tag will affect the protein yield. The LAP2/lipoic acid interaction will allow us to gain more insights of this specific enzyme activity and hopefully can be used as a therapeutic agent in the future.

5.2 E. coli Expresion Result

Same digestion and ligation methods are used in this chapter. After transforming the pGEM-4420-mRFP-LPETG-His6-cmyc plasmid to BL21(DE3) cells, the E. coli cells are induced with 0.1 mM IPTG, 20 degree Celsius and expressed for 24 hours. The nanodrop (Figure 27) and Western blot (Figure 28) results are shown below. The nanodrop results is promising since there is a small absorption peak around 550 nm which means that there should be mRFP protein. Based on the Beer's Law, the

concentration of the protein of interest shown in the nanodrop result is around 30 uM. The Western blot result show the right size band around 65kDa which matches the size of the protein of interest.

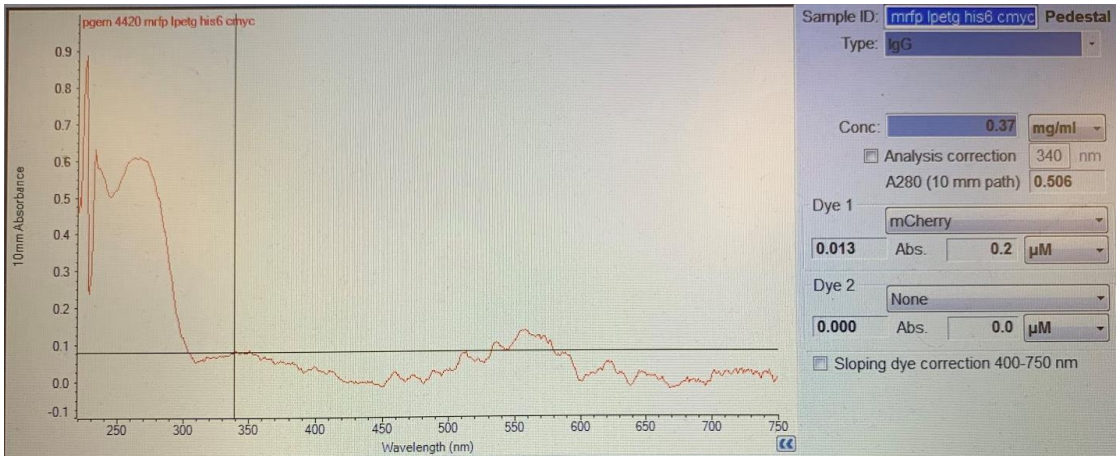


Figure 27. Nanodrop result for 4420-mRFP-LPETG-His6-cmyc protein. The result shows that there is a small absorption peak around 550 nm which shows the existence of mRFP protein. Based on the Beer's Law, the protein of interest has a concentration around 30 uM after calculation.

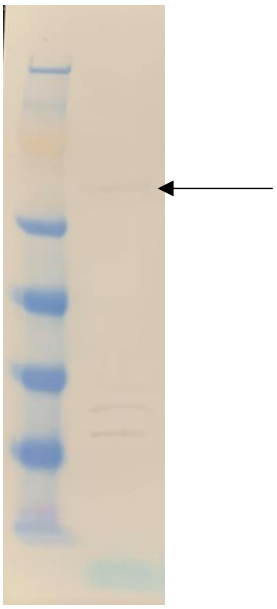


Figure 28. Western blot result for 4420-mRFP-LPETG-His6-cmyc protein. Ladder: Prestained See Blue Ladder Column 1. 4420-mRFP-LPETG-His6-cmyc protein after purification and elution through Ni-NTA column. The bands at the first column is around 65 kDa which is the right size of the protein of interest

The LAP2 tag is inserted to the construct using the same method mentioned in previous chapter. The full sequence pGEM-4420-mRFP-LAP2 sequence are transformed to BL21(DE3) cells. The transformed BL21(DE3) cells are then expressed in 20 degree Celsius with 0.1 mM IPTG induction. The nanodrop (Figure 29) and Coomassie staining (Figure 30) result are shown below. The protein of interest has a concentration of 50uM.

