MOLECULAR CLONING OF NITRIC OXIDE SENSOR *NORR* FROM *ESCHERICHIA COLI*

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MOLECULAR CLONING OF NITRIC OXIDE SENSOR *norR* FROM *Escherichia coli*

By

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ABSTRACT

MOLECULAR CLONING OF NITRIC OXIDE SENSOR *norR* FROM *Escherichia coli*

SOO SIAN SIU

Nitric oxide is a gaseous inorganic nitrogen compound that serves as a double edged sword. In low concentration, nitric oxide is essential in biochemical process and metabolism but is harmful in high concentration. NorR is a nitric oxide sensor found in prokaryotes, such as *Escherichia coli*. The gene *norR* codes for NorR which acts as a transcription factor that activates nitric oxide regulated genes. This study serves as a primary step towards the construction of a nitric oxide biosensor for eukaryotic systems, such as yeast. The full-length *norR* gene was amplified by polymerase chain reaction from the total DNA of *Escherichia coli,* and subsequently used as the template for PCR to generate three *norR* variants. The three variants, *norR (full-NLS), norR (HTH-NLS)* and *norR (SACT-NLS)* which were 1,537 bp, 1,049 bp and 1,263 bp, respectively, were cloned into the Gateway® vector, pENTR™-TOPO®.

ACKNOWLEDGEMENT

I owe my deepest gratitude to my supervisor, Dr. Wong Hann Ling, for his constant guidance, support and encouragement. Without him, the completion of this project and thesis would have been impossible. His inspiring, motivating, generous and patient character has been my pillar in facing the challenges encountered in this journey.

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Last and foremost, I would like to show my appreciation to everyone who have assisted and supported me in any aspect during the completion of this project.

DECLARATION

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

_____

Soo Sian Siu

APPROVAL SHEET

This project report entitled "**MOLECULAR CLONING OF NITRIC OXIDE SENSOR *norR* FROM *Escherichia coli***" was prepared by SOO SIAN SIU and submitted as partial fulfillment of the requirementsfor the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

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(Dr. Wong Hann Ling) Date:_____

Supervisor

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UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **SOO SIAN SIU** (ID No: **08ADB04594**) has completed this final year project entitled "**MOLECULAR CLONING OF NITRIC OXIDE SENSOR *norR* FROM *Escherichia coli***" supervised by Dr. Wong Hann Ling (Supervisor) from the Department of Biological Science, Faculty of Science.

I hereby give permission to my supervisors to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

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LIST OF ABBREVIATIONS

AAA^+	ATPase associated with various cellular activities
Amp	Ampicillin
bEBP	Bacterial enhancer binding protein
ddH_2O	Double distilled water/Deionized water
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
GAF	cGMP specific and stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *E. coli* FhlA
HCl	Hydrochloric acid
HTH	Helix-turn-helix
Kan	Kanamycin
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NO	Nitric oxide

PCI	Phenol/Chloroform/Isoamyl alcohol
PCR	Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
UASs	Upstream activated Sequences

CHAPTER 1

INTRODUCTION

1.1 Nitric Oxide and Problems Faced

Nitric oxide (NO) is a double-sided blade as it has both beneficial and harmful effects on the biological system (Gryglewski & Minuz, 2001; Jugdutt, 2004). It is suspected as a potential mutagenicity and cytotoxity mediator involved in cancer and tumour causing agents (Bonavida, 2010; Weber, 2007) besides playing an essential role in the well-being of organisms. Thus, it is important for scientists to unveil the many functions and roles, mechanism, effects of NO at the molecular and systemic level.

Fast, real-time, and accurate tools are needed to sense NO and to study its physiological response. Microarrays (Schoenfisch et al., 2006) and electrochemical biosensors are being developed in sensing and detecting NO (Zhang, Ju & Wang, 2007; Cragg, 2010). However, the problem faced is that detecting NO *in vivo* in both localized and systemic responses in a short-time scale is still difficult.

Thus, in this project, we utilized the regulatory protein, a NO sensing transcription factor, NorR derived from *Escherichia coli* as a means of constructing a NO biosensor (Tucker, Autreaux, Spiro & Dixon, 2005). However, inducing a functional prokaryotic enhancer binding protein in a eukaryotic system poses a problem.

1.2 Objectives of Project

- This project aimed to clone the gene, *norR* encoding a NO sensing transcription factor, NorR, into pENTR™/D-TOPO® through Gateway™ cloning by TOPO® reaction .

CHAPTER 2

LITERATURE REVIEW

2.1 Nitric Oxide and its Roles and Effects in Organisms

NO is produced endogenously in denitrifying bacteria and as an intermediate of denitrification (Tucker, Autreaux, Spiro, & Dixon, 2006) involving the stepwise reduction of nitrate to nitrite (Tucker et al., 2005). NO is also used for anaerobic respiration of nitrate and nitrite by enteric or intestinal bacteria such as *E. coli* (Ignora & Murad, 1995).

Besides, acting as messages and signaling molecules, NO plays an essential role in biological function, involving neuronal activity, immune response, blood pressure and vasodilatation and respiration in various eukaryotes consisting of animals, plants and humans (Lamas & Cadenas, 2006; Gryglewski & Minuz, 2001).

NO is commonly known as an air pollutant from automobile engines and power plants which contribute to acid rain and also from natural processes such as biological decay from organic materials (Butler & Nicholson, 2003; Mukhopadhyay, Zheng, Bedzyk, LaRossa, & Storz, 2004). NO is also a toxic reactive free radical gaseous compound (Ignarro & Murad, 1995; Tokada, Akihisa, Tanaka, Takayasu, & Nishino, 2006) that is very toxic to microorganisms (Zumft, 1997). NO is used for countering invading pathogens as it is produced exogenously by macrophages (MacMicking, Xie & Nathan, 1997; Mayer, 2000).

NO was proposed to have mutagenic properties in bacteria and also in rat primary lung cells leading it to be suspected as a potential carcinogen. Besides NO is an important mediator of cytotoxicity and mutagenicity (Tokada et al., 2003; Bonavida, 2010). Therefore, regulation of NO is important as it being a double-edged sword, beneficial in low concentration but harmful in high concentration (Mukhopadhyay et al., 2004).

2.2 Nitric oxide regulation and *norR*

NO regulation at the gene level is executed with signaling and transcription factors through NO targeting sensors upstream of NO regulatory genes. In mammalian systems, HIF-1, NF-κB, AP-1 and IRPs (Metzen, Zhou, Jelkmann, Fandrey & Brune, 2003; Kim, Hwang, Shin, Kang & Chun, 2002; Gudi, Casteel, Vinson, Boss & Pilz, 2000) are the few of the NO-sensitive regulators. As for bacteria, NO regulatory sensors are SoxR, Fur and NorR (Turpaev, Bouton, Diet, Glatigny, & Drapier, 2005; Nunoshiba, deRojas-Walker, Wishnok, Tannenbaum & Demple, 1993).

2.2.1 Function of NorR

Our focus here would be *norR* gene, which encodes the prokaryotic enhancer binding protein (bEPB) NorR, a σ^{54}-dependent transcription factor sensitive to NO. NorR is encoded by *norR,* which activates the transcription of *norVW* genes found in *E. coli* and itself in the presence of NO (Tucker, Autreaux, Studholme, & Spiro, 2004). *norVW* encodes the NO regulatory protein, flavorubredoxin and its associated oxidoreductase that converts NO to nitrous oxide in anaerobic conditions (Tucker et al., 2006).

However, it was proposed that NorR regulates other target(s) which have not been characterized as transcription is activated under both aerobic and anaerobic conditions (Tucker et al., 2005).

In other bacteria such as *Ralstonia eutropa* and *Pseudomonas aeruginosa*, NorR is also present for the regulation of *norAB,* encoding the respiratory NO reductase (Pohlmann, Cramm, Schmelz & Friedrich, 2000), and *fhp* encodes flavohaemoglobin (Arai, Hayashi, Kuroi, Ishii and Igarashi, 2005). Therefore, it is proposed that NorR is able to regulate genes encoding enzymes and proteins which use NO as a substrate as was found in *E.coli* (Tucker et al., 2006).

2.2.2 Structure of NorR

The structure of NorR is the core of its regulatory function, characterized by scientists whereby it consists of three domains, a tripartite structure, typical of a bacterial enhancer binding protein (bEBP) (Justino, Goncalves, & Saraiva, 2005). The N-terminal domain is the sensor itself called GAF (cGMP specific and stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *E. coli* FhlA) domain. The central domain is the AAA^+ (ATPase associated with various cellular activities) domain and the C-terminal domain is the DNA binding domain called helix-turn-helix (HTH) (Tucker et al., 2006) as shown in Figure 2.1.

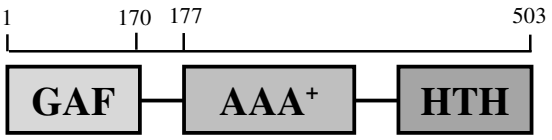


Figure 2.1: Diagrammatic representation of NorR structure

Consisting of GAF, AAA^+ and HTH domains with amino acid positions indicated (Autreaux, Tucker, Dixon, & Spiro, 2005).

2.2.3 Binding of NorR to target site

As an enhancer binding protein and transcription factor, NorR regulates transcription by binding to DNA upstream of the regulated genes, known as UASs (**u**pstream **a**ctivated **s**equences) (Rappas, Bose, & Zhang, 2007). Hence, it was proposed that NorR associates with three binding sites located at the *E. coli norR* and *norVW* integenic region located 75-140 bp upstream of *norV* transcription start site through the HTH domain (Justino et al., 2004).

