CLONING AND EXPRESSION OF SUPERFOLDER GREEN FLUORESCENT PROTEIN (*sfGFP*) AND ANTI-GFP NANOBODY (*NbGFP*) GENES IN YEAST

By

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A project report submitted to the Department of
Biological Science
Faculty of Science
Universiti Tunku Abdul Rahman
in partial fulfilment of the requirements for the
degree of
Bachelor of Science (Honours) Microbiology
April 2024

ABSTRACT

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Membrane protein-protein interactions play pivotal roles in cellular signalling, transport, and homeostasis. Deciphering the intricacies of cellular function and disease pathways requires thorough understandings of these interactions. The yeast two-hybrid system has been a popular choice of screening platform to examine the interactome in cells. It is a fast and convenient first-step analysis of interacting proteins. In this study, a variant of the yeast two-hybrid system, the split-ubiquitin membrane yeast two-hybrid system was used to examine protein-protein interactions involving anchored proteins on the membrane surface. The Gateway™ LR cloning technique was used to clone the target genes, which encode superfolder green fluorescent protein and anti-green fluorescent protein nanobody into yeast expression vectors pXN21-Dest and pMetYC-Dest, respectively. The expression clones were transformed and screened in *Escherichia coli* and verified by DNA sequencing. The recombinant plasmids were then co-transformed into yeast and analysed by yeast two-hybrid assay. Gateway™ LR cloning allowed successful transfer of genes of interest and was reflected in successfully transformed *E. coli* and yeast colonies when screened via colony PCR. The observed PCR product sizes were similar to

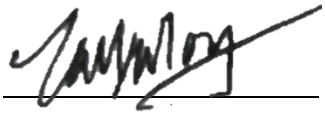
expected band sizes of 544 bp and 542 bp for pMetYC-NbGFP and pXN21-sfGFP, respectively. Productive yeast growth was observed on synthetic defined medium lacking leucine, tryptophan and histidine. The yeast cells were subjected to two-fold serial dilutions up to 7 times, but growth was still observed, which signifies the strong intensity of interaction. Hence, the screening platform is functional and may be used to detect interactions between other proteins of interest in the future.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor, Prof. Dr. Wong Hann Ling for his patience, trust and wisdom in guiding me in the completion of my final year project. I am grateful for the chance to take on this project. I would also like to extend my appreciation to the postgraduate students, Mr. Toh Wai Keat and Mr. Jordan Ooi Zhong Zeng for their guidance and encouragement throughout the project. I am indebted to them for their assistance. Last but not least, I would also like to take the chance to mention my family and close friends that have supported me during my time in completing the project.

DECLARATION

I hereby declare that this final year project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.



(TAN YU TONG)

APPROVAL SHEET

This final year project report entitled "**CLONING AND EXPRESSION OF SUPERFOLDER GREEN FLUORESCENT PROTEIN (*sfGFP*) AND ANTI-GFP NANOBODY (*NbGFP*) GENES IN YEAST**" was prepared by TAN YU TONG and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Honours) Microbiology at Universiti Tunku Abdul Rahman.

Approved by:

Date: 7 May 2024

(Professor Dr. Wong Hann Ling)

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Date: 6 APRIL 2024

PERMISSION SHEET

It is hereby certified that **TAN YU TONG** (ID No: **21ADB01776**) has completed this final year project report entitled "**CLONING AND EXPRESSION OF SUPERFOLDER GREEN FLUORESCENT PROTEIN (*sfGFP*) AND ANTI-GFP NANOBODY (*NbGFP*) GENES IN YEAST**" under the supervision of Prof. Dr. Wong Hann Ling from the Department of Biological Science, Faculty of Science.

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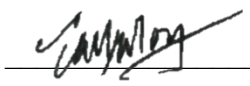

(TAN YU TONG)

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LIST OF ABBREVIATIONS

2× YT	2× Yeast-Tryptone
AD	activation domain
BD	binding domain
Cub	C-terminal of ubiquitin
g	gravitational force
GFP	green fluorescent protein
HA	hemagglutinin
HIS3	imidazoleglycerol-phosphate dehydratase
lacZ	beta-galactosidase
LEU2	beta-isopropylmalate dehydrogenase
NbGFP	anti-GFP nanobody
Nub*G*	N-terminal of ubiquitin with mutated glycine
POI	protein of interest
PPI	protein-protein interactions
SD	synthetic defined
sfGFP	superfolder GFP
TAE	Tris-acetic acid-EDTA
TF	transcription factor
TRP1	phosphoribosylanthranilate isomerase
UBPs	ubiquitin-specific protease
VP16	viral protein of herpes virus
Y2H	yeast two-hybrid
YPD	yeast peptone dextrose

CHAPTER 1

INTRODUCTION

1.1 Membrane Protein-protein Interaction Screening System

Protein-protein interactions (PPI) form the basis of cellular processes as they regulate biological functions such as signal transduction, gene regulation, and metabolic pathways (Lalonde et al., 2010). The complexities of cellular functions and disease mechanisms can be elucidated by understanding the intricate network of interactions between proteins. Initially, the yeast two-hybrid was developed by Fields and Song in 1989. Their innovation remains powerful in the detection and characterization of *in vivo* PPIs due to its time and cost efficiency, as well as uncomplicated identification methods (Paiano et al., 2018). The assay utilizes a 'bait and prey' model where interactions between two proteins would bring about the reassembly of the transcription factor (TF) by reassembling the DNA-binding domain (BD) with the activation domain (AD) that is attached to the interacting proteins, respectively. The functional restoration of the TF will be picked up by reporter genes, eventually resulting in gene expression (Paiano et al., 2018). As most Y2H systems are performed intracellularly, where the protein of interest (POI) is expressed in the nucleus or cytoplasm, a split-ubiquitin Y2H is introduced instead to study the PPIs of membrane proteins (Lehming, 2002). The Y2H system employs ubiquitin, a small and highly conserved protein, as the resembling functional protein with the N- and C-termini (Figure 1.1).

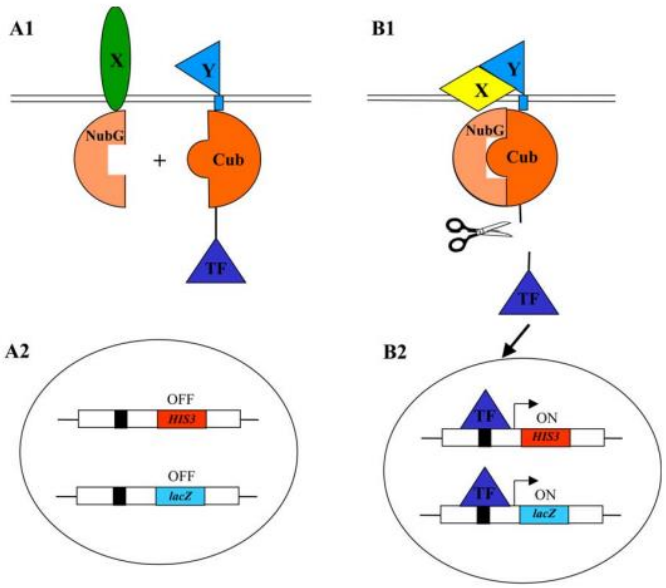

Figure 1.1: An illustration of the split-ubiquitin yeast two-hybrid assay. Interactions of bait and prey protein (X and Y) draw in proximity of Nub*G* and Cub. TF attached to Cub is cleaved and released. TF enters the nucleus and turns on reporter gene *HIS3* and *lacZ* expression, ultimately, allowing growth on medium without histidine (Fetchko and Stagljar, 2004).

1.2 Superfolder GFP

Originally identified from jellyfish, *Aequorea victoria*, the green fluorescent protein (GFP) is a powerful reporter molecule as it folds to form a chromophore, emitting green fluorescence that can be brought about internally, allowing rapid visual detection of tagged protein of interest without requiring external cofactors (Andrews et al., 2007). However, chromophore oxidation and slow folding rate limits fluorescence maturation. Hence, an improved variant, superfolder green fluorescent protein (sfGFP), has been developed to overcome limitations of proneness to aggregation, misfolding, and slow folding rate of

GFP, where its fluorescence remains independent of the abovementioned limitations (Andrews et al., 2007).

1.3 Anti-GFP nanobody

As a binding protein, antibodies are unpreferred due to the larger size that require the formation of disulfide bonds *in vivo*, and have high tendency to aggregate (Zhang et al., 2020). Nanobody is a single domain antibody that does not compromise binding affinity and selectivity despite being just a portion of a full-sized antibody (Aguilar et al., 2019; Zhang et al., 2020). Nanobodies are highly stable proteins that are resistant to denaturants and organic solvents, allowing them to withstand harsh conditions of purification and biochemical assays (Zhang et al., 2020). In addition, being able to be used in humans as therapeutic reagents, nanobody technology is the most promising technology to date (Zhang et al., 2020). This was a pilot study conducted with the interest to enhance anti-GFP nanobody affinity to sfGFP to form a more stable interaction in a future study.

1.4 Problem Statement

Most conventional Y2H screenings are performed to study nuclear PPI; however, the reducing intracellular conditions restrain proper protein folding. The extracellular space, which is more oxidizing, encourages disulfide bond formation and hence promotes better protein folding. PPI that occurs in the extracellular space requires the activation of intracellular signal cascade for

facilitating PPI detection. Hence, as a proof-of-concept for a yeast-based surface display system, known interacting proteins such as anti-GFP nanobody and superfolder GFP need to be expressed using this system, where the interaction of these proteins may be reported by the split ubiquitin reporter system.

1.5 Objectives of Study

The main purpose of this study was to clone and express *NbGFP* and *sfGFP* genes into yeast expression vectors and study the interactions between the two proteins of interest using a split-ubiquitin Y2H system.

Specific objectives to be achieved:

1. To insert *NbGFP* and *sfGFP* genes into pMetYC-dest and pXN21-dest vectors, respectively
2. To study extracellular protein-protein interaction between NbGFP and sfGFP using yeast two-hybrid assay

CHAPTER 2

LITERATURE REVIEW

2.1 Membrane Protein-protein Interaction

Interaction networks, which are mostly composed of proteins, are a crucial characteristic of complex biological systems. These are essential for cellular energy availability as well as for all other aspects of cellular function, such as signaling, metabolism, and architecture (Brückner et al., 2009; Hunke and Müller, 2012). Ligands and receptors, antibodies and antigens, are examples of PPIs that occur in the extracellular space (Brückner et al., 2009). As much as 60% of the available pharmaceuticals are targeting membrane proteins (Hunke and Müller, 2012). As extracellular PPIs are involved in numerous physiological functions, the urgency in comprehension of these interactions cannot be disregarded. Hence, screening systems that are specific, sensitive and high-throughput are in high demand.

2.2 Yeast as Host

Saccharomyces cerevisiae is among the most extensively studied eukaryotic model organisms and has been widely used as a host for genetic engineering in research on gene expression regulation (Kondo and Ueda, 2004; Karathia et al., 2011). In addition to its short life cycle with a doubling time of approximately 90 minutes, yeast can be readily grown to high densities in an affordable medium (Kondo and Ueda, 2004). Furthermore, not only do model organisms

help overcome the hurdles of ethical and experimental restrictions, but because yeasts are eukaryotes specifically, they are also fully believed to be representative of higher-class organisms, thereby providing a guide for the development and optimization of analytical procedures (Karathia et al., 2011).