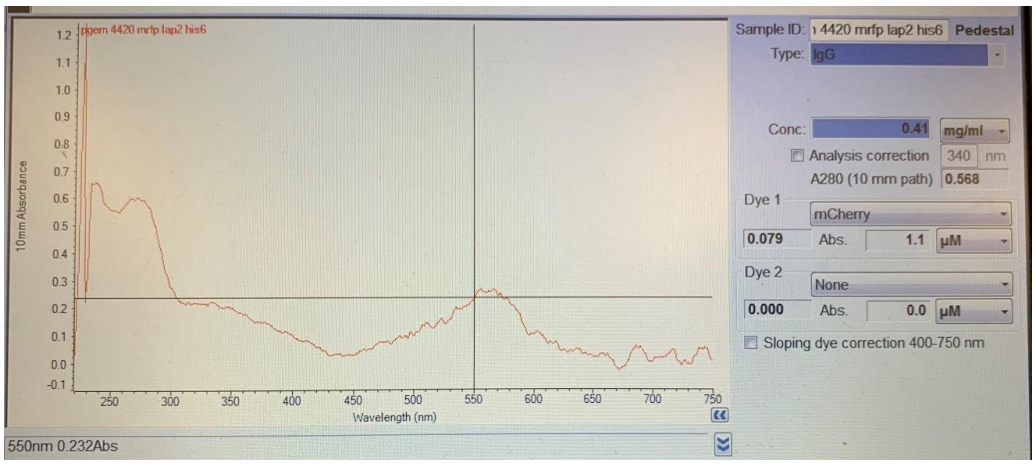


Figure 29. Nanodrop result for 4420-mRFP-LAP2-His6 protein. The result shows that there is a small absorption peak around 550 nm which shows the existence of mRFP protein. Based on the Beer's Law, the protein of interest has a concentration around 50 uM after calculation.

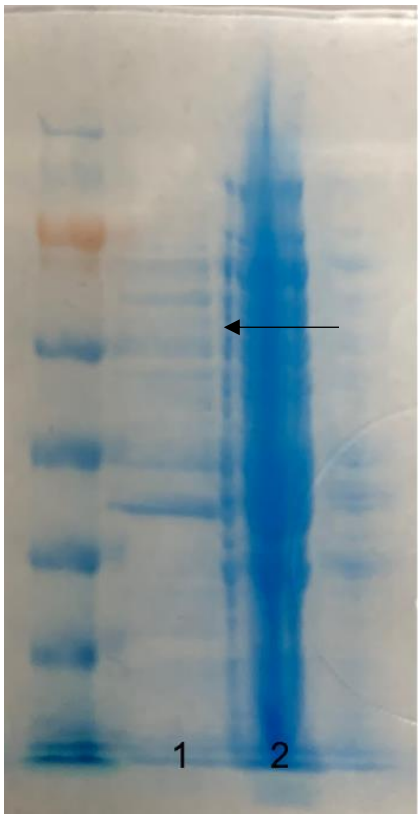


Figure 30. Coomassie gel staining result for 4420-mRFP-LAP2-His6 protein. Ladder: Prestained See Blue Ladder Column 1. 4420-mRFP-LAP2-His6 protein after purification and elution through Ni-NTA column. 2. 4420-mRFP-LAP2-His6 with BL21(DE3) cells. Western blot is not suitable for this protein since this protein does not have a cmyc tag. Though Coomassie gel staining cannot tag the specific protein, based on the size shown above (the arrow: 65kDa), The result is still promising. Note: cmyc tag is around 1.2 kDa.

5.2 Yeast Expresion Result

As for the yeast expression system, the plasmids with enzymatic tags has been successfully cloned into the pRS314 vector with the method shown in previous chapters. The Western Blot and Coomassie gel staining Result are shown below in figure 31 and 32. In this part, three different plasmids with enzymatic tag are expressed: pRS-4420-LPETG-His6-cmyc, pRS-4420-mRFP-LPETG-His6 and pRS-4420-mRFP-LAP2-His6. The first plasmid has a cymc tag which we can verify the protein expression with Western blot. The other two will be verified by Coomassie gel staining.