The proposed sequence at the binding site of NorR was GT-(N_7)-AC which is conserved among proteobacteria. Any disruption of the binding sites would result in transcriptional inactivation of the regulated genes (Tucker et al., 2006).

NorR can bind independently to the affinity sites but would require binding as a trimer for the activation of transcription (Justino et al., 2005). Another binding mechanism was proposed where NorR binds to the three sites as three dimmers (Rappas et al., 2007).

2.2.4 Regulatory Mechanism of NorR

The mechanism which controls the regulatory function of NorR has two prerequisites, the GAF domain and the ATPase activity of AAA^+ domain. An experiment shows that removal of the GAF domain causes constitutive activation of transcriptional activity independent of NO signaling activity (Tucker et al., 2006; Autreaux et al., 2005). Therefore, it was proposed that in an inactive state, when NO not present, GAF domain is suppresses the activity of the AAA^+ domain. This occurrence prevents it from interacting with the σ^{54} factor of RNA polymerase to initiate transcription by the formation of the close complex (Tucker et al., 2006).

The GAF domain contains a five or six coordinated mononuclear non-haem iron centre in its reduced state. The GAF domain senses NO by binding reversibly to NO forming a non-haem iron mono-nitrosyl complex, $\{Fe(NO)\}_7$. The iron centre is essential for transcriptional activation by inducing the formation of an open complex, releasing the suppression activity of GAF domain on the AAA^+ domain (Autreaux et al., 2005).

Once activated by NO and the suppressing activity of the GAF domain is removed, the bending and looping of the DNA is facilitated thus allowing the promoter to be located at the vicinity of the RNA polymerase (Rappas et al., 2007). Besides, the ATPase activity of AAA^+ domain in the presence of ATP promotes nucleotide hydrolysis (Adhya & Garges, 2003; Lengeler, Drews & Schlegel, 1999). Thus, energy is provided for the formation of the open complexes by σ^{54}-RNA polymerase and thus transcriptional initiation is able to proceed (Courey, 2008; Baumberg, 1999).

CHAPTER 3

MATERIALS & METHODS

3.1 Molecular Design

An overview of the molecular design of this project is shown in Figure 3.1, Figure 3.2 and Figure 3.3 as shown in the following pages. The project started off with the amplification of *norR(Full)* from *E. coli* strain DH5α genomic DNA through PCR. *norR(Full)* was used for the subsequent amplification of three variants of *norR* at three different lengths. The first would be a full length *norR* comprising of three sections coding for the GAF domain, AAA^+ domain and the HTH domain and named as *norR(full-NLS)*. The second fragment would include both the GAF domain and AAA^+ domain where the HTH domain would be removed, which was named as *norR(HTH-NLS).* The third would be the same as *norR(HTH-NLS)* but containing the intergenic region between the AAA^+ domain and HTH domain and named as *norR(SACT-NLS).*

For all three gene fragments, the nuclear localization sequence (NLS) and 5'-CACC-3' would be included at the upstream of each. However, only *norR(HTH-NLS)* and *norR(SACT-NLS)* would contain the restriction sites, *Spe*I and *Xho*I downstream of the gene. Hence, the expected length for *norR(full-NLS)*, *norR(HTH-NLS)* and *norR(SACT-NLS)* would be 1,537 bp, 1,049 bp and 1,263 bp, respectively.

Next, the cloning of *norR(full-NLS), norR(HTH-SACT)* and *norR(SACT-NLS)* into pENTR™/D-TOPO® through TOPO® reaction, a technique developed by Invitrogen™, was conducted. The gene-vector construct was transformed into *E. coli* and purified from the positive recombinant colony which was screened through selective kanamycin plate and colony PCR.

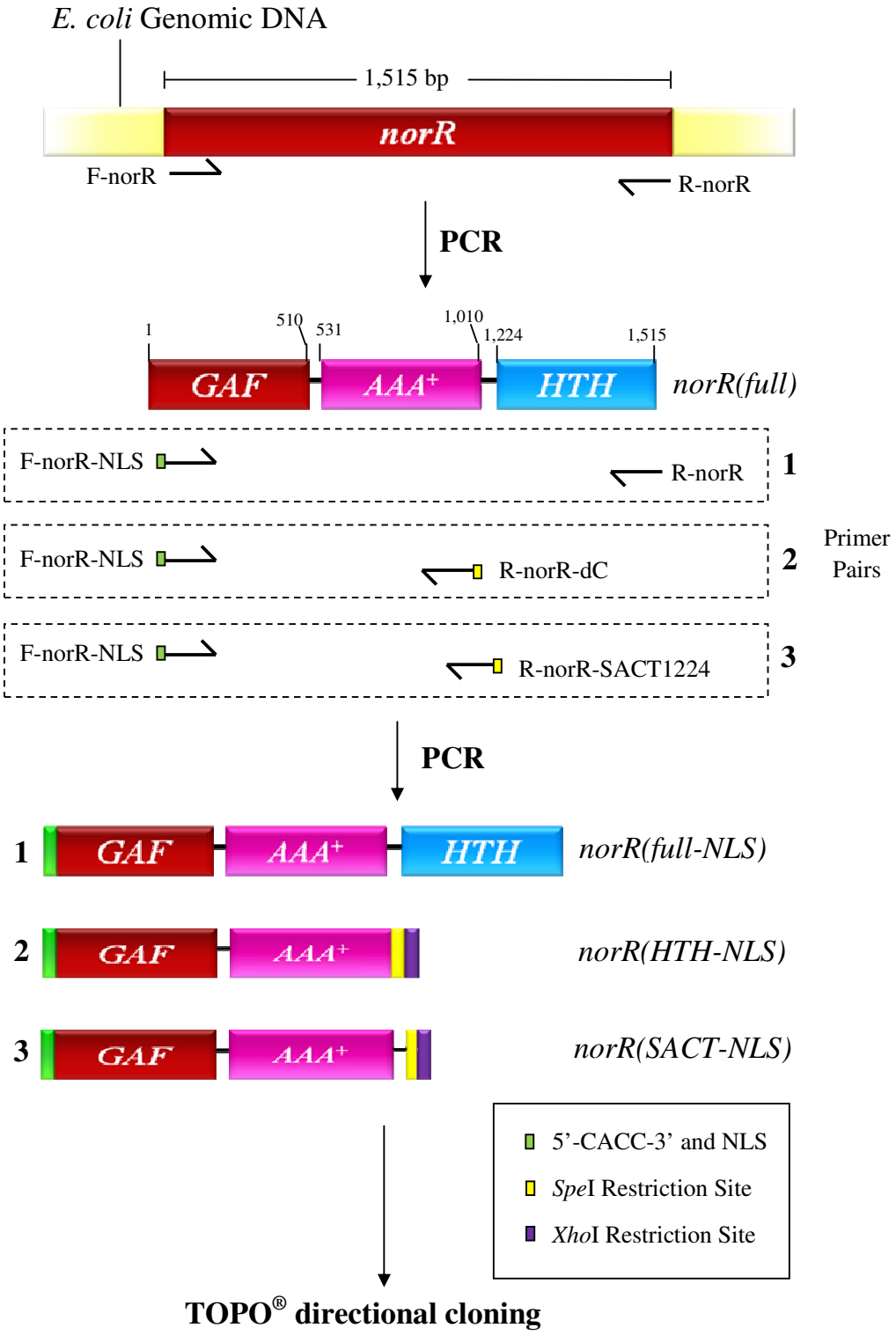

E. coli Genomic DNA
1,515 bp
norR
F-norR
R-norR
PCR
1
510
531
1,010
1,224
1,515
GAF
AAA+
HTH
norR(full)
F-norR-NLS
R-norR
1
F-norR-NLS
R-norR-dC
2
Primer Pairs
F-norR-NLS
R-norR-SACT1224
3
PCR
1
GAF
AAA+
HTH
norR(full-NLS)
2
GAF
AAA+
norR(HTH-NLS)
3
GAF
AAA+
norR(SACT-NLS)
5'-CACC-3' and NLS
SpeI Restriction Site
XhoI Restriction Site
TOPO® directional cloning

Figure 3.1: Diagrammatic representation of amplification of *norR* with different primers