2.3 Classic Yeast Two-Hybrid System

Protein binding is a useful parameter for understanding protein function, and the yeast two-hybrid (Y2H) system is an effective option for detecting protein-protein interactions. Initially developed to study nuclear PPI, the 'classic' Y2H system is based on the joining of complementary domains of transcription factor Gal4. The reassembly of the TF can only be brought about by PPI and hence trigger downstream signalling processes, to stimulate reporter gene expression, such as LacZ, which would result in blue *Escherichia coli* colonies due to ß-galactosidase activity. Y2H assays can be used to identify novel interacting proteins, verify interactions between two known proteins, and map interacting domains (Paiano et al., 2018). Y2H facilitates the detection and characterization of PPIs by manipulating the expression of enzymes to biosynthesize auxotrophic markers in yeast. Y2H also has the advantage of being able to detect weak or transient protein interactions, unlike other techniques (Bao et al., 2009). Y2H assay results were found to be reliable when compared with data obtained from mass spectrometry (Paiano et al., 2018). DNA sequences encoding specific tags were fused with yeast open reading frames for isolation and purification of pure protein complexes (Causier, 2004).

2.4 Split-ubiquitin Membrane Yeast Two-Hybrid

Since the establishment of the Y2H assay in 1989, it has been mostly used to detect PPI in the intracellular environment, where it is more reducing and unpreferred by extracellular proteins that require oxidative environments to promote protein folding. Ubiquitin is a highly conserved protein that plays a significant role in eukaryotic cellular regulation (Lehming, 2002). Ubiquitin exists in all eukaryotic cells in its free form or is covalently linked with other proteins according to Varshavsky (2000), as cited by Lehming in 2002. Thus, a split-ubiquitin system has been developed with the aid of PPI studies between membrane proteins and screening of new interacting partners for transcription factors (Lehming, 2002). Similar to Y2H systems, interactions of extracellular membrane proteins pull the intracellular ubiquitin halves into close proximity, allowing functional recovery that activates proteasomal degradation by ubiquitin-specific proteases (UBPs), ultimately releasing LexA-VP16, an artificial TF into the nucleus and eventually leading to the expression of reporter genes (Li et al., 2016; Paiano et al., 2018), which may be colorimetric, fluorescent readouts, or growth on selective media (Koegl and Uetz, 2007). Interactions between soluble proteins have been studied using the split-ubiquitin system, bringing advantages such as *in vivo* and *in situ* PPI detection; the small size of ubiquitin minimizes the possibility of steric hindrance and enable the detection of transient interactions (Stagljar et al., 1998). Most importantly, cleavage performed by UBP is the highlight of the system, rather than transcription of the classic Y2H (Stagljar et al., 1998).

2.5 Advancement of Nanobody Studies

Originally found in camelids and sharks, nanobodies carry only one of the two variable heavy fragments of an antibody that are crucial for antigen recognition and binding specificity. Thus, uncompromised stability, specificity, and affinity to bind targets make nanobodies a popular choice as a protein tag (Aguilar, 2019; Wendel et al., 2016). Furthermore, the small size of nanobodies, along with the lack of essential disulfide bond formation, contributes to the ease of production in *E. coli* (Wendel et al., 2016). Nanobodies have been used in developmental studies of protein function manipulation. The use of chromobodies, a fusion protein of a fluorescent protein, and a nanobody in the recognition and binding to functional domains in target proteins would, for example, induce degradation or alter its locations (Aguilar et al., 2019).

2.6 Anti-GFP Nanobody and Superfolder GFP Interaction

The structure of NbGFP and sfGFP fusion proteins explain the high affinity and specificity of the protein complex (Kubala et al., 2010). Strong stabilization is notably contributed by hydrophobic interactions as well as polar interactions at the binding site, where the burial of the Trp47 sidechain of NbGFP essentially leads to high complex affinity. Meanwhile, the burial of hydrophilic side chains and salt bridges is responsible for spatial alignment, which gives rise to high binding specificity (Kubala et al., 2010). The structural stabilization achieved by the complex gives rise to thermostability and enhancement in fluorescence intensity (Kakasi et al., 2023). The nanomolar affinity interaction between the pair contributes to the formation of a complex that remains stable over a wide

pH (pH 7.0 to 8.5) and only denatures when temperature exceeds 70 °C (Kakasi et al., 2023).

2.7 NbGFP and sfGFP as Surface Display Platform

With the aid of antibodies and enzymes, established surface display platforms include yeasts, phages, and bacteria (Gai and Wittrup, 2007; Wendel et al., 2016). Research has increasingly shown the ease of visualization of proteins that are displayed on the cell surface using chromobodies (Wendel et al., 2016). A drawback of using GFP as a reporter is that GFP-producing cells emit fluorescence regardless of they are extracellularly displayed or not, causing confusion between intracellular and surface-displayed GFP (Wendel et al., 2016). Hence, anchoring GFP with surface proteins such as autotransporters or outer membrane proteins would overcome this problem (Wendel et al., 2016), where they showed the efficiency and functionality of the Nb:GFP platform of different anchoring proteins and the display of Chitinase A respectively.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Workflow of Project

The workflow of the project was illustrated in a flowchart (Figure 3.1).

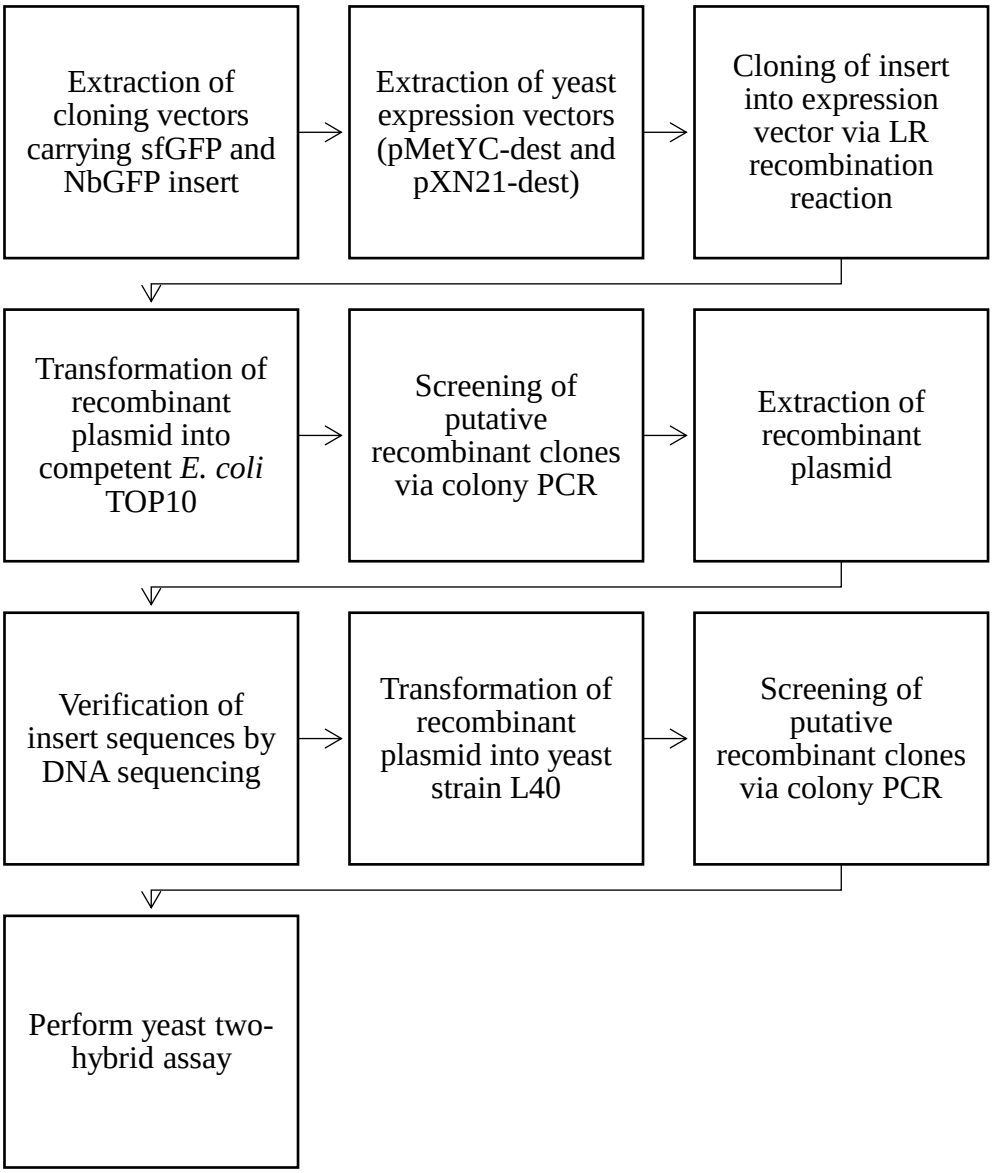

Figure 3.1: Workflow of molecular cloning of NbGFP into the pMetYC-dest vector and sfGFP into the pXN21-dest vector into *E. coli* and yeast.

3.2 Preparation of Media and Buffers

3.2.1 2×YT Medium

An amount of 2 g yeast extract, 3.2 g tryptone, and 1 g sodium chloride were added and dissolved in 200 mL of distilled water to prepare 2×YT broth. An amount of 3 g agar was added to the same composition for 2×YT agar. The mixture was then autoclaved. Antibiotics were added to the cooled-down medium (~50°C) with 200 µL of 50 mg/mL kanamycin or 100 mg/mL carbenicillin.

3.2.2 10x Tris/Acetic acid/EDTA (TAE) Buffer

EDTA powder (3.7 g) was dissolved in 25 mL distilled water and adjusted to pH 8.0 with NaOH solution. Tris powder (48.4 g) and acetic acid (11.4 mL) were dissolved in 700 mL distilled water. Both solutions were mixed and the 10x TAE buffer stock solution was topped up with distilled water to 1 L.

3.2.3 Synthetic Defined (SD) Medium

An amount of 1.67 g of yeast nitrogen base without amino acids was added to 210 mL of distilled water. The solution was then autoclaved at 121°C for 15 minutes. A volume of 25 mL of dropout solution and 12.5 mL of 40% (w/v) glucose were added to the medium. Adenine and histidine were added to a final concentration of 0.002% to prepare SD/-L-W. Tryptophan was added for SD/-

L, leucine was added for SD/-W. The amino acids were filtered before being added to the solution.

3.2.4. YPD Medium

Two grams of yeast extract, 4 g of peptone, and 4 g of dextrose were dissolved in 200 mL of distilled water to prepare a medium. The medium was then autoclaved before use.

3.2.5 Materials and Chemicals Used

The full list of materials and chemicals used are listed in Appendices A1 and A2.

3.3 Plasmid Extraction with NucleoSpin® Plasmid Kit

Plasmid DNA extraction from both entry vectors and destination vectors was performed using NucleoSpin® by Machery-Nagel. A volume of 3 mL of overnight *E. coli* culture was subjected to centrifugation at 11,000 × *g* for 30 s to obtain the cell pellet. Cell lysis was performed by resuspending the cell pellet in 250 µL of A1 solution. An equal volume of A2 solution was added and inverted gently to mix before adding 300 µL of A3 solution. The mixture was thoroughly mixed and centrifuged at 11,000 × *g* for 5 min until a clear lysate was obtained. The supernatant was carefully transferred into a column and placed in a collection tube. The column was washed with 500 µL of AW solution and 600 µL of A4 solution by centrifugation in between, where the conditions

remained consistent at 11,000 × *g* for 1 min and flowthrough was discarded. The column was then centrifuged to remove any remaining ethanol until it appeared dry before being transferred into a 1.5 µL microcentrifuge tube, and 20 µL of pre-warmed elution buffer was added. The concentration and purity of the extracted vectors were measured using a Nanodrop® 2000 spectrophotometer.

3.4 LR Recombination of Gateway™ Cloning

The mixture of the entry and destination vectors was prepared according to the manufacturer's instructions (Invitrogen, 2003) and stated in Tables 3.1 and 3.2. The mixture was then incubated overnight at room temperature. A volume of 0.5 µL of proteinase K was added and incubated in a 37°C water bath for 15 min to inactivate the LR reaction.

Table 3.1: Composition of the reaction for LR cloning of pMetYC-NbGFP.