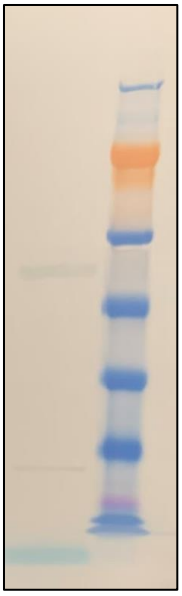


Figure 31. Western blot result for 4420-LPETG-His6-cmyc protein. The 4420-mRFP-LPETG-His6-cmyc protein is around 65 kDa. Since our expressed protein has no mRFP protein and the size of the mRFP protein is around 25 kDa. The estimated expressed protein size is around 40 kDa. The band on the blot result is higher than what we expected therefore we will have to reverify it again,

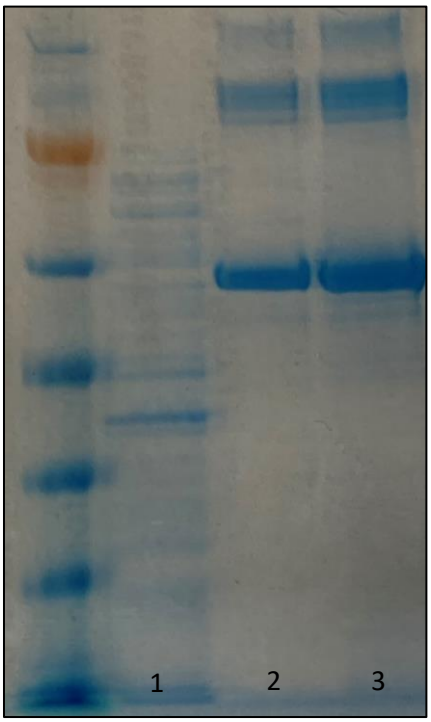


Figure 32. Coomassie gel staining result for 4420-mRFP-LAP2-His6 and 4420-mRFP-LPETG-His6 proteins. Ladder: SeeBlue prestained ladder. Column1: E. coli expression of 4420-mRFP-LAP2-His6 protein. Column 2: 4420-mRFP-LAP2-His6 after purification and elution. Column 3: 4420-mRFP-LPETG-His6 after purification and elution. According to Expasy translation, both proteins should be around 65kDa which matches the results in the blot.

Conclusion and Future Direction

Based on the results shown above, both E. coli and yeast expression system has expressed the protein successfully based on the size of our protein of interest. The enzymatic tags: S6, LPETG and LAP2 will also not affect the protein yield. According to the last Coomassie staining gel results (figure 32), we found out that the proteins expressed by yeast are cleaner after purification and elution through the Ni-NTA column. Also, since yeast expression system will not encounter the protein misfolding problem after the proteins have been expressed. We are drawing a conclusion that yeast expression system will be a better choice for the future experiments.

All plasmids (pRS and pGEM backbone with mRFP and moxGFP, and enzymatic tags LAP2 and LPETG) have been cloned successfully and been verified by sending out for sequencing and the plasmids are transformed into cells (pRS vector to BJ5464 and pGEM vector to BL21 cells. For further experiments, enzymatic tests such as Sortase A, Sfp synthase and lipoic acid reaction needs to be done therefore the result can help with the construction of the protein and nanoparticle adhesion system. The conjugation can be tested with flow cytometry.

As for long-term research, fusing multiple fluorescent proteins into one plasmid can be a direction for research. Also, scaling up protein production for nanoparticle conjugation and perform a flow chamber kinetic measurement. Once we have all data from fluorescent protein testing, enzymatic test, different combinations of linkers and fluorescent protein, a library can be created and easily provide insights and data for targeting therapeutic nanoparticles.