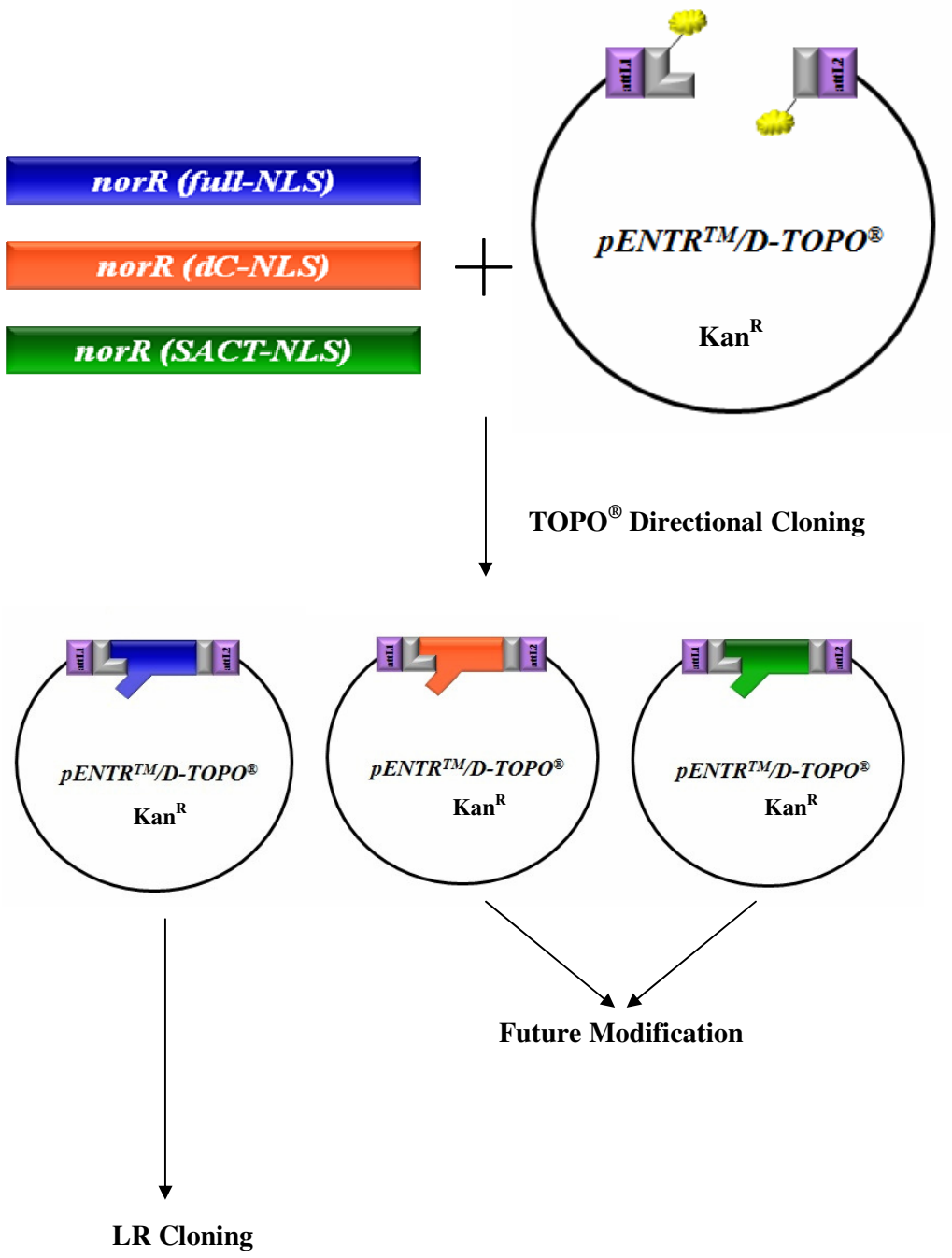


Figure 3.2: Diagrammatic representation of TOPO® directional cloning of *norR* gene fragments into pENTR™/D-TOPO®

3.2 Materials

All materials, equipment and tools were provided by the Department of Biological Science in the Faculty of Science, University Tunku Abdul Rahman and project supervisor, Dr. H. L. Wong.

3.2.1 Tools & Equipment

Table 3.1 shows the list of manufacturers and models regarding the tools and equipment used in this project

Table 3.1: List of tools and equipment

Equipment	Manufacturer	Model
Desktop Refrigerated Centrifuge	Dynamica	Velocity 14R centrifuge
Electroporator	Eppendorf	Electroporator 2510
Evaporator	EYELA	Centrifugal Evaporator cve-2000
Incubator	Memmert	
Incubator shaker	N-Biotek	HandyLAB® System
Microcentrifuge	Thermo Electron Corporation	Sorvall Legend Micro 17 Centrifuge
Nanospectrometer	Implen	
PCR cycler	Eppendorf	Mastercycle gradient
PCR cycler	BIOER	Gene Pro
pH meter	Mettler Toledo	Delta 320
Spectrometer	BIO-RAD	SmartSpec™
UV Transilluminator	UVP	MultiDoc-It Digital Imaging System
Water bath	Memmert	

3.2.2 General Materials & Reagents

In addition, Table 3.2 shows the general materials and reagents utilized.

Table 3.2: List of general materials and reagents

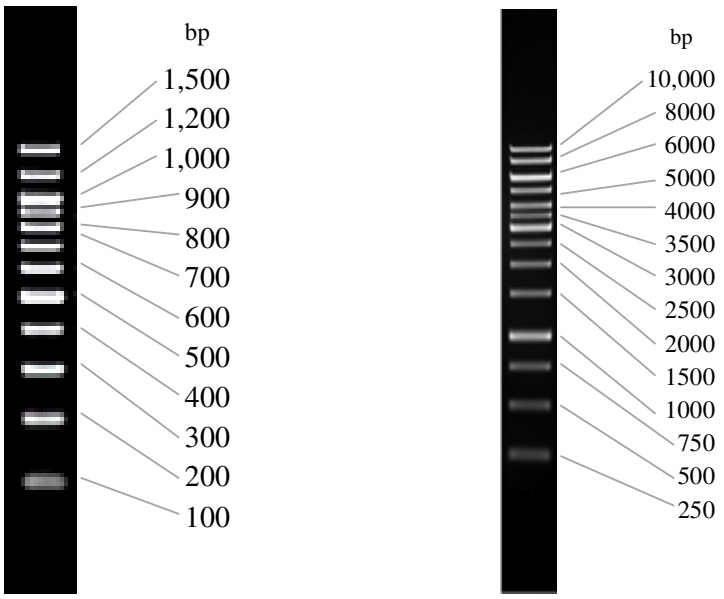
Reagents	Manufacturer	Form	Storage
100 % Glycerol	QRëC™	Solution	RT[1]
95 % Ethanol	Copens Scientific (M) Sdn. Bhd.	Solution	RT
Absolute Ethanol	HmbG® Chemical	Solution	RT
Acetic Acid	SYSTERM®	Solution	RT
Agar-agar Powder	R & M	Powder	RT
Agarose Powder	Vivantis	Powder	RT
Boric Acid	QRëC™	Powder	RT
Calcium Chloride Dehydrate	QRëC™	Powder	RT
EDTA	QRëC™	Powder	RT
Ethidium Bromide	BIO BASIC INC.	Solution	RT (Dark)
Glucose (Dextrose)	Rdeh	Powder	RT
Hydrochloric Acid	MERCK	Solution	RT
Isopropanol	MERCK	Solution	RT
Magnesium Chloride	QRëC™	Powder	RT
PCI	CALBIOCHEM	Solution	4°C
Potassium Acetate	MERCK	Powder	RT
Sodium Acetate	SYSTERM®	Powder	RT
Sodium Chloride	SYSTERM®	Powder	RT
Sodium Hydroxide	R & M	Powder	RT
Tris	Vivantis	Powder	RT
Tris-HCl	BIO BASIC INC.	Powder	RT
Tryptone	CONDA pronadisa	Powder	RT
Yeast Extract	Scharlau	Powder	RT

[1]Room Temperature

3.2.3 Molecular Reagents

3.2.3.2 DNA Ladder

DNA ladders used for agarose gel electrophoresis were GeneRuler™ 1 kb DNA ladder from Fermentas and 100 bp DNA Ladder One from Nacalai Tesque. Both were stored in 4°C for short-term storage and -20°C for long term storage. Figure 3.5 shows the size of each band for the DNA ladders.



(a) (b)

Figure 3.5: Bands size of DNA ladders

(a) 100 bp DNA Ladder One (Nacalai Tesque, n.d.) and (b) GeneRuler™ 1 kb DNA ladder (Thermo Fisher Scientific Inc., 2011)

3.2.3.3 PCR reagents

The reagents needed for PCR were Taq polymerase, buffers, $MgCl_2$, primers and dNTPs. $MgCl_2$ was obtained from Fermentas at 25 mM which was used along with Taq DNA Polymerase (recombinant). The dNTPs utilized was at 2 µM from the

manufacturer, TOYOBO and TAKARA. The details for Taq polymerase and primers are shown in Table 3.3 and 3.4 respectively.

Table 3.3: Details on Taq Polymerase used for PCR

Taq Polymerase	Buffer	Manufacturer	Storage
Taq DNA Polymerase (recombinant)	10X Taq Buffer with $(NH_4)_2SO_4$	Fermentas	-20°C
Ex Taq DNA Polymerase	10X Ex Taq Buffer + $MgCl_2$	Takara	-20°C
LA Taq DNA Polymerase	10X LA Taq Buffer (GC II)	Takara	-20°C
PrimeSTAR HS DNA polymerase	5X PrimeSTAR Buffer- Mg^{2+} plus	Takara	-20°C

3.2.3.4 Sequencing Primers

Only the primer, attB1 Reverse manufactured by Invitrogen® was sent along with the samples to 1st BASE Laboratories at 10 µM in solution form. The other primers were provided by 1st BASE Laboratories as they were considered as universal primers. The details of the primers are shown in Table 3.6.

Table 3.6: Details on sequencing primers

Plasmid	Concentration	bp
M13 Forward (-20)	5'-GTA AAA CGA CGG CCA G-3'	16
M13 Reverse – pUC (-26)	5'-CAG GAA ACA GCT ATG AC-3'	17
T7 Promoter Forward	5'-TAA TAC GAC TCA CTA TAG GG-3'	20
attB1 Reverse	5'-CTT TGT ACA AGA AAG CTG GGT-3'	21

3.2.3.5 Kits

Table 3.7: Details on Kits used in this project

Kits	Manufacturer	Catalog No.
Montage® PCR Centrifugal Filter Devices (Montage® Life Science Kits)	MILIPORE	P36461
pENTR™/D-TOPO® Cloning Kit	Invitrogen®	K2400-20

3.2.3.6 Restriction Enzymes and Buffers

All restriction enzymes and buffers were purchased from Roche Diagnostics and stored in -20°C. Restriction enzymes used were *Not*I and *Eco*RV. Buffers used were Sure/Cut Buffer H for Restriction Enzymes and Sure/Cut Buffer B for Restriction Enzymes, respectively.