Components	**Volume (µL)**
pTwist-NbGFP (269.9 ng/µL)	1.0
pMetYC-Dest (272.8 ng/µL)	1.8
Distilled water	2.7
5× LR Clonase™ II	1.0
Proteinase K (2 µg/µL)	0.5
Final volume	7.0

Table 3.2: Composition of the reaction used for LR cloning of pXN21-sfGFP.

Components	Volume (µL)
pTwist-sfGFP (258.1 ng/µL)	1.5
pXN21-Dest (279.2 ng/µL)	1.8
Distilled water	2.2
5X LR Clonase™ II	1.0
Proteinase K (2 µg/µL)	0.5
Final volume	7.0

3.5 Preparation of *E. coli* Competent Cells

An isolated colony of *E. coli* Top10 was inoculated into 3 mL of 2× YT broth and incubated overnight at 37°C with shaking at 220 rpm. A volume of 500 µL of the overnight liquid culture was transferred into 50 mL of freshly prepared 2× YT broth and incubated at 37°C with shaking at 220 rpm for about 2.5 hours until the OD_{600} reached 0.4-0.8. The culture broth was centrifuged at 3,500 × *g* at 4°C for 5 min. The supernatant was discarded, and the pellet was resuspended in 5 mL ice-cold 0.1 M $CaCl_2$ added with 10% (v/v) glycerol. The cell suspension was then incubated on ice for 20 min. The cell suspension was then aliquoted evenly (100 µL) into 1.5 mL microcentrifuge tubes and kept on ice until use, while the remaining competent cells were stored at -80°C for long-term storage.

3.6 Heat Shock Transformation of *E. coli*

The LR reaction mixture prepared in Section 3.4 was added and mixed thoroughly by resuspension in 100 µL of competent *E. coli* cells. The cell suspension was incubated on ice for 10 min before being transferred into a 42°C water bath for 30 s and finally on ice for 5 min. A volume of 700 µL of 2×YT broth was added to the cells and incubated at 37°C with shaking at 220 rpm for 1 h. The cells were then centrifuged at 3,500 × *g* for 5 min to obtain the cell pellet. The supernatant was discarded, leaving 100 µL for resuspension of the pellet. The cells were then plated on carbenicillin-supplemented 2× YT agar and incubated overnight at 37°C.

3.7 Screening of *E. coli* Positive Clones by Colony PCR

A total of 8 colonies were randomly selected from the plate. A toothpick was used to pick up the individual colonies, streaked lightly on a master plate, and dipped into the microcentrifuge tubes containing the PCR mix with compositions stated in Table 3.5 and Table 3.6. The tubes were then subjected to PCR with conditions stated in Table 3.7, using primer sets indicated in Table 3.4. The sequences of each primer are stated in Table 3.3.

Table 3.3: Sequences of primers used in colony PCR.

Primer	Sequence (5'-3')
F-Met17-198	TGGCACCTTGTCCAATTGAAC
R-NbGFP-ColPCR	TAAACAGTGTTACGCGCGTC
F-ADHpro-531	GGCCTTCCTTCCAGTTACTTG
R-sfGFP-ColPCR	TTTCATGTGGTCCGGGTAAC

Table 3.4: Primer sets used for *E. coli* and yeast colony PCR.

Recombinant plasmid	Primer sets
pMetYC-NbGFP	F-Met17-198 & R-NbGFP-ColPCR
pXN21-sfGFP	F-ADHpro-531 & R-sfGFP-ColPCR

Table 3.5: Composition of colony PCR for screening *E. coli* colonies harboring pMetYC-NbGFP.

Reagent	Volume (μL)
10× buffer	1.0
25 mM $MgCl_2$	0.8
10 mM dNTPs	0.8
10 μM F-Met17-198	0.4
10 μM R-NbGFP-ColPCR	0.4
DNA *Taq* polymerase	0.2
ddH_2O	6.4
Total	10.0

Table 3.6: Composition of colony PCR for screening *E. coli* colonies harboring pXN21-sfGFP.

Material	Volume (µL)
10× buffer	1.0
25 mM $MgCl_2$	0.8
10 mM dNTPs	0.8
10 µM F-ADHpro-531	0.4
10 µM R-sfGFP-ColPCR	0.4
DNA *Taq* polymerase	0.2
ddH_2O	6.4
Total	10.0

Table 3.7: Thermocycling conditions for *E. coli* colony PCR screening.

Step	Temperature (°C)	Time (seconds)	Cycles
Initial denaturation	95	600	1
Denaturation	95	15	30
Annealing	52	30	
Extension	72	45	
Final extension	72	120	1
Hold	12	∞	

3.8 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed on PCR products. Agarose 1.5% (w/v) was prepared according to the expected size of DNA bands. Agarose powder was added to the 10× TAE buffer, as described in Section 3.2.2, and the mixture was heated until the agarose powder was fully dissolved. The electrophoresis setup was set at 100 V in 1× TAE buffer for approximately 30 min.

3.9 Verification of DNA by Sequencing

Putative positive clone colonies carrying the gene of interest were selected from the master plate for plasmid extraction, as described in Section 3.3. Extracted recombinant DNA from putative positive clones was sent to Apical Scientific Sdn. Bhd. for DNA sequencing. Sequencing results were interpreted and compared with the expected sequence by using the Blastn by National Institute of Health.

3.10 Preparation of Yeast Competent Cells

Competent yeast cells were prepared using the Frozen-EZ Yeast Transformation II^{TM} kit (Zymo Research, 2021). A single colony of yeast strain L40 was picked, inoculated into 3 mL of YPD broth supplemented with 0.01% (v/v) adenine hemisulfate, and incubated overnight at 30°C with shaking at 220 rpm. The overnight culture was diluted to an OD_{600} value of 0.25 by inoculating into 20 mL of fresh 0.01% (v/v) adenine hemisulfate-supplemented YPD broth and

incubated at 30°C with shaking at 220 rpm until the absorbance reached OD_{600} 0.8-1.0. The cell culture was then centrifuged again under the same conditions as described above. The supernatant was discarded and the pellet was washed with 10 mL of 1× SD/-H-L-M-W. The cell suspension was centrifuged and washed once again with SD/-H-L-M-W and twice with 10 mL of Solution 1. The cell suspension was centrifuged at 500 × *g* for 4 min to pellet the cells, and the supernatant was discarded. The pellet was resuspended in 1 mL of Solution 2 and divided into 50 µL aliquots. The competent cells were immediately used for yeast transformation.

3.11 Transformation of Recombinant Plasmid into Yeast L40

A volume of 50 µL of yeast competent cells was added to 1 µg of recombinant plasmid extracted and verified in Section 3.9 and 500 µL of Solution III (Frozen EZ Yeast Transformation II ™). For co-transformation, 1 µg of each recombinant plasmid was added to 50 µL competent cells. The mixture was then incubated in a water bath at 30°C for 1 h, and the cell suspension was vortexed briefly in 15-min intervals. pMetYC-NbGFP yeast cells were plated on SD/-L agar, and pXN21-sfGFP yeast cells (100 µL) were plated on SD/-W agar, SD/-L-W agar was used for co-transformed yeast cells. The plates were incubated at 30°C for 3 days.

3.12 Screening of Yeast Positive Clones by Colony PCR

Colonies from the yeast transformant plate were picked up using a sterile toothpick, streaked on a master plate, and dipped in 10 µL of A1 solution from NucleoSpin® by Machery-Nagel. The tubes were heated at 80°C for 5 min. Then, the tubes were added with 9.4 µL of A1 solution and 0.6 µL of zymolase (500 unit/mL) were added to lyse the yeast cell walls. The solution was then incubated at 37°C for 30 min and left on ice. A volume of 40 µL of A2 solution was added to the solution, vortexed, and incubated on ice for 5 min. A volume of 30 µL of A3 solution was added, vortexed, and incubated on ice for 5 min before centrifugation at 4°C at 3,500 × *g* for 10 min. The supernatant was transferred to a new tube, and an equal volume of absolute ethanol was added before incubation on ice for 10 min and centrifuged under the same conditions. The supernatant was discarded and the pellet was resuspended in 70% (v/v) ethanol and centrifuged again. The supernatant was discarded and the pellet was subjected to vacuum drying at 60°C for 2 min before being resuspended in 10 µL distilled water. The DNA extracted from yeast cells (5 µL) was added to the same composition as in Table 3.5. The thermocycling conditions were identical to those of *E. coli* colony PCR (Table 3.6).

3.13 Yeast Two-Hybrid Assay

A single co-transformed yeast colony was picked from the master plate and inoculated in 5 mL of SD/-H-L-M-W at 30°C with shaking at 220 rpm overnight. The culture was then centrifuged at 500 × *g* for 1 min. The supernatant was discarded. The pellet was resuspended with 5 mL of SD/-H-L-M-W as a

washing step to clear residual salt. The cell suspension was centrifuged and resuspended twice with SD/-H-L-M-W. The absorbance reading was measured and incubated to get OD_{600} value of 0.1. The cells were diluted in two-folds serial dilution until it reached 128×. A volume of 10 µL of each dilution nwas spotted onto SD/-L-W and SD/-H-L-W agar. pTwist-Ost1 and pTwist-Wbp1 co-transformed yeast cells served as positive control, while empty destination vectors, pMetYC-dest and pXN21-dest co-transformed yeast cells, served as negative control. The plates were incubated at 30°C for 2 days

CHAPTER 4

RESULTS

4.1. Construction of pMetYC-NbGFP

Gene of NbGFP was transferred from entry clone, pTwist-NbGFP, to yeast expression vector, pMetYC-dest. The expression clone was then transformed into *E. coli* via heat shock. Colony PCR was performed to screen and select positive clones. The recombinant plasmid was extracted and transformed into yeast cells after sequence verification.

4.1.1 Plasmid Extraction from Entry and Destination Vectors

Both pTwist-NbGFP and pMetYC-Dest were subjected to plasmid extraction from isolated *E. coli* colonies, following the procedures described in Section 3.3. The concentration and purity are shown in Table 4.3

Table 4.1: Concentration and purity of extracted pTwist-NbGFP and pMetYC-Dest.

Vector	Concentration (ng/µL)	Purity ($A_{260/280}$)
pTwist-NbGFP	258.1	1.82
pMetYC-Dest	279.2	1.88

4.1.2. LR Cloning of pMetYC-NbGFP

The expression clone in Figure 4.1 is the expected recombinant plasmid after LR cloning using the Gateway™ cloning method. The *att* sites were expected to permit the exchange of Gateway™ cassettes between the entry clone, pTwist-NbGFP, and the expression vector pMetYC-dest.

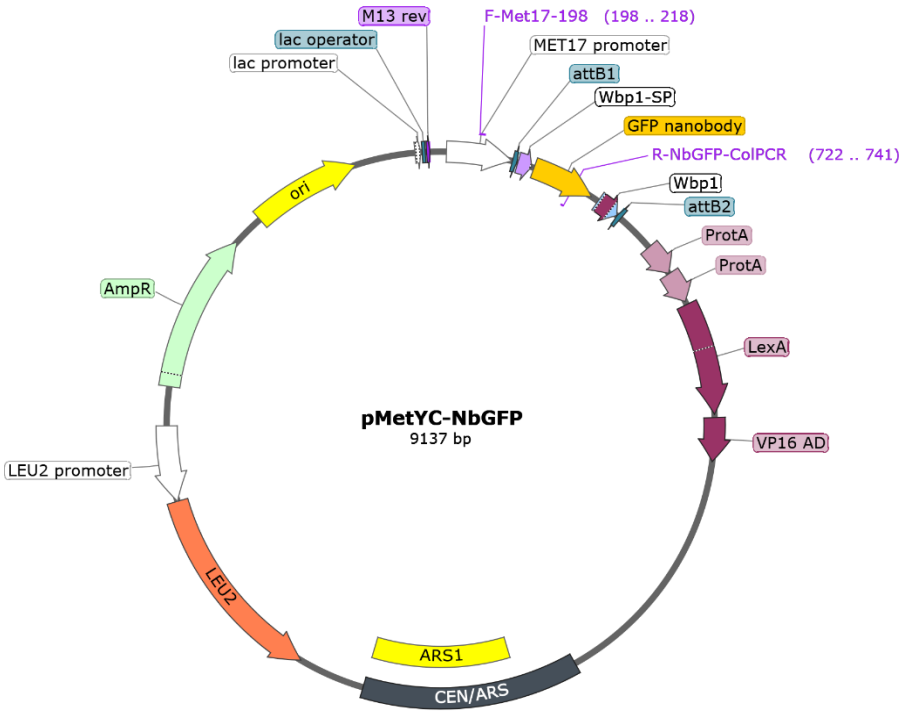

Figure 4.1: Recombinant plasmid map of pMetYC-NbGFP generated using the SnapGene software.