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Appendix 1: Gene Cloning and Propagation

D.1 PCR (Optimized for Vent Polymerase)

1. Prepare Master Mix- (Note: prepare on ice from highest volumes first)

40 ul	Ultrapure Water
5 ul	Thermopol buffer
1 ul	dNTPs
1 ul	FWD Primer (100 uM Concentration)
1 ul	REV Primer (100 uM Concentration)
1 ul	Plasmid DNA
1 ul	Vent Polymerase

---

50 ul total

2. Thermocycling:

a. 94°C	2 min (denature)
b. 94°C	30 sec
c. 60°C	30 sec (anneal- note: each insert has an optimal anneal temp)
d. 72°C	45 sec (polymerization)
e. 72°C	2 min
f. 4°C	Infinite time (Hold)

Repeat steps b-d 30x (PCR time should be approximately 1 hour 30 min)

D.2 Electrophoresis and Gel Extraction

Electrophoresis

1. Prepare Gel (0.5 grams agar gel powder/ 50 mL 1x TAE buffer)
   Note: Melt agarose in microwave
   Pour gel into gel mold after addition of the comb, 2.5 uL of 10 mg/ml Ethidium Bromide
2. Add purple loading dye to the samples
   Ladder prep = 8 ul ultrapure DI water, 1 ul Gel loading dye, Purple (6x) SDS Dye, 1 ul DNA Ladder
   Sample (including Backbone) to +SDS Dye = 3 : 1 (ie 15 ul to 5 ul)
   -example: (For samples of 50 ul, add 17 ul of SDS Dye)

Note: 8-well comb can hold up to 25 uL of sample, 10-well comb can hold up to 15 uL of sample

3. Load ladder, samples and negative control (backbone) to the gel
4. Run gel at 105 Volts for 50 mins

Gel Extraction

While gel is running-

5. Weigh and label empty vials to calculate mass of cut out bands

After gel is done running-
6. Cut out bands with a clean razor blade and place samples into the labelled microcentrifuge tubes to weigh
7. Extract the bands using the Qiagen Gel Extraction Kit per instructions.
-Note: Make sure gel is completely dissolved during step 3 incubation with QG Buffer!

D.3 Plasmid Digestion

1. Prepare Master Mix- (Note: prepare on ice from highest volumes first)

1000ng DNA	Insert from Gel Extraction
5 ul	Digestion Buffer
Up to 50 ul	Ultrapure water
2 ul each	Enzyme(s)

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50 ul total

2. Incubate at 37°C for 30 min - 1 hour (Note: Each enzyme has its own optimal conditions. Pay attention to the temperature. Ie. Digest DNA with Xbal and BsiWI. BsiWI requires 55°C Set the temperature at 37°C for 1 Hour with XbaI and then add BsiW1 and then increase it to 55°C for another 1 hour. MUST BE IN WATER BATH INCUBATOR. Also Select correct buffer ie 2.1, 3.1 or cutsmart)

3. Purify vector by electrophoresis (refer to D.2). (Note: Run the gel for at least 1 hour. Recommended: 115V)
Inserts can then be purified using the gel extraction protocol via the Qiagen Gel Extraction Kit.

D.3 Ligation

1. Prepare Master Mix- (Note: prepare on ice from highest volumes first)

9 ul	Ultrapure water
4 ul	Ligase Buffer
5 ul total	3:1 molar ratio of insert to vector
1ul	T4 DNA ligase

20 ul total
Note: Room temperature for 2 hours or 16°C overnight

D.4 Transformation into E. coli (based on DH5α from Invitrogen)
Prep: Pre warm LB Broth (see media recipes below)
1. Place 50 ul DH5α cells on ice for 10 min until thawed
2. Add 1-5 ul containing 1 pg - 100 ng of plasmid DNA to the cell mixture
a. Miniprep (conc 400+): 1-2 ul
b. Ligation: 5 ul
Carefully flick the tube 4-5 times to mix **DO NOT VORTEX**
3. Heat shock at 42°C for exactly 30 sec, and immediately transfer back to ice for 5 min
4. Add 500 ul prewarmed LB broth media

5. Incubate on shaker at 250 rpm at 37°C for 60 minutes
Prep: Warm LB Plates to 37°C 10 minutes before next step-
6. Spread each dilution onto selection plate and incubate overnight at 37°C