3.2.4 *Escherichia coli* strains

Two strains of *E. coli* were used in this project. Strain DH5α (F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(rk^-, mk^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-) (Invitrogen™, 2006b) was used for the extraction of total DNA and also transformation with pENTR™/D-TOPO® inserted with *norR(full-NLS)*. On the other hand, One Shot® Mach1™-$T1^R$ Chemically Competent *E. coli* (F- ϕ 80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R(rK^-mK^+) Δ*rec*A1398 *end*A1 *ton*A) (Invitrogen™, 2006a) were utilized for the heat shock transformation of pENTR™/D-TOPO® inserted with *norR(HTH-NLS)* or *norR(SACT-NLS)* inserted with *norR(Full-NLS)*.

3.3.5 Formulation of Solutions and Medium used

3.3.5.1 Culture Medium

Two types of medium were used in this project were yeast extract and tryptone (2x YT) and super optimal broth with catabolite repression (S.O.C) medium.

The ingredients mentioned in Table 3.8 were dissolved in 1 L of distilled water and the resulting solution's pH was adjusted to 7.0 and autoclaved. Three grams of agar-agar powder was added to solution before autoclaving to make agar medium. 2x YT medium were used for the cultivation of transformed and untransformed *E. coli*.

Table 3.8: Recipe of 2x YT medium

Content	Volume (g)
Tryptone	16.0
Yeast Extract	10.0
NaCl	5.0

Table 3.9: Recipe of S.O.C medium

Content	Volume (g)
Tryptone	20.0
Yeast Extract	5.0
NaCl	0.5

The ingredients mentioned in Table 3.9 were dissolved in 990 ml of distilled water and 10.0 ml of 250 mM KCl was added. The pH of the resulting solution was adjusted to 7.0 and autoclaved. Before used, 5.0 ml of sterile solution of 2 M of $MgCl_2$ and

20.0 ml of sterile 1 M of glucose was added. S.O.C medium was used to revive transformed competent cells before plating.

3.3.5.2 Antibiotics

Antibiotics were used for selecting transformed colonies by antibiotic selective plates.

Table 3.10: Antibiotic stock solutions

Antibiotic	Salt	Form	Manufacturer	Stock Concentration (mg/µl)	Volume of stock per 100 ml medium (µl)	Final Concentration (µg/ml)
Kan	Kanamycin sulfate	Powder	Wako	50	100	40
Amp	Ampicilin sodium	Powder	Wako	100	100	100

The antibiotics were prepared by dissolving in distilled water and filter sterilized. Appropriate volume of stock solution was added to the culture medium to obtain the desired concentration. The antibiotics were stored in 4°C.

3.3.5.3 Solutions for Total DNA and Plasmid Extraction

One molar of Tris-HCl, 0.5 M EDTA, 1 N NaOH, 1% SDS, 5 M Potassium acetate and 1 N NaOH were obtained through dissolving the pure powdered form of the chemical with appropriate volume of distilled water and autoclaved. The pH of the solutions was adjusted accordingly. Table 3.11 shows the recipe of solutions used for total DNA extraction and plasmid extraction.

Table 3.11: Recipe of solutions used in total DNA and plasmid extraction

Solutions	Content	Final Concentration	pH	Sterilization	Storage
TE Buffer	1 M Tris-HCl (pH 8.0)	10 mM	8.0	Autoclaved	RT[1]
	0.5 M EDTA (pH 8.0)	1 mM			
Solution 1	Glucose	50 mM	-	Autoclaved	RT
	1 M Tris-HCl (pH 8.0)	25 mM			
	0.5 M EDTA (pH 8.0)	1 mM			
Solution 2	1 N NaOH	0.2 N	-	Filter sterilized	4°C
	15 % SDS	1 %			
Solution 3	5 M Potassium acetate	3 M	-	Autoclaved	RT
	Acetic acid (glacial)				
DNA extraction solution	1 M Tris-HCl (pH 8.0)	200mM	-	Filter sterilized	4°C
	1 M NaCl	250mM			
	0.5 M EDTA	25mM			
	15% SDS	0.5 %			

[1] RT = Room temperature

3.4 Methodology

3.4.5 *Escherichia coli* Genomic DNA Extraction

The basic procedures for genomic DNA extraction were illustrated in Figure 3.6. Firstly, *E. coli* strain DH5α was utilized as the total DNA would serve as a template for the PCR of *norR*. Besides, the cell culture prepared should utilize DH5α from a fresh plate, about one to three days old to ensure the genetic stability of the cells.

The crucial part for DNA isolation would be the utilization of PCI, which contain phenol, chloroform and isoamyl alcohol at 25:24:1, for separation and purification. PCI serves to separate the mixture into a biphasic mixture through centrifugation to give an upper aqueous phase and lower organic phase. DNA and RNA which are soluble in aqueous solution cover the upper phase while protein will be separated through the organic phase. At this stage, pippetting technique should be precise to avoid any contact with the inter-phase of both layers.

Isopropanol served to precipitate the DNA including RNA and 70% chilled ethanol was used to wash the pellet to remove any salt and other contaminating solutions. The drying of the pellet was conducted with a vacuum evaporator. However, before PCR could be carried out, the genomic DNA was subjected to RNase treatment to remove any RNA which may interfere with subsequent steps.

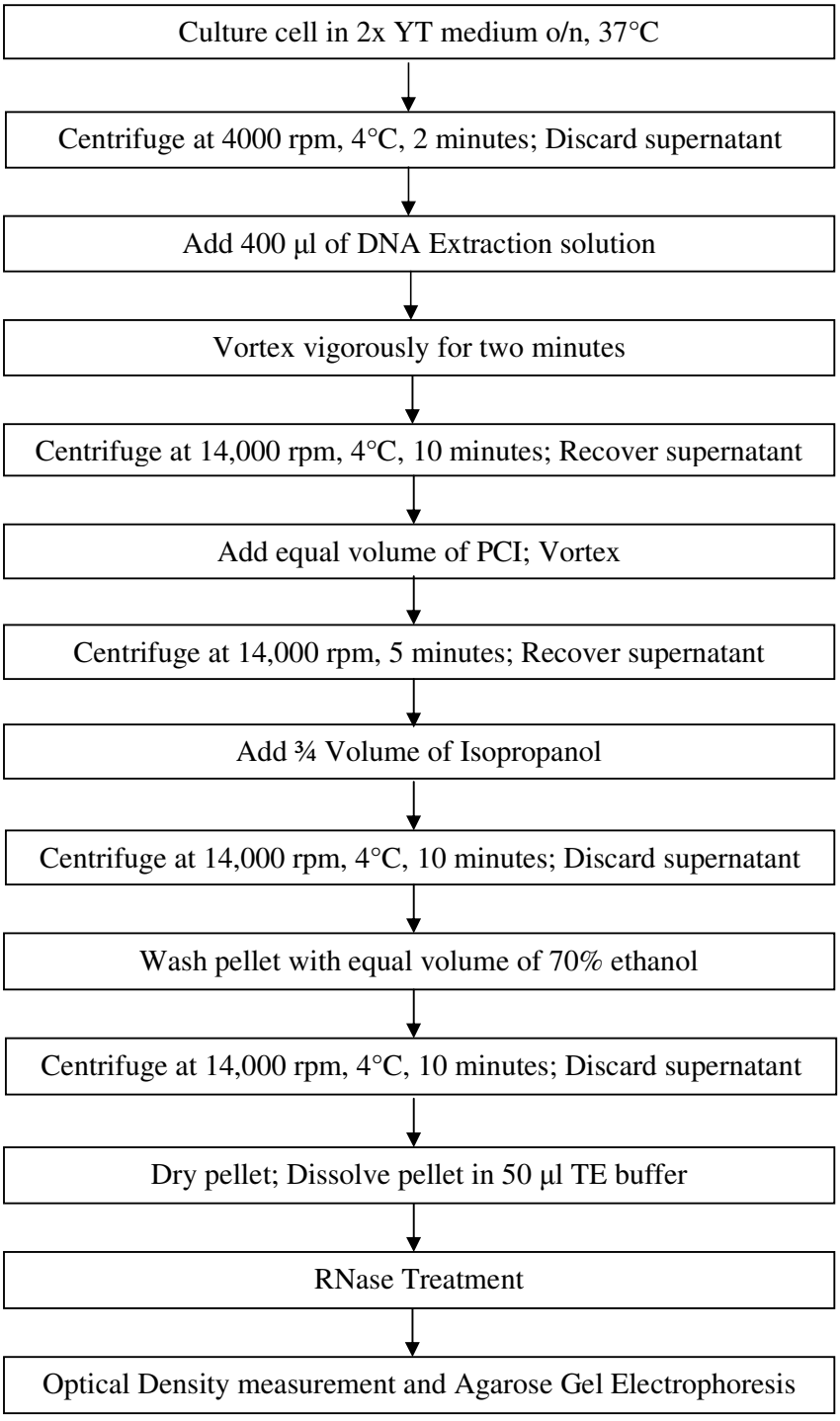


Figure 3.6: Illustrative flow of procedures for genomic DNA extraction from *Escherichia coli*

3.4.6 Polymerase Chain Reaction (PCR)

Firstly, a master mix was prepared by adding the solutions as stated in Table 3.12 into a microcentrifuge tube and the DNA template and primers used were added according to Table 3.13.