4.1.3 Transformation of *E. coli* Top10 with pMetYC-NbGFP

The LR mixture was transformed into competent *E. coli* cells by heat shock with procedures stated in Section 3.6. The *E. coli* cells were plated on 2× YT agar

supplemented with Carbenicillin and incubated overnight at 37°C. The colonies formed in Figure 4.2 were transferred onto a master plate (Figure 4.3).

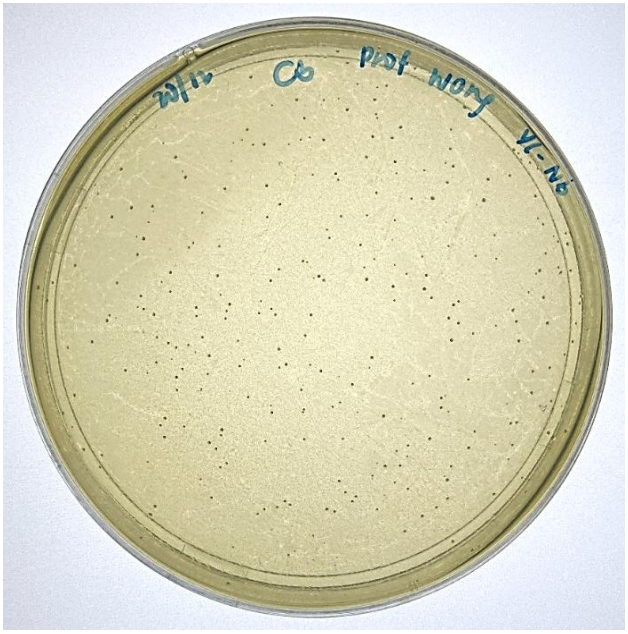

Figure 4.2: *E. coli* transformation plate with pMetYC-NbGFP.

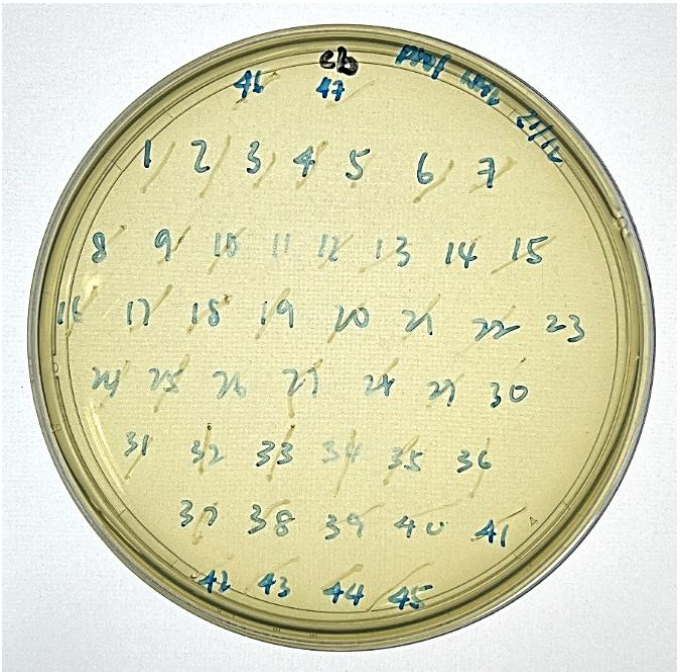

Figure 4.3: Master plate of *E. coli* transformants harboring pMetYC-NbGFP.

4.1.4 Screening of pMetYC-NbGFP by Colony PCR

Of the eight colonies screened, only two were positive with the expected band size of 544 bp. Colonies 3 and 8 (Figure 4.3) obtained band sizes of approximately 550 bp as seen as in Figure 4.4.

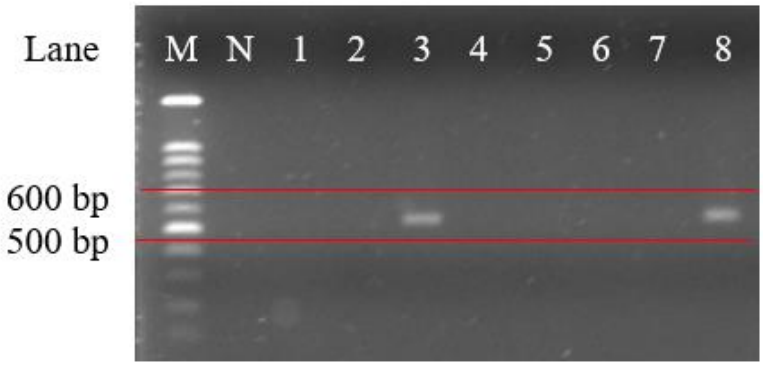

Figure 4.4: Colony PCR screening of *E. coli* transformants carrying pMetYC-NbGFP using primers F-Met and R-sfGFP.
Lane M: 100 bp ladder; Lane N: No-template control; Lane 1-8: Colonies 1-8.

4.1.5 Plasmid Extraction of Positive Clone pMetYC-NbGFP

Putative positive colony 3, screened by gel electrophoresis (Figure 4.4), was picked from the master plate (Figure 4.3) and extracted following the procedures described in Section 3.3. The concentration of the extracted recombinant plasmid was 382.9 (ng/µL) with purity ($A_{260/280}$) of 1.91.

4.1.6 Verification of pMetYC-NbGFP by DNA Sequencing

DNA extracted as described in Section 4.1.4 was sent to Apical Scientific Sdn. Bhd. for DNA sequencing. The targeted DNA fragment was 544 bp in length. As shown in Figure 4.4, the sequencing results showed 100% identity and 0% gaps, indicating that no nucleotide changes occurred during the transfer of the DNA fragment into the destination vector, pMetYC-dest.

Score	Expect	Identities	Gaps	Strand
1005 bits(544)	0.0	544/544(100%)	0/544(0%)	Plus/Plus

```
Query  372  TAAACAGTGTTACGCGCGTCGTCACGGCTAATGGTGAAACGGCCCTTGACGCTATCCTCA  431
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TAAACAGTGTTACGCGCGTCGTCACGGCTAATGGTGAAACGGCCCTTGACGCTATCCTCA  60

Query  432  TAGCTGCTACGGTCACCCGCGCTGCTCATGCCTGCTACCCACTCACGTTCCTTGCCCGGA  491
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TAGCTGCTACGGTCACCCGCGCTGCTCATGCCTGCTACCCACTCACGTTCCTTGCCCGGA  120

Query  492  GCCTGACGATACCAACGCATGCTGTAACGGTTGACCGGGAAACCGCTCGCTGCACAGCTC  551
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GCCTGACGATACCAACGCATGCTGTAACGGTTGACCGGGAAACCGCTCGCTGCACAGCTC  180

Query  552  AGACGCAAGCTGCCGCCCGGCTGGACCAGGGCACCGCCGCTTTCGACCAACTGAACGGTC  611
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AGACGCAAGCTGCCGCCCGGCTGGACCAGGGCACCGCCGCTTTCGACCAACTGAACGGTC  240

Query  612  TCCGTCTCCGTTCTTGAAGTTTGTGTGCCCACAACGAAAATGGCCTGCAGAAGGATACAA  671
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TCCGTCTCCGTTCTTGAAGTTTGTGTGCCCACAACGAAAATGGCCTGCAGAAGGATACAA  300

Query  672  AAGAAAAAATTCCAATCGGTCCGCATGGTGCAGCCTGCTTTTTTGTACAAACTTGTGATT  731
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  AAGAAAAAATTCCAATCGGTCCGCATGGTGCAGCCTGCTTTTTTGTACAAACTTGTGATT  360

Query  732  GTATGGATGGGGGTAATAGAATTGTATCTATGTATCTGACGACCCTGTATTACACGAAGG  791
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GTATGGATGGGGGTAATAGAATTGTATCTATGTATCTGACGACCCTGTATTACACGAAGG  420

Query  792  AAATAGTAGATGAAAAGACAAGAGAGCAAGAAAAAGGAAAAACTTCCTTTCTAACAGACG  851
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AAATAGTAGATGAAAAGACAAGAGAGCAAGAAAAAGGAAAAACTTCCTTTCTAACAGACG  480

Query  852  CTTTACTTAACCTTATATATATCTTAttttttCATCGAGCGTGTTCAATTGGACAAGGT  911
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CTTTACTTAACCTTATATATATCTTATTTTTTTCATCGAGCGTGTTCAATTGGACAAGGT  540

Query  912  GCCA  915
            ||||
Sbjct  541  GCCA  544
```

Figure 4.5: DNA sequencing results compared with the expected sequence of pMetYC-NbGFP with the Blastn program by National Institute of Health.

4.1.7 Yeast Transformation with pMetYC-NbGFP

Positive recombinant plasmid of pMetYC-NbGFP was extracted and transformed into competent yeast strain L40 by heat shock method. The transformed yeast cells were plated on SD/-W agar and incubated at 30°C for 3 days (Figure 4.6) and individual colonies were randomly selected and streaked on a master plate (Figure 4.7).

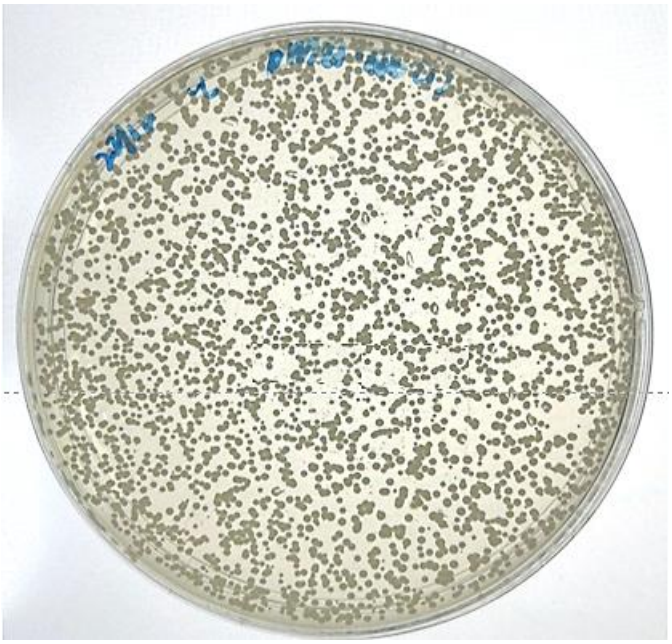

Figure 4.6: Yeast transformation plate carrying pMetYC-NbGFP on SD/-W agar.