D.5 Colony PCR
1. Scrape a single colony using a pipet tip or inoculation loop and dip into 50 ul ultrapure water
2. Perform a typical PCR except using half the volumes listed above-

20 ul	Ultrapure water
5 ul	Cell suspension (previous step)
2.5 ul	Thermopol Buffer
0.5 ul	dNTP
0.5 ul	FWD Primer
0.5 ul	Backbone Primer
0.5 ul	Vent Polymerase

29.5 ul total

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3. Check for desired product on a 1% agarose gel
4. Positive samples can be plated (5 ul) and grown up (remaining 40 ul) in 3 mL LB- Amp for later use and sequencing, respectively

Media recipes

LB Broth
Base media (for 1L): 25 g LB powder, filter sterilize or autoclave
Supplement: Ampicillin or other antimicrobial agents, typically 50 ug/ ml

LB plates
40 g LB agar powder/ L, autoclave
Supplements: Add ampicillin or other antimicrobial agents (typically at 50 ug. ml) once the media has cooled to 50°C

Item	Company	Catalog Number
LB Broth	Difco	244620
LB Agar	Difco	244520
Ampicillin	Fisher Bioreagents	11593-027
SOC Media		
DNA LoBind Tubes, DNA LoBind, 1.5 mL, PCR clean	eppendorf	22431021
NEB 5-alpha Competent E. Coli (High Efficiency)	New England Biolabs	C2987H
100 bp DNA Ladder	New England Biolabs	N3231S
Gel Loading Dye, Purple (6x), no SDS	New England Biolabs	B7025S
QIAquick Gel Extraction Kit	QIAGEN	28704
Vent DNA Polymerase	New England Biolabs	M0254S
ZipTip with 0.6 uL C18 resin	EMD Millipore	ZTC18S008
MluI	New England Biolabs	R0198S
NotI	New England Biolabs	R0189S
BsiWI	New England Biolabs	R0553S

Appendix 2: Yeast Culture and Recombinant Protein Production

Prepare: YPD/ SD-CAA Plates

YPD, SDCAA, SGCAA, SDCAA + URA, SGCAA + URA + 0.1% BSA media
See Media Recipes

D.1 Growth

1. Streak a single colony on YPD and SD-CAA plates and incubate at 30°C for 2 days.
2. Inculate 3 mL liquid culture with a single colony in YPD overnight or 1.5 days in SDCAA at 30°C while shaking at 200-225 RPM.

D.2 Transformation (Lithium Acetate method from Gietz RD and RA Woods, Method in Enzymology, 2002)

1. Inoculate 3 mL YPD with BJ5464 (transformation with pRS plasmid) or EBY100 (pCT plasmid) and grow overnight

D.3 Transformation Cont.

2. Heat single stranded carrier DNA (2mg/ml) in a boiling water bath for 5 min and then place on ice.
3. Spin yeast at 3000 RPM for 1 min, resuspend in 1 mL ultrapure water and transfer to a microcentrifuge tube. Spin again and discard the supernatant.
4. 4. Layer the following on top of the pellet: 240 uL PEG 3500 (50% w/v), 36 uL LiAc (1.0 M), 50 uL carrier DNA (after vortexing) and 34 uL plasmid DNA (0.1 to 1 ug mixed in ultrapure water, typically use 2 uL). Vortex to mix and resuspend cells (pipet if necessary).
5. Incubate at 42°C for 1 hr before centrifugation and aspiration of the transformation mix. Add 1 mL ultrapure water, resuspend and plate 100 ul on selective media (SD-CAA) + URA.

D.4 Surface Display of Recombinant Protein

1. Inoculate 3 mL of SD-CAA with EBY 100 yeast transformed with a pCT-based plasmid. Grow Overnight at 30°C while shaking at 225 RPM.

D.5 Surface Display of Recombinant Protein cont.