Table 3.12: Master Mix preparation for the PCR of *norR (full), norR (full-NLS), norR (HTH-NLS),* and *norR (SACT-NLS)* with PrimeStar Taq Polymerase

Solution	**[ ]Initial**	**[ ]Final**	**Volume (µl)**
PCR buffer	5x	1x	4.0
dNTPs	2.0mM	0.2mM	2.0
Primer 1	10µM	0.4µM	0.8
Primer 2	10µM	0.4µM	0.8
PrimeStar Taq Polymerase	-	-	0.2
ddH_2O	-	-	Top up
Total	-	-	20.0

Next, 9.5 µl of the master mix was pipetted into a PCR tube and 0.5 µl of the DNA template was added in. The tube was placed in a PCR thermal cycler and the PCR was carried out with the following condition for amplification of *norR* (all four fragments) according to Table 3.14 .

Table 3.14: PCR condition for amplification of *norR*

Temperature	**Time**	**Cycle**
96.0	15 seconds	1
96.0	15 seconds	

55.4	5 seconds	25
70	2 minutes 30 seconds	
70	2 minutes	1
10	∞	1

3.4.7 Purification of PCR Products

There were two ways of purifying the PCR products. Firstly was the use of a purification kit, Montage^{TM} life science kit to remove any primer dimmers as the principle of the kit was to remove any nucleotides at the size below of 200 bp. The protocols for Montage^{TM} life science kit was followed according to the instruction manual provided by Milipore (2005). This purification step only applied to PCR product of *norR(Full)* which was used as template for subsequent PCR.

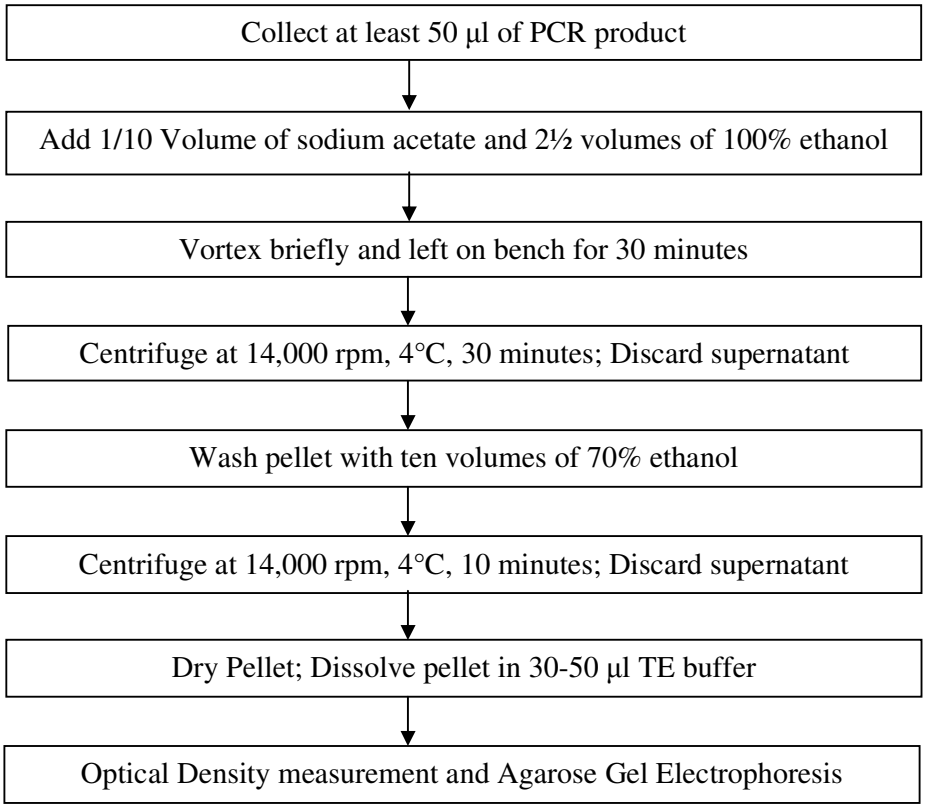


Figure 3.7: Illustrative flow of the procedure for purification of PCR product through ethanol precipitation

3.4.8 Directional TOPO® Cloning Reaction

The protocol for directional TOPO® Cloning was obtained from pENTR™ Directonal TOPO® Cloning Kits instruction manual (Invitrogen™, 2006). The TOPO® Cloning was set up according to Table 3.16.

Table 3.16: Reaction mixture for TOPO® cloning reaction with pENTR™/D-TOPO®

Solution	**Volume (µl)**	
	Chemically competent *E. coli*	**Electrocompetent *E. coli***
Purified PCR Product	0.5	0.5
Salt solution	1	-
Diluted salt solution (1:4)	-	1
ddH_2O	3.5	3.5
TOPO® vector	1	1
Total	6	6

The diluted salt solution was used if TOPO® reaction would be used for transformation with electroporation to prevent arching. The diluted salt solution was prepared using 1 µl of salt solution and was diluted with 4 µl of ddH_2O.

As a precaution, the mixture was mixed gently and not vortexed or shaken vigorously. Then, the mixture was incubated at 22°C for 30 minutes. After that, the reaction mixture was placed on ice and was used to transform with *E. coli* competent cells.

3.4.9 Transformation by Electroporation

Firstly, 1 µl of the TOPO® cloning reaction were added into 40 µl of *E. coli* electrocompetent cells and transferred into a chilled 0.1 cm electroporation cuvette. As a precaution, the cells were not mixed by pipetting up and down. The cuvette was incubated on ice for 6 minutes.

Next, the cuvett were placed into the electroporator and electroporated at 1600 V. Immediately, 250 µl of 37°C S.O.C. medium was used to re-suspend the cells. The mixture was transferred into a microcentrifuge tube and agitated at 37°C for 1 hour. A volume of 100 µl to 150 µl of the transformation reaction was spread onto pre-warmed 50 µg/ml Kanamycin selective plate and incubated at 37°C overnight.

3.4.10 Transformation by the Heat Shock Method

Firstly, 1 µl of the TOPO® cloning reaction was added to 25 µl of *E. coli* chemically competent One shot® Mach 1™ cells and mixed gently in a microcentrifuge tube. The mixture was incubated on ice for 30 minutes.

Next, the cells were heat shocked for 90 seconds by being placed into a 42°C water bath. Immediately, the tube were transferred back into ice and incubated for 10 minutes. After that, 250 µl pre-warmed S.O.C medium was added to the cells and incubated at 37°C for 1 hour with agitation.

Similar to transformation with electroporation, 100 µl to 15 0µl of the transformation reaction was plated onto its respective selective plate, where 40 µg/ml Kanamycin for pENTR™/D-TOPO®.The plates were incubated at 37°C overnight.

3.4.11 Colony PCR

Firstly, colonies that were growing on antibiotic selective plate were picked by using a yellow pipette tip and touched onto a Master plate before being inserted into a PCR tube. The end of the pipette tip was pressed against the inside wall of the PCR tube gently. Next, the PCR tube was placed in boiling water for 10 minutes to lyse the cells.

Meanwhile, a master mix was prepared by adding the solutions according to Table 3.18 when Ex-Taq DNA Polymerase was used and Table 3.19 when LA Taq DNA polymerase was used. LA-Taq DNA polymerase was used when screening vectors recombinant with *norR(full-NLS)* and Ex-Taq DNA polymerase was used for *nor(dC-NLS)* and *norR(SACT-NLS).*

Table 3.18: Reaction mixture for colony PCR using Ex-Taq Polymerase

Solution	**[ ]$_{Initial}$**	**[ ]$_{Final}$**	**Volume (µl)**
PCR Buffer	10x	1x	1.00
dNTP	2.5 mM	0.2 mM	0.60
Primer 1	10 µM	1 µM	1.00
Primer 2	10 µM	1 µM	1.00
Ex-Taq Polymerase	-	-	0.02
ddH_2O	-	-	Top up
Total	-	-	10.00

Table 3.19: Reaction mixture for colony PCR using LA Taq Polymerase

Solution	**[ ]Initial**	**[ ]Final**	**Volume (µl)**
PCR Buffer (GCII)	2x	1x	5.00
dNTP	2.5 mM	0.2 mM	0.80
Primer 1	10 µM	0.8 µM	0.80
Primer 2	10 µM	0.8 µM	0.80
LA Taq Polymerase	-	-	0.02
ddH_2O	-	-	Top up
Total	-	-	10.00

Primer 1 and Primer 2 were used according the vector that was screened as shown in Table 3.20

Table 3.20: Primers used for colony PCR screening of each vector

Vector	**Primer 1**	**Primer 2**
pENTR™/D-TOPO®	M13 Forward	M13 Reverse
	M13 Forward	Reverse Primer for Insert

Then, 10 µl was pipetted into the previous PCR tube and placed in a PCR cycler where PCR were carried out at the following conditions as shown in Table 3.21.

Table 3.21: Condition for PCR colony

Temperature	Time	Cycle
94°C	5 minutes	1
94°C	30 seconds	35
55°C	1 minute	
72°C	2 minute	
72°C	5 minutes	1
10°C	∞	1

The final PCR product were analysed by agarose gel electrophoresis.

3.4.12 Small Scale Plasmid Extraction (Miniprep)

Plasmid extraction was conducted to recover the recombinant plasmid from positive transformants selected through selective plates and colony PCR. The methods are illustrated in Figure 3.8.

The crucial part of plasmid extraction was to avoid genomic DNA contamination. As cells were lysed to obtain the DNA, both plasmid and genomic DNA would be released. Thus, in this procedure, the lysis procedure with Solution II should be carried out gently by inverting the tube without vortex. The purpose was to prevent the release and fragmentation of genomic DNA causing difficulties in purification in the later steps.