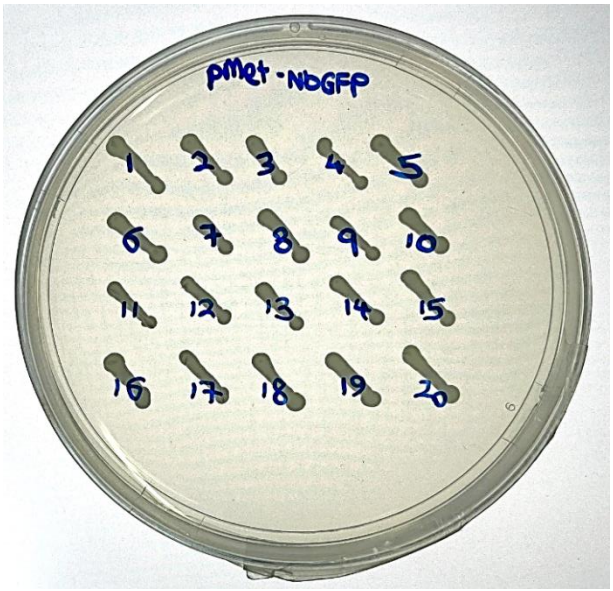

Figure 4.7: Master plate of yeast transformants carrying pMetYC-NbGFP on SD-/W agar.

4.1.8. Screening of Yeast with pMetYC-NbGFP

Colonies 1, 2, 3, and 4 (Figure 4.7) were positive clones with an observed band size of approximately 550 bp, similar to the expected PCR product of 544 bp (Figure 4.8).

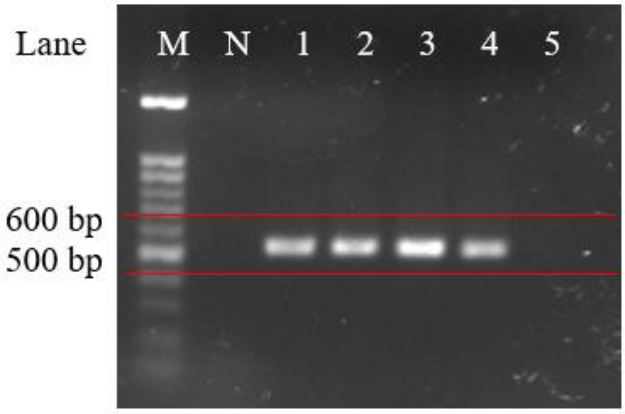

Figure 4.8: Colony PCR screening of yeast transformants carrying pMetYC-NbGFP with primers F-Met and R-NbGFP.
Lane M: 100 bp ladder; Lane N: No-template control; Lanes 1-5: Colonies 1-5.

4.2 Construction of pXN21-sfGFP

Gene of sfGFP was transferred from entry clone, pTwist-sfGFP, to yeast expression vector, pXN21-dest. The expression clone was then transformed into *E. coli* via heat shock. Colony PCR was performed to screen and select positive clones. The recombinant plasmid was extracted and transformed into yeast cells after sequence verification.

4.2.1 Plasmid Extraction from Entry and Destination Vectors

Both pTwist-sfGFP and pXN21-Dest were subjected to plasmid extraction from isolated *E. coli* colonies, following the procedures described in Section 3.3. The concentration and purity are shown in Table 4.2

Table 4.2: Concentration and purity of extracted pTwist-sfGFP and pXN21-Dest.

Vector	Concentration (ng/µL)	Primary purity ($A_{260/280}$)
pTwist-sfGFP	269.9	1.88
pXN21-Dest	272.8	1.83

4.2.2. LR cloning of pXN21-sfGFP

The expression clone shown in Figure 4.9 is the expected recombinant plasmid after LR cloning using the Gateway™ cloning method. The *att* sites were expected to permit the exchange of Gateway™ cassettes between the entry clone, pTwist-sfGFP, and the expression vector, pXN21-dest.

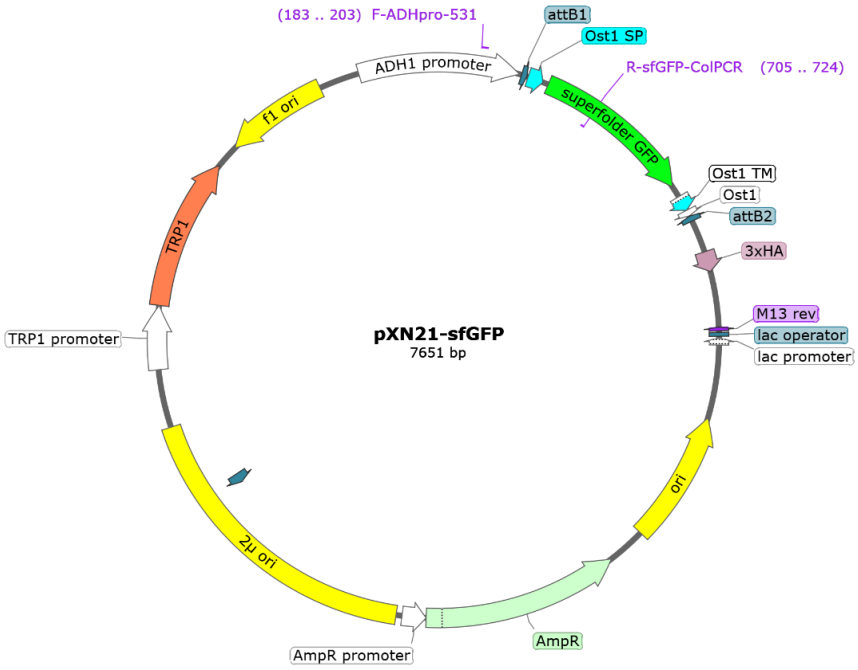

Figure 4.9: Recombinant plasmid map of pXN21-sfGFP generated using the SnapGene software.

4.2.3 Transformation of *E. coli* with pXN21-sfGFP

The LR mixture was transformed into competent *E. coli* cells by heat shock with procedures stated in Section 3.6. The *E. coli* cells were plated on 2× YT agar supplemented with Carbenicillin and incubated overnight at 37°C. The colonies formed in Figure 4.10 were transferred onto a master plate (Figure 4.11).

Figure 4.10: *E. coli* transformation plate carrying pXN21-sfGFP.

Figure 4.11: Master plate of *E. coli* transformants harboring pXN21-sfGFP.

4.2.4 Screening of pXN21-sfGFP by Colony PCR

Of the eight colonies screened, only colonies 3, 4, and 5 (Figure 4.11) were positive clones, with PCR products of approximately 550 bp, similar to the expected band size of 542 bp (Figure 4.12).

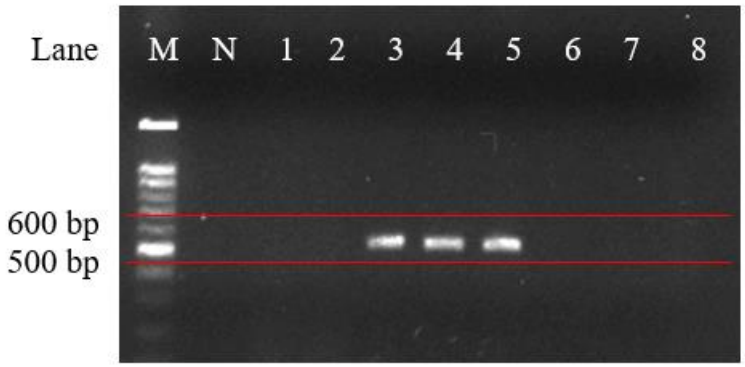

Figure 4.12: Colony PCR screening of *E. coli* carrying pXN21-sfGFP with primers F-XN and R-sfGFP.
Lane M: 100 bp ladder; Lane N: No-template control; Lanes 1-8: Colonies 1-8.

4.2.5 Plasmid Extraction of Positive Clone pXN21-sfGFP

Putative positive clone colony 3 screened in Section 4.2.4, was picked from the master plate (Figure 4.11) for recombinant plasmid extraction following the procedure described in Section 3.3. The concentration of extracted recombinant plasmid was recorded at 352.5 ng/µL with purity ($A_{260/280}$) of 1.87.

4.2.6 Verification of pXN21-sfGFP by DNA Sequencing

DNA extracted as described in Section 4.2.4 was sent to Apical Scientific Sdn. Bhd. for DNA sequencing. The targeted DNA fragment was 542 bp long. The sequence showed 100% identity and 0% gaps, indicating that no nucleotide was changed during the transfer of the DNA fragment into the destination vector pXN21-dest (Figure 4.13).

Score	Expect	Identities	Gaps	Strand
1002 bits(542)	0.0	542/542(100%)	0/542(0%)	Plus/Plus

```
Query  23   GGCCTTCCTTCCAGTTACTTGAATTTGAAATaaaaaaaaGTTTGCTGTCTTGCTATCAAG  82
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GGCCTTCCTTCCAGTTACTTGAATTTGAAATAAAAAAAAGTTTGCTGTCTTGCTATCAAG  60

Query  83   TATAAATAGACCTGCAATTATTAATCTTTTGTTTCCTCGTCATTGTTCTCGTTCCCTTTC  142
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TATAAATAGACCTGCAATTATTAATCTTTTGTTTCCTCGTCATTGTTCTCGTTCCCTTTC  120

Query  143  TTCCTTGTTTCTTTTTCTGCACAATATTTCAAGCTATACCAAGCATACAATCAACTCATA  202
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TTCCTTGTTTCTTTTTCTGCACAATATTTCAAGCTATACCAAGCATACAATCAACTCATA  180

Query  203  TGTGAACAAGTTTGTACAAAAAAGCAGGCTGCACCATGAGGCAGGTTTGGTTCTCTTGGA  262
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TGTGAACAAGTTTGTACAAAAAAGCAGGCTGCACCATGAGGCAGGTTTGGTTCTCTTGGA  240

Query  263  TTGTGGGATTGTTCCTATGTTTTTTCAACGTGTCTTCTGCTGCCCAATACGAGCTCCCTA  322
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TTGTGGGATTGTTCCTATGTTTTTTCAACGTGTCTTCTGCTGCCCAATACGAGCTCCCTA  300

Query  323  GGGAGACCAGCAAAGGTGAAGAACTGTTCACTGGTGTAGTGCCGATTCTGGTAGAGTTGG  382
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GGGAGACCAGCAAAGGTGAAGAACTGTTCACTGGTGTAGTGCCGATTCTGGTAGAGTTGG  360

Query  383  ACGGTGACGTGAATGGCCACAAATTTAGCGTTCGTGGTGAAGGCGAAGGTGACGCGACCA  442
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  ACGGTGACGTGAATGGCCACAAATTTAGCGTTCGTGGTGAAGGCGAAGGTGACGCGACCA  420

Query  443  ACGGTAAATTGACTCTGAAATTCATCTGCACTACGGGCAAATTGCCGGTACCGTGGCCGA  502
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  ACGGTAAATTGACTCTGAAATTCATCTGCACTACGGGCAAATTGCCGGTACCGTGGCCGA  480

Query  503  CCTTGGTTACTACCCTGACTTATGGCGTCCAATGCTTCAGCCGTTACCCGGACCACATGA  562
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CCTTGGTTACTACCCTGACTTATGGCGTCCAATGCTTCAGCCGTTACCCGGACCACATGA  540

Query  563  AA  564
            ||
Sbjct  541  AA  542
```

Figure 4.13: DNA sequencing results compared with the expected sequence of pXN21-sfGFP with the Blastn program by National Institute of Health.

4.2.7 Yeast Transformation with pXN21-sfGFP

Positive recombinant plasmid of pXN21-sfGFP was extracted and transformed into competent yeast strain L40 by heat shock method. The transformed yeast cells were plated on SD/-L agar and incubated at 30°C for 3 days (Figure 4.14) and individual colonies were randomly selected and streaked on a master plate (Figure 4.15).