2. Measure concentration based on absorbance at 600 nm, which is typically 4-6 absorbance units for an overnight culture. 1 absorbance unit is approximately 10^7 cells/ mL.
3. Trasnfer 3x10^7 cells to a microcentrifuge tube, centrifuge, resupsend in 1 mL SG-CAA media, spin, and resuspend in 3mL SG-CAA.
4. Grow for 16 hours at 20°C while shaking at 225 RPM.

D.6 Soluble Expression of Recombinant Protein (small scale assessment)

1. Inoculate 3 mL of SD-CAA + Ura with BJ54464 Yeast transformed with a pRS-based plasmid. Grow 24 hours at 30°C while shaking at 225 RPM.
2. Centrifuge cells, resuspend in 1 mL SG-CAA media, spin, and resuspend in 3 mL SG-CAA + Ura + 0.1% BSA.
3. Grow for 3 days at 20°C (or 2 days at 30°C) while shaking at 225 RPM.
4. Recover supernatant by centrifuging at 3000xg for 10 min.

Media Recipes

YPD Plates (1L)

1. 10g Yeast Extract, 20g peptone, 20g agar, dissolve in 900 mL Ultrapure Water *
2. 20 g dextrose in 100 mL Ultrapure Water **

Let autoclaved solution cool and combine, pour into plates

SDCAA Plates (1L)

1. 5.4g Na2HPO4 Dibasic, 8.56g Na2HPO4 monobasic, 182g sorbitol, 15g Agar in 900 mL Ultrapure Water*
2. 20g dextrose, 6.7g yeast N2 base w/o Amino Acids, 5g Casamino Acid **

Let autoclaved solution cool and combine, pour into plates

YPD Media (1L)

1. 10g yeast extract, 20g peptone, 20g dextrose **

SDCAA Media (1L)

1. 20g dextrose, 6.7g Yeast N2 Base w/o Amino Acids, 5 g Casamino Acids, 5.4 g Na2HPO4 dibasic, 7.46g NaH2PO4 monobasic, add Ultrapure Water up to 1L **

SDCAA + URA Media (1L)

1. 20g dextrose, 6.7g Yeast N2 Base w/o Amino Acids, 5 g Casamino Acids, 5.4 g Na2HPO4 dibasic, 7.46g NaH2PO4 monobasic, 20mg Uracil, add Ultrapure Water up to 1L **

SGCAA Media (1L)

1. 20g galactose, 6.7g Yeast N2 Base w/o Amino Acids, 5 g Casamino Acids, 5.4 g Na2HPO4 dibasic, 7.46g NaH2PO4 monobasic, add Ultrapure Water up to 1L **

SGCAA + URA + 0.1% BSA Media (1L)

1. 20g dextrose, 6.7g Yeast N2 Base w/o Amino Acids, 5 g Casamino Acids, 5.4 g Na2HPO4 dibasic, 7.46g NaH2PO4 monobasic, 20 mg Uracil, 1g BSA add Ultrapure Water up to 1L **

Autoclave - *
Filter Sterilize - **

Appendix 3: BL21 (DE3) Protein Expression

1. Transform expression plasmid into BL21. Plate on antibiotic selection plates and incubate overnight at 37°C.
2. Resuspend a single colony in liquid culture with antibiotic to produce a starter culture. Inoculate starter culture at a 1:100 dilution into expression media containing antibiotic.
3. Incubate at 37°C with shaking until OD_{600} reaches 0.4–0.8.
4. For most vector systems, induce with 40 or 400 μM IPTG and express protein for 3 hours at 37°C, 5 hours at 30°C or overnight at 16°C or 23°C. (optimized IPTG concentration and incubation temperature: final IPTG concentration 0.1 mM and 30 degree Celsius overnight)
5. For large scale, inoculate 1 Liter of liquid medium (with antibiotic) with a freshly grown colony or 10 ml of freshly grown culture. Incubate at 37°C until OD_{600} reaches 0.4–0.8. Add 40 or 400 μM IPTG and express protein using optimal time/temperature determined in a small-scale trial.