Besides, precise timing of incubation in ice and addition of Solution III should be taken note of. Furthermore, PCI purification step should be repeated to completely remove genomic DNA.

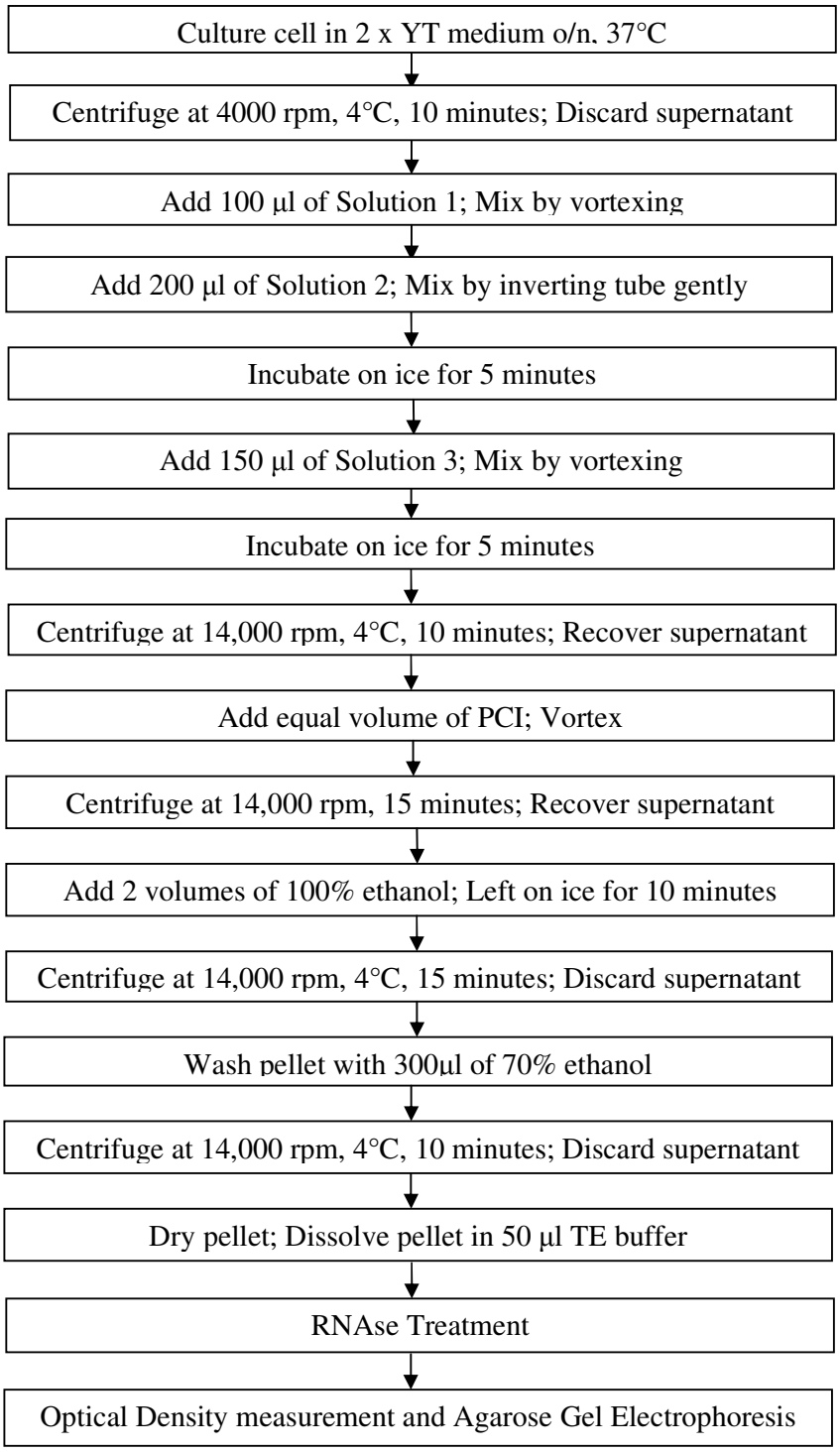


Figure 3.8: Illustrative flow of the procedures for plasmid extraction

3.4.13 Restriction Enzyme Digestion

Restriction enzyme digestion was used as part of the screening of recombinant plasmid, pENTR™/D-TOPO®. The purpose of this step was to ensure the plasmid contained the desired gene. Both enzymes were used for single and double digestion of the plasmid. The restriction site for these enzymes and the position of these sites on pENTR™/D-TOPO® is shown in Table 3.22.

Table 3.22: Sequence and position of restriction site of restriction enzyme for digestion on pENTR™/D-TOPO®

Restriction Enzyme	Restriction Site	Position on pENTR™/D-TOPO®
*Not*I	5'-GC'GGCCGC-3'	Base 673
*Eco*RV	5'-GAT'ATC-3'	Base 818

Thus, in order to conduct a restriction enzyme digestion, the mixture as shown in Table 3.23 for single digestion or Table 3.24 for double digestion was prepared. Then, the mixture was incubated at 37°C, which is the optimal temperature for the activity of both enzymes.

Table 3.23: Reaction mixture for single digestion of either *Not*1 or *Eco*RV

Solutions	Volume (µl)
Restriction Enzyme[4]	0.5
Buffer[5]	1.0
Plasmid	1.0
ddH_2O	7.5
Total	10.0

[4] *Not*I or *Eco*RV
[5] Buffer H for *Not*I and Buffer B for *Eco*RV

Table 3.24: Reaction mixture of double digestion of *Not*I and *Eco*RV

Solutions	Volume (µl)
*Not*I	0.5
*Eco*RV	0.5
Buffer H	1.0
Plasmid	1.0
ddH_2O	7.0
Total	10.0

3.4.14 RNAse Treatment

RNase treatment was carried out by adding 1 µl of RNAse into 50 µl of sample and incubated at 37°C in a water bath for one hour to remove any RNA in a DNA extraction step.

After incubation, any traces of RNase were removed by phenol chloroform treatment and ethanol precipitation whereby the solution was added with equal volume of PCI and vortexed briefly. Next, the mixture was centrifuged at 14,000 rpm for 5 minutes at room temperature with a desktop centrifuge.

As in any phenol chloroform purification in this project, the upper aqueous layer was obtained and transferred to a new microcentrifuge tube. 1/10 volume of sodium acetate and 2½ volumes of 100% ethanol was added, vortexed, followed by being left to stand on the bench for 30 minutes. Later, the mixture was centrifuged at 14,000 rpm for 30 minutes at 4°C.

Next, the pellet was washed with ten volumes of 70% ethanol and centrifuge at 14,000 rpm, for 10 minutes at 4°C. The supernatant was discarded and the pellet was dried. Lastly, the dried pellet was dissolved with 30 µl to 50 µl of TE buffer depending on the size of the pellet.

3.4.15 Optical Density Measurement

The measurement of optical density at specific wavelength through the use of an ultraviolet (UV) spectrometer served as a reference for the determination of concentration, yield and purity of DNA.

The samples were measured by using a Nanospectrometer, which provides the ease of using smaller volumes (1µl) of nucleic acid samples compared to a conventional UV spectrometer.

The parameters for Nanospectrometer was set at double stranded DNA and at lid factor ten for purified PCR products and lid factor of fifty for purified *E.coli* Genomic DNA and plasmid. Firstly, 1 µl of TE buffer was used to blank the instrument. Then, 1 µl of each samples were used to obtain the absorbance at A_{260} and A_{280}, concentration in ng/µl and A_{260}/A_{280} ratio. A_{260}/A_{280} ratio of 1.8 to 2.0 indicated a relatively pure DNA.

3.4.16 Agarose Gel Electrophoresis

Agarose gel electrophoresis provide a means of viewing the results of genomic DNA extraction, PCR and plasmid extraction. The agarose concentration and DNA ladder varied with each sample besides of the duration for the electrophoresis as shown in

Table 3.26. The parameters of the electrophoresis were set to 80 V and 200 mA and the buffer used in the electrophoresis tank was 1 x TBE buffer.

Once the running process was completed, the gel was transferred to ethidium bromide solution for staining for five to fifteen minutes. Then, the gel was de-stained by submerging it in clean water and thus it was able to be viewed under a UV transilluminator.

3.4.17 Electrocompetent Cells Preparation

In order to achieve ease of transformation, competent cells should be prepared to give high transformation efficiency which can be aided by making sure that all materials and solutions used were sterilized. Besides, it was especially important to keep all materials and solutions chilled. An additional level of sterility can be achieved by washing all microcentrifuge tubes and centrifuge tubes with sterilized deionised water before usage to remove any residues left during manufacturing.

Firstly, *E. coli* strain, DH5α, was inoculated into 2 ml 2 x YT broth and incubated at 37°C with agitation overnight. After that, the optical density of the cell culture was measured with a spectrometer at 600 nm and the cell culture was diluted to absorbance 0.1 with appropriate amount of 2 x YT broth. The culture was incubated again at 37°C until the optical density reached 0.4 to 0.8.

Next, the cell culture was placed in ice for about 30 minutes. Then, the cells were collected by centrifugation at 3000 g at 4°C for 10 minutes and the supernatant was discarded as much as possible.

The crucial part of preparing electrocompetent cells is to make sure that no salt should remain or be introduced to avoid arcing during electroporation. Therefore, the cell pellet were washed with 10% glycerol and centrifuged at 3000 g at 4°C for 10 minutes where the supernatant was discarded. This step was repeated two times where each time the volume of the 10% glycerol was reduced from equal to half to a quarter.