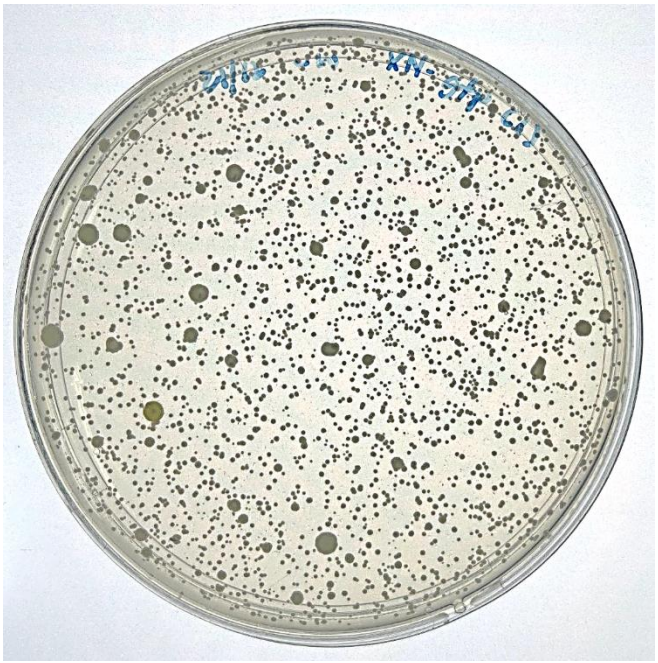

Figure 4.14: Yeast transformation plate carrying pXN21-sfGFP on SD/-L agar.

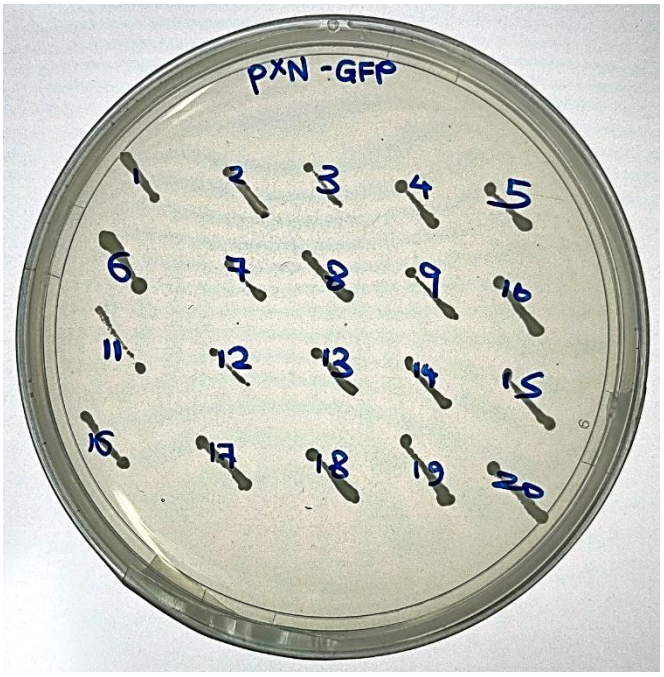

Figure 4.15: Master plate of yeast transformants carrying pXN21-sfGFP on SD/-L agar.

4.2.8 Colony PCR Screening of Yeast Transformants Carrying pXN21-sfGFP

Out of 10 colonies screened by PCR, only colony 6 (Figure 4.15) failed to generate the expected band of 542 bp. All other colonies obtained PCR products were approximately 550 bp long (Figure 4.16). Hence, all colonies were positive clones, except for colony 6.

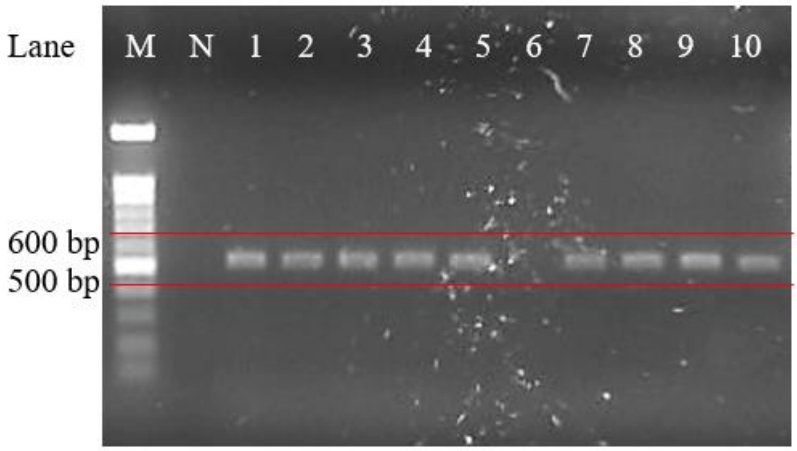

Figure 4.16: Colony PCR screening of transformed yeast carrying pXN21-sfGFP with primers F-XN and R-sfGFP.
Lane M: 100 bp ladder; Lane N: No-template control; Lanes 1-10: Colonies 1-10, respectively.

4.3 Yeast Co-transformation of pMetYC-NbGFP and pXN21-sfGFP

Both recombinant plasmids were co-transformed into competent yeast L40 cells and plated on SD/-L -W agar. The plates were incubated at 30°C for 3 days (Figure 4.17) and individual colonies were randomly selected and streaked onto a master plate (Figure 4.18)

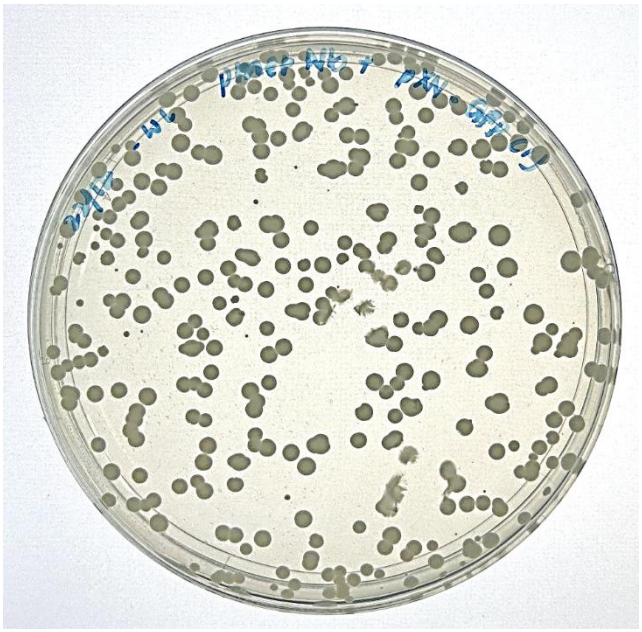

Figure 4.17: Yeast co-transformation plate of pMetYC-NbGFP and pXN21-sfGFP on SD/-L-W agar.

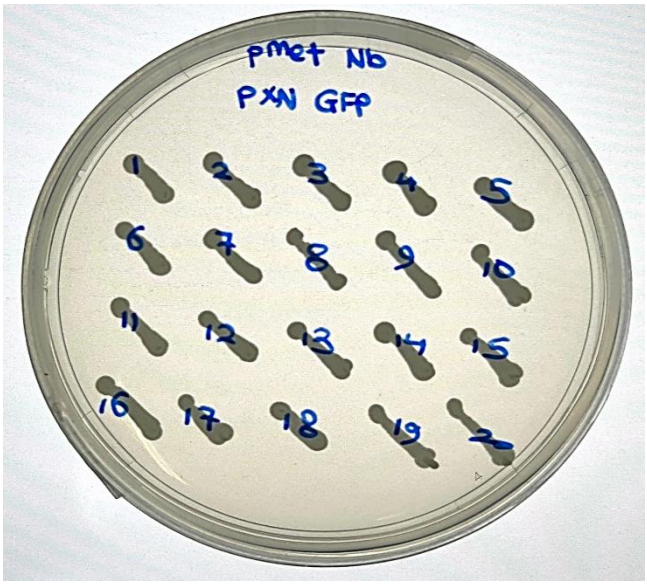

Figure 4.18: Yeast co-transformation master plate carrying pMetYC-NbGFP and pXN21-sfGFP on SD/-L-W agar.

4.3.1 Colony PCR Screening of Yeast Co-transformants Carrying pMetYC-NbGFP and pXN21-sfGFP

Of the 5 colonies screened, only colony 4 was not successfully co-transformed (Figure 4.18). Colonies 1, 2, 3, and 5 were successfully co-transformed as shown in Figure 4.19, where the observed band sizes of approximately 550 bp met the expected band sizes of pXN21-sfGFP and pMetYC-NbGFP, 542 bp and 544 bp, respectively. Duplex PCR would not have been able to differentiate amplicons with similar band sizes. The primers used to target NbGFP in pMetYC-dest failed to amplify the gene of interest, as shown in Lane 8 of Figure 4.19.

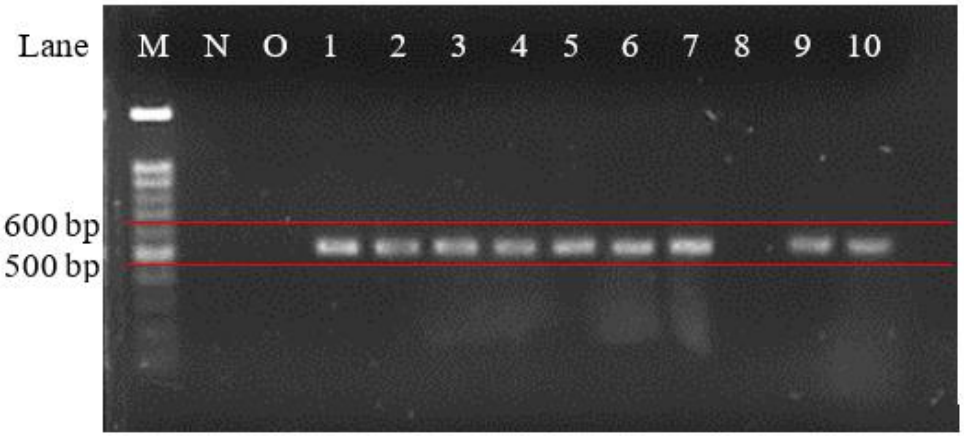

Figure 4.19: Colony PCR screening of co-transformed yeast carrying pMetYC-NbGFP and pXN21-sfGFP on 1.5% (w/v) agarose.
Lane M: 100 bp ladder; Lane N: No-template control for F-XN and R-sfGFP;
Lane O: No-template control for F-Met and R-NbGFP;
Lanes 1, 3, 5, 7, 9: Colonies 1-5 with primers F-XN and R-sfGFP, respectively;
Lanes 2, 4, 6, 8, 10: Colonies 1-5 with primers F-Met and R-NbGFP, respectively;

4.4 Yeast Two-Hybrid Assay

The positive control pair, Wbp1 and Ost1, are interactive proteins, full length sequence of both proteins (Stagljar et al., 1998) were allowed to interact and served as a positive control for this assay. Co-transformed yeast cells expressing Wbp1 and Ost1 were spotted on SD/-L-W as a standard to ensure the functionality of the assay, including yeast viability and medium preparation in Figure 4.20.

On SD/-L-W, yeast growth for all three sets were consistent and comparable (Figure 4.20). The spots of yeast were full until 16× dilution and small spots of medium without growth was observed from 32× dilution onwards for all three sets of interacting proteins. On SD/-L -W -H, empty vectors without interacting proteins could not trigger auxotrophic growth of yeast, while positive control pair, Wbp1 and Ost1, showed slight scarcity in yeast growth even at 1× dilution. Area covered by yeast growth decreased with dilution and the area spotted with 128× diluted yeast cells only showed little growth when compared against with interacting proteins of interest, NbGFP and sfGFP. Subject pairing, NbGFP and sfGFP, biosynthesis of the essential amino acids, leucine, tryptophan, and histidine was suggested by the auxotrophic growth. The spotted area was covered in yeast and evident sparse areas without growth was observed from 16× dilution onwards.