Appendix 4: Western Blot

Buffer Recipe:

MOPS Running Buffer: 50 mL of 20x MOPS stock + 950 mL of DI water

PBS: Dilute 50x PBS to 1x PBS using water

PBST: 1x PBS with 10% w/v Tween20

Sample Preparation:

Prep: Sample Buffer (SB), Reducing Agent (RA), Sterile water, Protein

Add 5uL of SB, 2uL of RA, 3 uL of water to 10 uL of protein. (Total Volume for each sample: 20 uL)

Western Blot Protocol (for NuPAGE MOPS)

1) Grab gel wells, Clamp, gel (Gel size depends – Typically 4-12%)

2) Slide gel frames into compartments by pulling the gray lever

3) Pour MOPS running buffer in the front of the gel

4) Get rid of bubbles using a syringe to flush out air

5) Spin denatured peroteins and put in water bath for 10 minutes

6) Grab antioxidant and seeblue plus 2 prestained (leave in ice bucket)

7) Load 20 uL sample from halfway down the wells pipetting slowly

8) load 10 ul ladder

9) add 500 uL antioxidant to each Blot compartment

10) Top off buffer SLOWLY, being careful not to disturb samples.

11) Put lid on and run at 130V until bands are visible.

Western Blot Transfer

Follow iBlot 2 blot transferring system instructions. The transfer process takes around 7 mins.

Adding Primary Antibody

1) Grab Plate and label with antibody/ protein

2) Take out the sandwich from transfer and open, removing the membrane from the gel

3) Place membrane side touching gel facing down

4) Quick PBST wash on gyrator 3x 5 min

5) Add 10 mL skim milk 60 mins

#see Milk Preparation Protocol

6) Wash with PBST 3x 5 min each

7) Add primary antibody

8) Incubate for 1 hr on gyrator in 4-degree Celsius cold room

Adding Secondary Antibody

1) Save primary antibody for future use (pour into a 50 mL tube)

2) Wash 3x 5 min in PBST on gyrator

3) Add secondary antibody (1:5000 PBST)

4) Leave on gyrator for 1 hr incubating in 4-degree Celsius cold room

5) Save antibody in 50 mL tube

6) Wash 3x 5 min PBST on gyrator

Ready for 1-Step TMB blotting

See appendix 4 for TMB blotting.

Appendix 5: 1-Step TMB-Blotting (Thermo Scientific)

34018 1-Step TMB-Blotting, 250mL
Storage: Upon receipt store product at 4°C. Product is shipped at ambient temperature.
Introduction
The Thermo Scientific 1-Step TMB-Blotting is a one component horseradish peroxidase (HRP) substrate for Western blotting and immunohistochemistry. TMB is highly sensitive and generates a blue-purple precipitate. This substrate is provided ready to use for maximum convenience.
Example Procedure for Western Blot Detection
This protocol is a general guideline for using 1-Step TMB-Blotting in a Western blot. Optimal conditions for each specific system must be determined empirically.
Note: Overdevelopment may cause the substrate precipitate to flake off the membrane. Color development rates vary depending on the amount of protein and detecting antibodies present and the blocking solution used. For best results, use Thermo Scientific SuperBlock Blocking Buffer − Blotting (Product No. 37517) and carefully monitor color development.
A. Materials Required
• Phosphate Buffered Saline with Tween®-20 Detergent (PBS-T): 0.1M sodium phosphate, 0.15M sodium chloride; pH 7.2 (Product No. 28372) with 0.05% Tween-20

Note: Use only high-quality Tween-20 such as Thermo Scientific Surfact-Amps Detergent Solution (Product No. 28320), which is a specially purified Tween-20 that is free of peroxides and carbonyls that may interfere in some systems.
• SuperBlock® Blocking Buffer − Blotting (Product No. 37517) containing 0.05% Tween-20

• Antigen-specific primary antibody diluted with Blocking Buffer. For best results, empirically determine the optimal dilution for each specific system.

• HRP-conjugated secondary antibody diluted with Blocking Buffer. For best results, empirically determine the optimal dilution for each system being tested.