After the final centrifugation, the pellet was re-suspended with 400 µl of 10% glycerol and 40 µl of the mixture was aliquot into microcentrifuge tubes. Next, the tubes were frozen in liquid nitrogen and stored at -80°C.

CHAPTER 4

RESULTS

4.1 *Escherichia coli* Genomic DNA Extraction

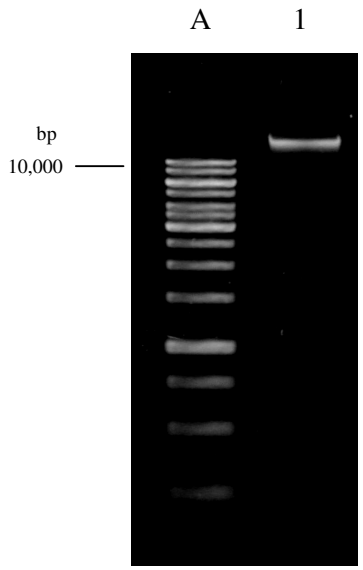


Figure 4.1: Agarose gel electrophoresis result on genomic DNA extracted from *E. coli* strain DH5α

Lane A : 1 kb ladder

Lane 1 : *E. coli* strain DH5α Genomic DNA

Total DNA of *E.coli* strain DH5α was extracted to give a single band above 10 kb. $A_{260/280}$ ratio of 1.833 indicated that the DNA extracted was relatively pure. The estimated DNA concentration was 38.5 ng/µl.

4.2 Polymerase Chain Reaction

4.2.1 *norR(full), norR(full-NLS), norR(HTH-NLS)* and *norR(SACT-NLS)*

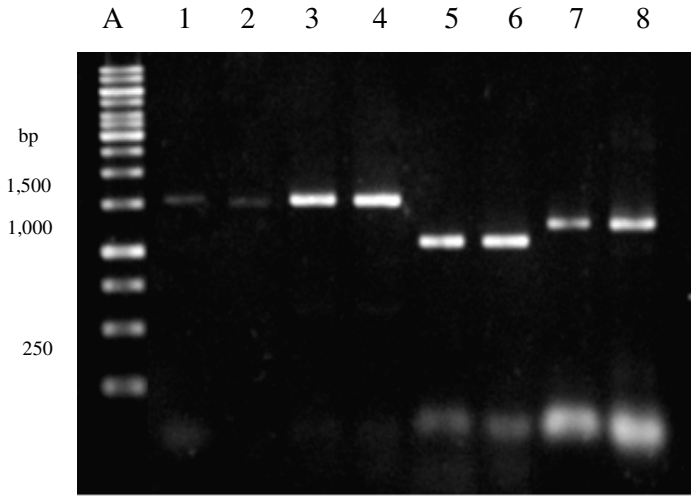


Figure 4.2: Agarose gel electrophoresis results of PCR amplified and purified *norR* gene fragments

Lane A : 1 kb ladder

Lane 1, 3, 5, 7 : PCR products of *norR (Full), norR(full-NLS), norR(HTH-NLS)* and *norR(SACT-NLS),* respectively

Lane 2 : Purified *norR(full)* PCR product with Montage^{TM} life science kit

Lane 4, 6, 8 : Purified *norR(full-NLS), norR(HTH-NLS)* and *norR(SACT-NLS)* PCR products with ethanol precipitation, respectively

PCR product of *norR(full)* was obtained at the expected size of more than 1.5 kb. Besides, it was used as DNA template for the PCR amplification of *norR(full-NLS), norR(HTH-NLS)* and *norR(SACT-NLS)*. Thus, PCR product of *norR(full)* was purified with Montage^{TM} life science kit to remove any primer dimmers as can be seen from the absence of bands lower than 250 bp in Lane 3.

Beforehand, in order to successfully obtain PCR products of *norR(full-NLS), norR (HTH-NLS)* and *norR(SACT-NLS)* from the DNA template, PCR product of *norR(full)*, PCR optimization was performed. Only PCR optimization of *norR(full-NLS)* was shown as all three fragments utilized the same optimized PCR condition as shown in Figure 4.3.

PCR products of *norR(full-NLS), norR(HTH-NLS)* and *norR(SACT-NLS)* was obtained at the expected sizes, more than 1.5 kb, 1 kb and 1.2 kb respectively. The purpose of ethanol precipitation was to remove any salt, primers, enzymes, residues from the PCR reaction and also to concentrate the products. $A_{260/280}$ measured on the purified PCR products indicated all three to be relatively pure showing 1.772, 1.698 and 1.714 respectively and estimated concentration was 158 ng/µl, 168 ng/µl and 164 ng/µl respectively. Thus, TOPO® cloning with these products could proceed.

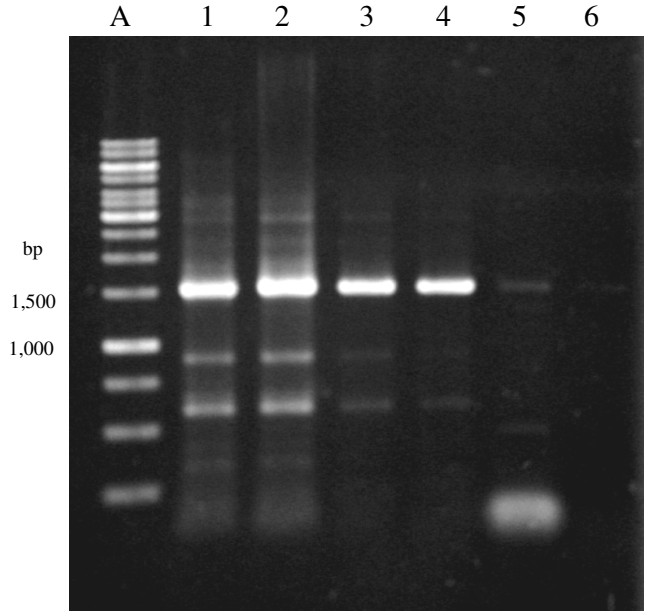


Figure 4.3: Agarose gel electrophoresis results for PCR optimization of *norR(full-NLS)* with serial dilution and gradient PCR

Lane A : 1 kb ladder

Lane 1, 2, 3, 4 : PCR products of *norR(full-NLS)* from undiluted, 10x, 100x and 1000x dilution of DNA template, respectively

Lane 5, 6 : PCR products of *norR(full-NLS)* at annealing temperature of 54°C and 56.8°C, respectively

A 10x serial dilution was carried out on the DNA template with TE buffer. The results showed that at 1000x dilution, a single band of the expected size of more than 1.5 kb of *norR(full-NLS)* was obtained. Where else, for 1x, 10x and 100x dilution, non-specific bands above and below the expected band were obtained. Besides, a gradient PCR from 54°C to 60°C was carried out. However, only annealing temperature at 56.8 °C showed a single band at the expected size while 54°C showed non-specific bands and temperature above 56.8°C showed no band.

4.3 TOPO® Cloning Reaction & Transformation

4.3.1 pENTR™/D-TOPO® and *norR(full-NLS)* Construct

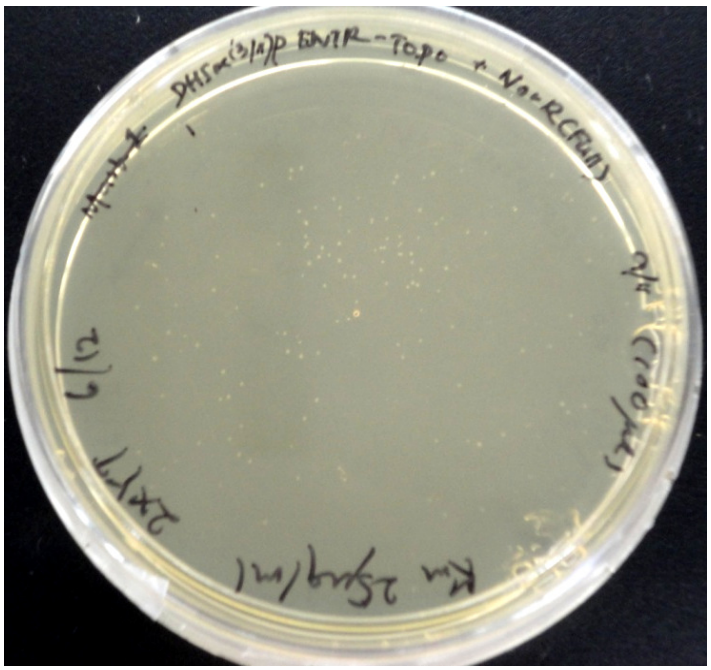


Figure 4.5: pENTR™/D-TOPO® recombinant with *norR(full-NLS)* transformed DH5α colonies on 25µg/ml Kanamycin added 2xYT selective plate [Plate 1]

TOPO® cloning of *norR(full-NLS)* into pENTR™/D-TOPO® was performed and the recombinant plasmid construct was transformed into *E. coli* strain DH5α electrocompetent cells with electroporation and positively selected on Kanamycin plate. As shown in Figure 4.5, about 143 colonies had grown on the selective plate. Next, colony PCR was carried out to select positive recombinants with the correct orientation.