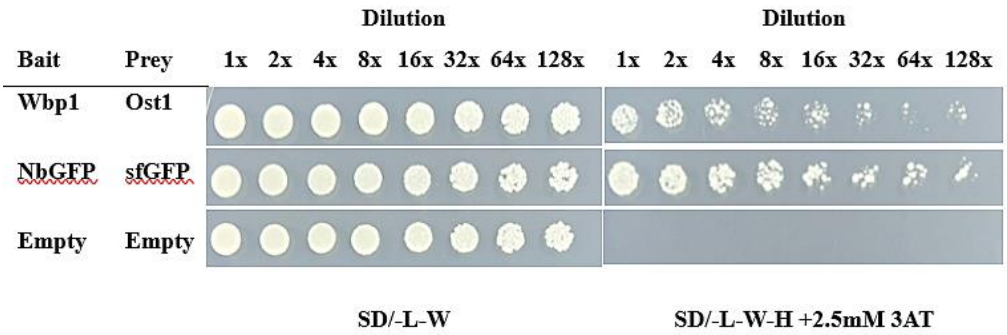

Figure 4.20: Yeast two-hybrid analysis of interactions between sfGFP and NbGFP on SD/-L-W agar and SD/-L-W-H agar. Wbp1 and Ost1 were used as positive controls and empty destination vectors served as negative controls.

CHAPTER 5

DISCUSSION

5.1 Features of Gateway™ Vectors Used

The Gateway™ technology allows quick and reversible transfers of gene of interest between entry clones with multiple destination vectors. The Gateway™ vectors used in this project include pTwist-NbGFP, pTwist-sfGFP, pMetYC-dest, and pXN21-dest.

5.1.1 pTwist-NbGFP

The entry clone has a simpler map than the expression vectors, as it only functions as an intermediate vector to harbor the gene of interest. In Figure 5.1, the pTwist-NbGFP is 2,971 bp in length, harboring the *attL* sites, the essence of Gateway™ cloning, to flank the Gateway™ cassette, and allow exchange with the destination vector. *Wbp1-SP* encodes for the signal peptide portion of the Wbp1 protein, that would drive the localization of NbGFP to the extracellular space, while the anchorage of the protein to the membrane is responsible by the transmembrane domain of Wbp1. The possession of the Kan^R gene aids in *E. coli* selection, whereas replication is initiated at the *ori* site.

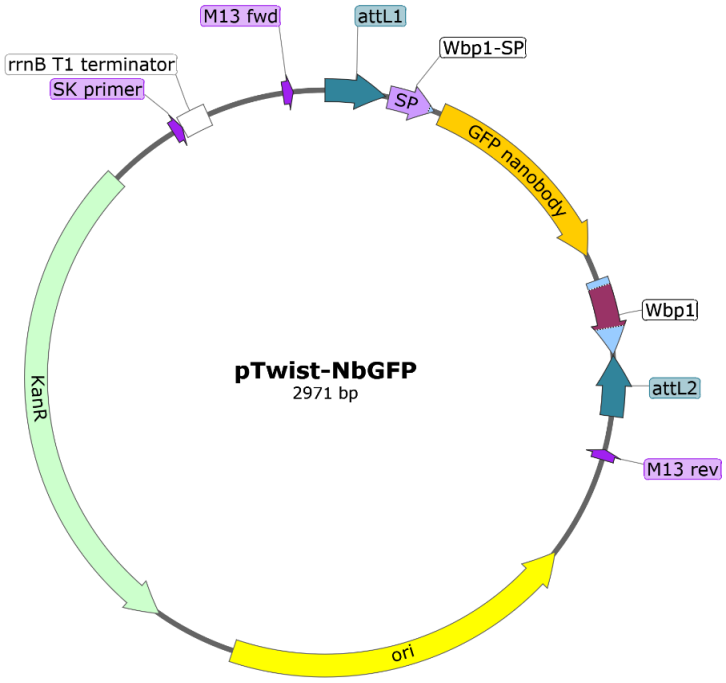

Figure 5.1: Plasmid map of the entry clone, pTwist-NbGFP highlighting features of *attL* sites, *Wbp1-SP*, *NbGFP*, *Wbp1*, *ori* site, and Kan^R. The plasmid map was generated using the SnapGene software.

5.1.2 pMetYC-Dest

Yeast expression vectors are much more complex than entry clone vectors, as they carry promoters to facilitate the expression of the gene of interest, selection markers, regulatory elements, and fusion tags to facilitate downstream processes such as purification and analysis. In figure 5.2, the pMetYC-dest is 10,186 bp in length and carries the *attR* sites for LR recombination, 'suicide' *ccdB* gene to aid in the selection of positive clones, C-terminal of the ubiquitin (Cub), activating domain of transcription factor VP16, polyA signal for post-transcriptional modification, and stabilization of the mRNA during processing (Patrick, 2014). Fusion proteins ProtA (Protein A) and LexA, a transcriptional

repressor, facilitate downstream processes such as protein purification and expression, respectively. The origins of replication of *E. coli* and yeast are represented by *ori* and *ARS*. The yeast auxotrophic marker *LEU2* is responsible for leucine biosynthesis, which enables yeast growth in leucine-deficient medium, whereas Amp^R confers ampicillin resistance in *E. coli*.

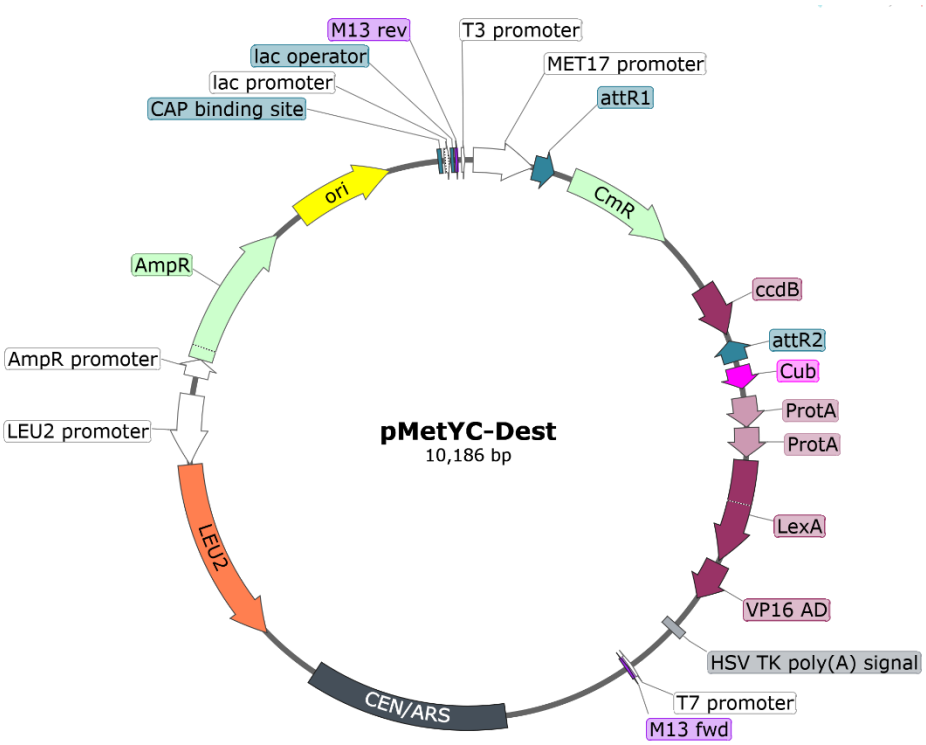

Figure 5.2: Plasmid map of yeast expression vector pMetYC-Dest highlighting features of *attR* sites*, ccdB, Cub, VP16 AD, poly(A) signal, ARS, LEU2,* Amp^R *ori* site, and *Met17* promoter. The plasmid map was generated using the SnapGene software.

5.1.3 pTwist-sfGFP

Similar to pTwist-NbGFP, pTwist-sfGFP (Figure 5.3) carries the signal peptide sequence of the Ost1 protein to aid in extracellular localization of sfGFP and is anchored on the membrane by the transmembrane domain of Ost1 for PPI. *KanR* confers resistance to kanamycin, and the *ori* site serves as an initiation point for *E. coli* replication.

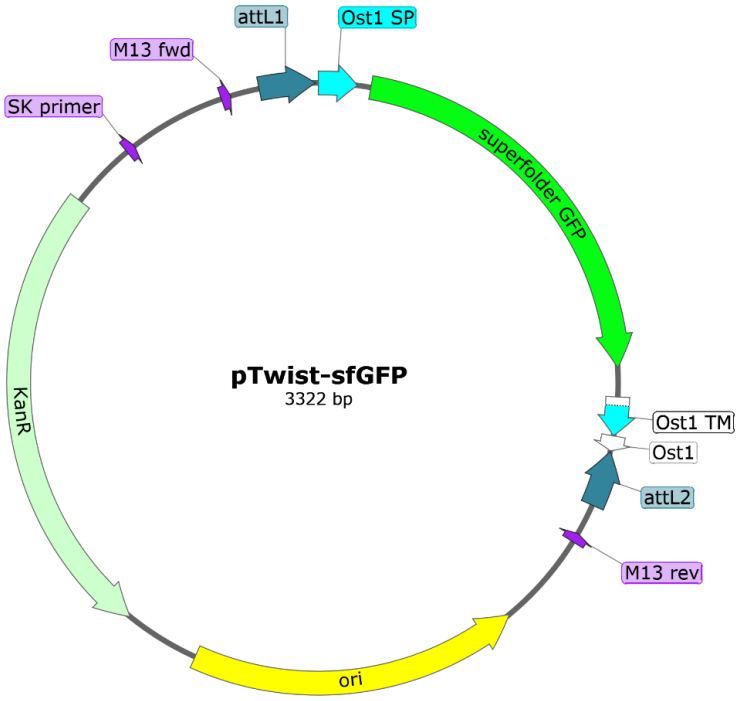

Figure 5.3: Plasmid map of the entry clone, pTwist-sfGFP highlighting features of *attL* sites, *Ost1-SP, sfGFP, Ost1-TM, Kan^R,* and *ori* sites. The plasmid map was generated using the SnapGene software.

5.1.4 pXN21-Dest

The second construct, pXN21-sfGFP, is a recombination reaction between pTwist-sfGFP (Figure 5.3) and pXN21-Dest (Figure 5.4). The destination vector is 8,517 base pairs in length and features the ADH1 promoter, which is constitutive, to drive high expression levels of the prey protein sfGFP, while the ADH1 terminator sequence downstream terminates transcription (Patrick, 2014; Paiano et al., 2018). Outside the Gateway^{TM} cassette lies a three-tandem hemagglutinin (HA) epitope tag to enable protein purification with the aid of anti-HA antibodies and quantification via Western blotting (Grefen, Lalonde and Obrdlik, 2007). Other features of the vector include the *TRP1* gene, which encodes phosphoribosylanthranilate isomerase, which is essential for tryptophan biosynthesis and functions as a yeast auxotrophic marker. The origins of replication for f1 bacteriophage, *E. coli*, and yeast are present in the backbone of the vector, represented by *f1 ori*, *2μ ori* and *ori*. The presence of drug-resistant gene, *AmpR*, confers *E. coli* resistance against ampicillin and carbenicillin.