B. Method

Note: Equilibrate the 1-Step TMB-Blotting to room temperature before use.
1. Remove blot from the transfer apparatus and block nonspecific sites with Blocking Buffer for 10-30 minutes at room temperature with shaking.
2. Add the primary antibody and incubate membrane for 1 hour with shaking.
3. Wash the membrane with PBS-T.
4. Add the HRP-conjugated secondary antibody and incubate membrane for 1 hour at room temperature with shaking.
5. Wash membrane with PBS-T.
6. Add the 1-Step TMB-Blotting to the membrane and carefully monitor color development. Stop the reaction by rinsing membrane with water.

Example Procedure for Immunohistochemical Staining

This protocol is a general guideline for using 1-Step TMB-Blotting in an immunohistochemical application. Optimal conditions for each specific system must be determined empirically.

A. Important Procedural Notes

• To minimize potential microbial contamination, carefully handle reagents and use ultrapure water in all solutions. Avoid touching slides and do not allow dust or other debris to contaminate samples, tissues or other material.

• Do not use sodium azide as a preservative for buffers as it inhibits HRP activity.

• Use a humidity chamber set at 20-25°C for all incubations to prevent evaporation. Additionally, completely cover the tissue section with solution during incubations to prevent drying.

• Adjust the standard protocol according to antigen concentrations. High antigen concentrations will require less incubation time to obtain optimal staining. When reducing incubation times, increase incubation temperature to 37°C.

• An ABC complex system, such as the Thermo Scientific ABC Standard Peroxidase Staining Kit (Product No. 32020) or Ultra-Sensitive ABC Standard Peroxidase Staining Kit (Product No. 32050), can increase sensitivity if necessary.

B. Materials Required

• Phosphate Buffered Saline (PBS): 0.1M sodium phosphate, 0.15M sodium chloride; pH 7.2 (Product No. 28372) containing 0.05% Tween-20

Note: Use only high-quality Tween-20 such as Surfact-Amps® 20 Detergent Solution (Product No. 28320), which is a specially purified Tween-20 that is free of peroxides and carbonyls that may interfere in some systems.

• Blocking Buffer: Thermo Scientific StartingBlock (PBS) Blocking Buffer (Product No. 37538) with 0.05% Tween-20 or StartingBlock™ T20 (PBS) Blocking Buffer (Product No. 37539), which is pre-formulated with Tween-20

• Antigen-specific primary antibody diluted with Blocking Buffer. For best results, empirically determine the optimal dilution for each specific tissue/antigen type being tested.

• HRP-conjugated secondary antibody diluted with Blocking Buffer. For best results, empirically determine the optimal dilution for each system being tested.

C. Method

7. Fix cryostat sections in acetone for 10 minutes and allow them to air-dry.

Note: Paraffin sections must be de-paraffinated with xylene and rehydrated with descending ethanol washes. If picric acid was used during fixation, incubate overnight in PBS followed by several PBS washes.

8. Quench endogenous peroxidase activity by incubating tissue for 30 minutes in 0.3% hydrogen peroxide in methanol. Omit this step if endogenous activity is not a problem or if the antigen will not survive exposure to H2O2.

Note: Use a humidity chamber set at 20-25°C for all incubations to prevent evaporation. Additionally, completely cover the tissue section with solution during incubations to prevent drying.

9. Wash tissue with PBS. Add Blocking Buffer and incubate for 30-60 minutes at room temperature.

10. Apply the primary antibody to the tissue and incubate for 30-90 minutes.

11. Rinse tissue three times for 10 minutes with PBS. Apply the HRP-labeled secondary antibody to the tissue and incubate for 30 minutes.

12. Equilibrate the 1-Step TMB-Blotting to room temperature. For best results, filter the substrate solution using a glass fiber filter paper immediately before use. Do not use hydrophobic membrane filters, which will retain the TMB substrate and reduce sensitivity.
13. Rinse slide three times for 10 minutes with PBS. Apply 1-Step TMB-Blotting solution to the tissue and incubate until significant color develops. To stop the reaction, wash section for 5 minutes with water.

Note: The precipitate is soluble in alcohol and organic solvents, therefore, use an aqueous counterstain