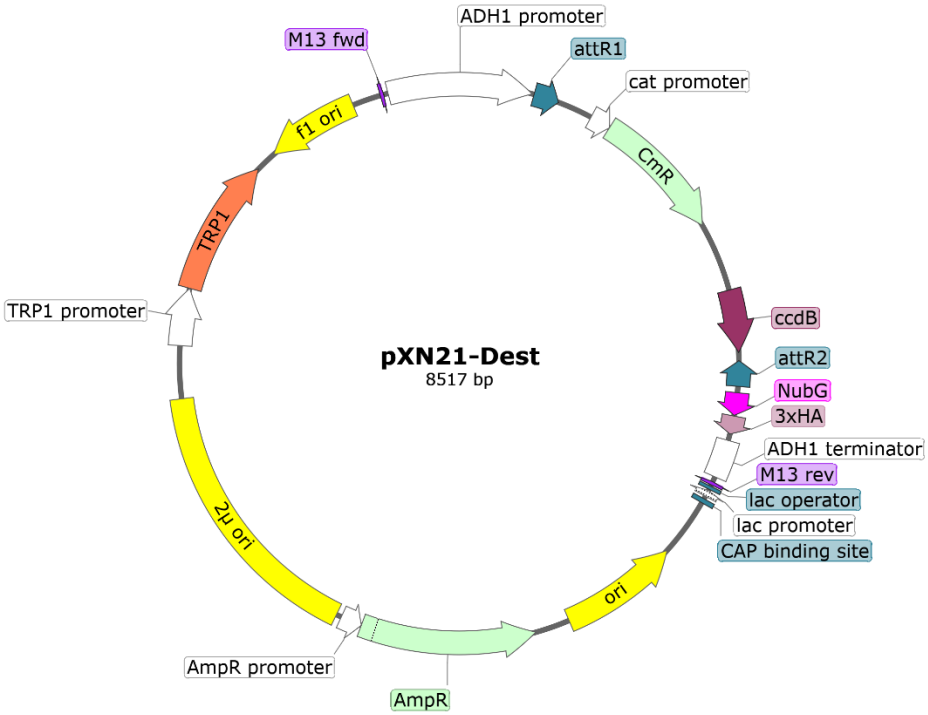

Figure 5.4. Plasmid map of pXN21-Dest vector highlighting the features of *attR* sites, *CmR, ccdB*, *Cub*, *3xHA*, *ori* site, *AmpR*, *2µ ori* site, *TRP1* and *f1 ori* site. The plasmid map was generated using the SnapGene software.

5.2 LR Cloning

The LR cloning method allowed easy and convenient DNA recombination of both the constructs, pMetYC-NbGFP and pXN21-sfGFP. Accurate and precise integration of genes of interest into the respective destination vectors was possible because of the site-specific recombination method of LR cloning. The DNA fragments were inserted with correct orientation and location as expected with high fidelity, as shown by DNA sequencing in Section 4.1.6 (Figure 4.5) and Section 4.2.6 (Figure 4.13).

The Gateway™ recombination system utilizes bacteriophage lambda, which allows DNA fragments to be excised and integrated between shuttle vectors using only one enzyme. The "Gateway™ cassette" flanks the gene of interest on the entry clone, and antibiotic-resistant gene and *ccdB* gene on the destination vector with *att* sites, that function as sites of recombination. *att* sites have longer sequences than restriction sites, therefore removing concerns of coincidental sequence similarity with the DNA fragments of interest. The long sequences of *att* sites also allow highly specific integration, resulting in only one possible outcome (Reece-Hoyes and Walhout 2018). Unlike the conventional DNA recombination technique, in which excision and ligation of DNA fragments require specific restriction enzymes, the Gateway™ system saves more time and cost, and with a one-step exchange of Gateway™ cassettes between the entry and destination vectors, only one enzyme is required to confer recombination (Reece-Hoyes and Walhout, 2018). The specificity of the *att* sites lies between the presence of "arms" adjacent to the 25-bp recognition site that controls the cutting and reconnecting of DNA fragments, which is essential for ensuring that DNA cloning only occurs in a single orientation (Reece-Hoyes and Walhout, 2018). Additionally, one of the key advantages of the one-step directional cloning and transfer method is its reversibility, giving it an edge over other cloning systems that can only perform either directional cloning or directional transfer, but cannot do both in a reversible manner (Katzen, 2007). Moreover, generation of complex constructs by multifragment cloning is deemed possible with the Gateway™ technology (Katzen 2007; Reece-Hoyces and Walhout, 2018).

The selection process of desired recombination product is expedited with the aid of the Gateway™ cassettes present in Gateway™ vectors. In all Gateway™ cassettes, they carry the *ccdB* 'suicide' gene, that results in cell death when the *ccdB* gene is not substituted by the gene of interest. Other genes that facilitate the selection of recombinant clones include antibiotic-resistant genes, which eliminate the background while allowing the growth of recombinant clones on antibiotic-supplemented medium.

5.3 Yeast Two-Hybrid Assay

From the Y2H assay shown in Figure 4.20 of Section 4.4, the growth of yeast cells on SD/-L-W-H agar plates indicated the expression of reporter genes, *LEU2*, *TRP1,* and *HIS3,* which are brought about by the cleavage of the transcription factor VP16-AD from the C-terminal of ubiquitin upon sfGFP and NbGFP interaction (Koegl and Uetz, 2008; Paiano et al., 2018). A pair of interacting proteins, Wbp1 and Ost1, were used as a positive control to validate the assay, while empty destination vectors, pMetYC-dest and pXN21-dest pair served as a negative control to eliminate doubts of contamination. As shown in Figure 4.20, more growth was observed in the co-transformed yeast cells than in the positive control, indicating that better interaction was achieved by the constructs compared to the positive control. As mentioned before, in Y2H assays, yeast growth is representative of *HIS3* gene expression, which is triggered by 'bait' and 'prey' protein interactions.

Yeast strain L40 has auxotrophic selectable markers for leucine, tryptophan, and histidine, where the activation of transcription may be monitored by their respective reporter genes, *LEU2, TRP1,* and *HIS3* (Paiano et al., 2018). Therefore, the yeast growth observed on SD/-L-W-H indicates the ability of the yeast to biosynthesize the essential amino acids for growth, which is a result of the successful interaction between sfGFP and NbGFP that pulled in proximity to ubiquitin. The restored ubiquitin is recognized by ubiquitin-specific proteases, where the VP16 activation domain of the transcription factor is cleaved to allow the transcription of reporter genes, *LEU2, TRP1,* and *HIS3*. Although the yeast cells were diluted up to 128×, all yeast spots showed growth on the SD/-L-W-H medium, indicating the intensity of the PPI (Grefen, Lalonde, and Obrdlik, 2007), though the number of yeast cells was lowered by dilution.

5.4 Strategies for Assay Stringency

In the pMetYC-destination vector, the *MET17* promoter efficiently and strongly represses transcription in the presence of methionine (Patrick, 2014; Møller et al., 2017). NbGFP expression was controlled by the methionine-regulated Met17 promoter. In this experiment, 0.15 mM methionine was used in accordance with the protocol suggested by Grefen, Lalonde, and Obrdlik (2007), where the amount of methionine was supplied adequately for the growth of yeast without over-repressing the bait protein, NbGFP.

The incorporation of 3-AT, short for 3-amino-1,2,4-triazole, also contributed to the enhanced assay stringency (Joung, Ramm and Pabo, 2000; Bashline and Gu,

2015). Imidazoleglycerol-phosphate dehydratase, the product of HIS3, is competitively inhibited by organic compounds (Bashline and Gu, 2015). Hence, growth of yeast would only be allowed when the threshold is overcome with higher transcription activation levels that can be brought about by bait and prey interactions, further reducing the background on selective medium lacking histidine while highlighting the intensity of PPI (Joung, Ramm and Pabo, 2000).

Furthermore, as ubiquitin is widely found in the cytoplasmic space of yeast cells, it raises concerns about its self-reassembly, which would eventually lead to false-positive results. However, modification of the N-terminal of ubiquitin has resulted in significantly diminished affinity to the C-terminal moiety compared to wild-type Nub (Stagljar et al., 1998; Bao et al., 2009; Lalonde et al., 2010; Bashline and Gu, 2015). Modifications of Nub by replacing the isoleucine at position 13 with glycine, the mutated Nub*G,* showed that ubiquitin reconstitution can only be brought about solely by a specific membrane PPI (Stagljar et al., 1998). Therefore, the possibility of false positives resulting from the spontaneous reconstitution of ubiquitin is eliminated.

5.5 Future Approaches

Despite the success of the PPI screening system, further testing is encouraged to validate the assay. Western blotting using epitopes targeting hemagglutinin of pXN21-sfGFP and Protein A of pMetYC-NbGFP should be performed. Other than serving as validation purposes, further analysis can also quantitate the

expressed genes to test the level and efficiency of gene expression (Grefen, Lalonde and Obrdlik, 2007).

When provided with sufficient proof of the functionality of the assay, the system can further elucidate protein-protein interactions and even map the binding interface of interacting proteins (Grefen, Lalonde and Obrdlik, 2007). Additionally, modifications to the system may be performed to investigate relationships between interacting proteins, such as replacing the proteins of interest, signal peptides, or transmembrane domains to elucidate PPI intensity or efficiency localization and anchorage, respectively. In the light of the fact that the split-ubiquitin Y2H is semi-quantitative of the intensity of PPI, it can be used as a tool in the first-step elucidation of a future study involving mutated NbGFP to test for further enhancement in affinity to sfGFP.

CHAPTER 6

CONCLUSIONS

This project aimed to provide a pilot study for the detection of membrane protein-protein interactions using the split-ubiquitin yeast two-hybrid technique. LR recombination reaction of Gateway™ technology was used to clone genes of interactive proteins, sfGFP and NbGFP, into yeast expression vectors pXN21-Dest and pMetYC-Dest, respectively. The test proteins were targeted to the extracellular space by translational fusion with signal peptides and anchored to the transmembrane domain of yeast cells, while they are linked to the N- and C-termini of an intracellular ubiquitin protein, respectively. Proteases that specialize in ubiquitin identify interactions between the proteins of interest that enable ubiquitin reassembly. This recognition causes the transcription factor that is bound to the C-terminal of ubiquitin to be cleaved off, allowing transcription and ultimately expression of yeast auxotrophic marker reporter genes, *TRP1, LEU2,* and *HIS3,* which conferred yeast growth on synthetic defined medium supplemented with essential amino acids, except for tryptophan, leucine, and histidine. The concerns of false-positives were addressed with strategies to enhance stringency, such as addition of 3-amino-1,2,4-triazole into the selective medium to act as a competitive inhibitor of *HIS3*, the presence of the *Met17* promoter that is repressed by methionine concentration, and modification of the N-terminal of ubiquitin to avoid spontaneous reconstitution. The findings of the project have led to the conclusion that the split-ubiquitin Y2H system is a functional platform in

detecting extracellular PPI between POI, NbGFP and sfGFP. The system can be modified by altering POI, signal peptides or anchor proteins for further elucidation on PPI. Despite the success of the PPI screening platform, further validation testing, such as Western blotting, is suggested.

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APPENDIX A

Table A1: List of equipment used in this project including manufacturers.

Equipment	Manufacturer
-80°C deep freezer	Thermo Scientific
4°C refrigerator	LinDen
Analytical balance	Copens Scientific (M) Sdn. Bhd.
Autoclave	Hirayama
Gel electrophoresis tank	Major Science
Gel imaging system	Bio-Rad
Incubator	Memmert
Laminar flow hood	Esco Lifesciences
Microcentrifuge	Beckman Coulter Inc.
Micropipettes	Accumax
Microwave oven	Panasonic
Nanodrop™ spectrophotometer	Thermo Scientific
PCR machine	Eppendorf
pH meter	Eutech instrument
Power supply for electrophoresis	Major Science
Refrigerated centrifuge	Atlantis Bioscience
Shaking incubator	Infors
Vacuum concentrator	Eppendorf
Water bath	Memmert

APPENDIX B

Table A2: List of chemicals used in this project including manufacturers.

Chemical	Manufacturer
100 bp DNA ladder	Vivantis Technologies
95% ethanol	Copens Scientific
Agar-agar powder	R&M Chemicals
Agarose powder	HydraGene
Chloramphenicol	Bio Basic
DNA loading dye	Vivantis Technologies
dNTPs	Sigma
EDTA	QRec™
Frozen-EZ Yeast transformation kit	Zymo Research
Glycerol	Fisher Scientific
Kanamycin	Bio Basic
LR Clonase™ II enzyme	Invitrogen
Magnesium chloride	Systerm
NucleoSpin® plasmid extraction kit	Macherey-Nagel
Proteinase K	Invitrogen
Sodium chloride	MERCK
Taq polymerase	Klentaq
Tris	Bio Basic
Tryptone	Condalab
Yeast extract	Condalab
Yeast nitrogen base without amino acids	Becton, Dickinson