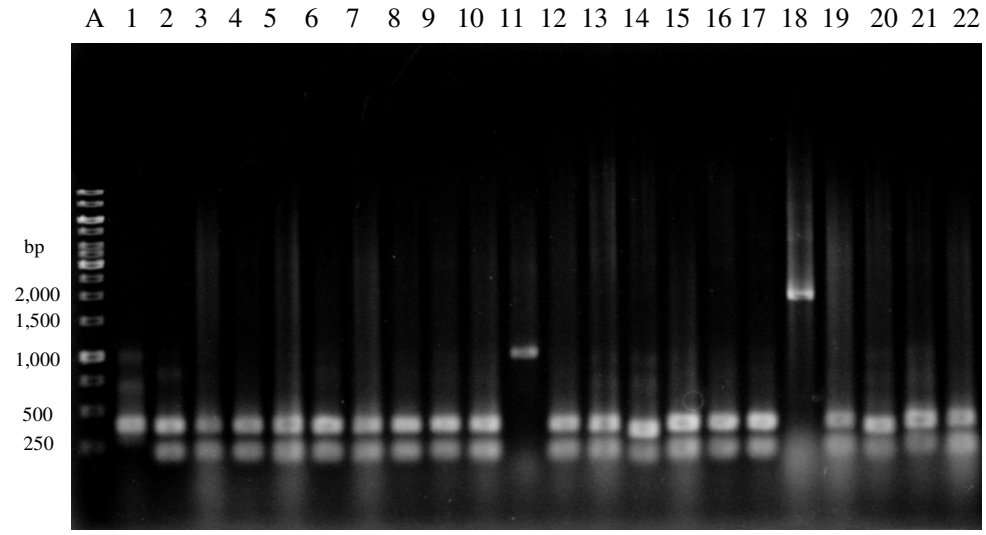


Figure 4.6: Agarose gel electrophoresis results on colony PCR conducted on colonies of Plate 1 by using primers F-M13 and R-M13

Lane A : 1 kb ladder

Lane 1-22 : Colony PCR products for colonies 1-22 of Plate 1

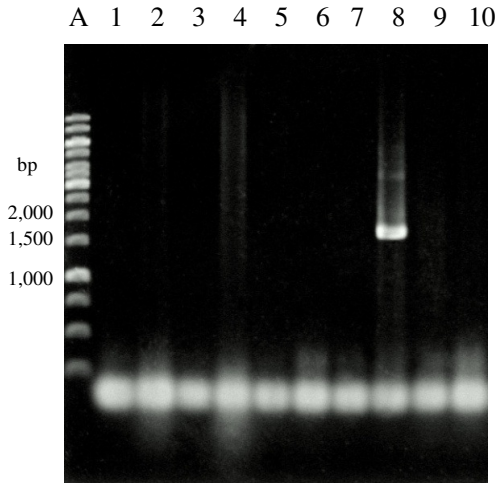


Figure 4.7: Agarose gel electrophoresis results on colony PCR conducted on colonies of Plate 1 using primers F-norR-NLS and R-M13

Lane A : 1 kb ladder

Lane 1-10 : Colony PCR products for colonies 11-20 of Plate 1

Colony PCR was performed twice on the colonies on Plate 1. The first round of colony PCR was performed on 22 randomly selected colonies with primers F-M13 and R-M13. The second round was performed on colonies 11 to 20 of the first round with primers F-norR-NLS and R-M13.

From the results as shown in Figure 4.6 and 4.7, the desired bands at more than 1.8 kb and 1.6 kb with different sets of primers, respectively were found in both rounds for colony 18. Thus, the colony successfully transformed with *norR(full-NLS)* recombinant into pENTR™/D-TOPO® was colony 18 and was designated as B8.

4.3.2 pENTR™/D-TOPO® and *norR(HTH-NLS)* Construct

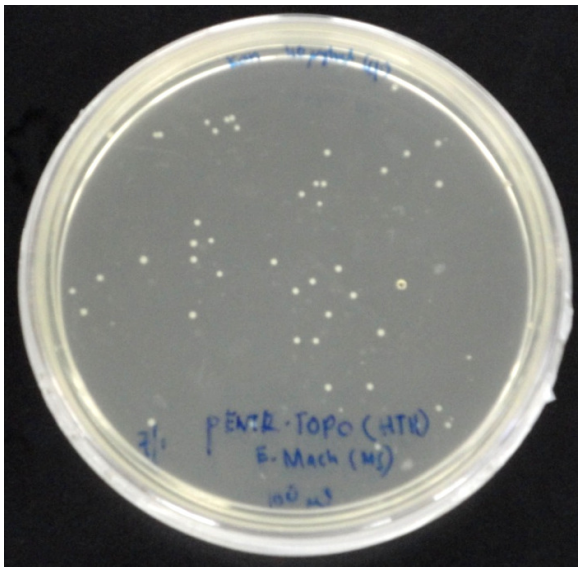


Figure 4.8: pENTR™/D-TOPO® recombinant with *norR(HTH-NLS)* transformed Mach1 colonies on 40µg/ml Kanamycin added 2xYT selective plate [Plate 2]

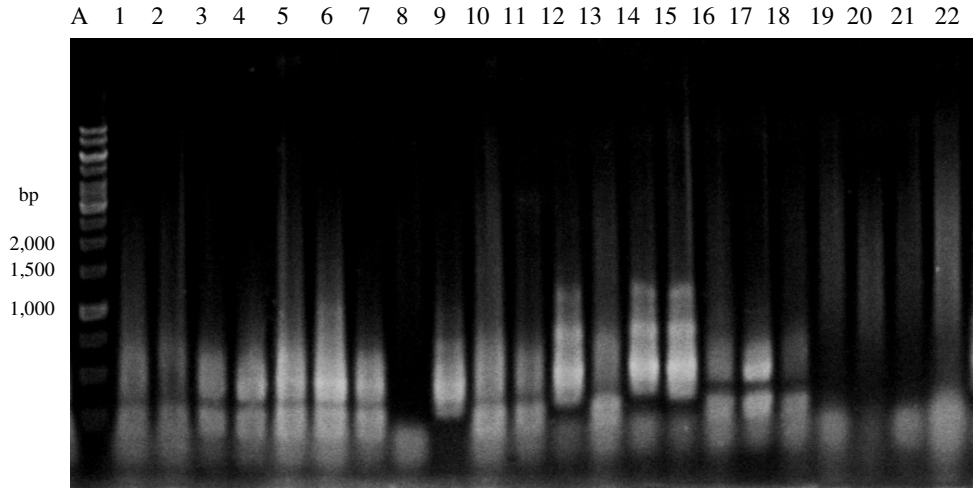


Figure 4.9: Agarose gel electrophoresis results on colony PCR conducted on colonies of Plate 2 using primers F-M13 and R-M13

Lane A : 1 kb ladder

Lane 1-22 : Colony PCR products for colonies 1-22 of Plate 2

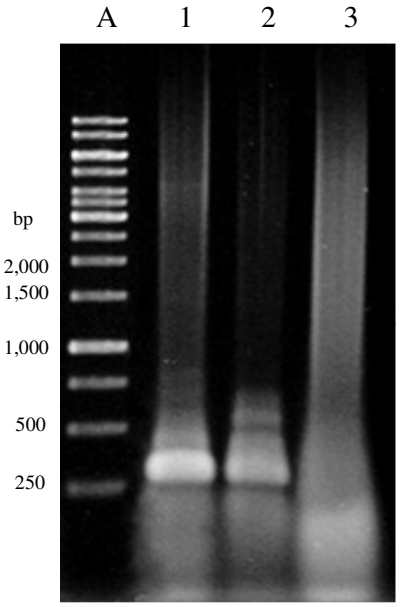


Figure 4.10: Agarose gel electrophoresis results on colony PCR conducted on colonies of plate 2 using primers F-norR-NLS and R-M13

Lane A : 1 kb ladder

Lane 1,2, 3: Colony PCR product for colonies 12, 14 and 15 of Plate 2, respectively

TOPO® cloning of *norR(HTH-NLS)* into pENTR™/D-TOPO® was performed and the gene-plasmid construct was transformed into Mach1 competent cells with heat shock transformation and positively selected on Kanamycin plate. As shown in Figure 4.5, 51 colonies had grown on the selective plate.

Colony PCR was performed twice on the colonies in Plate 2. The first round was performed on 22 randomly selected colonies with primers F-M13 and R-M13. The second round was performed on colonies 12, 14 and 15 for having the desired band at around 1.3 kb in the first round with primers F-norR-NLS and R-M13.

However, from the results as shown in Figure 4.9 and 4.10, colonies 12, 14 and 15 did not show any bands at the desired size, at aproximately 1.2 kb. Therefore, positive colonies transformed with *norR(HTH-NLS)* recombinant into pENTR™/D-TOPO® with the correct orientation could not be selected.

4.3.3 pENTR™/D-TOPO® and *norR(SACT-NLS)* Construct

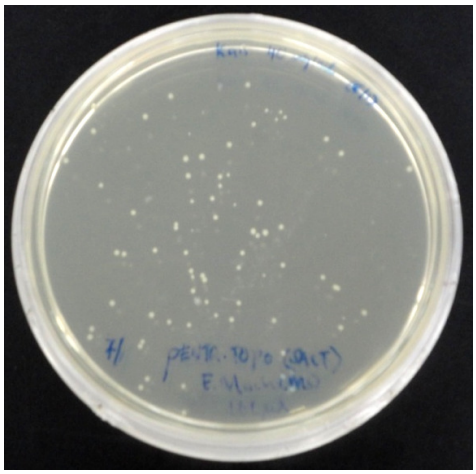


Figure 4.11: pENTR™/D-TOPO® recombinant with *norR(SACT-NLS)* transformed Mach1 colonies on 40µg/ml Kanamycin added 2xYT selective plate [Plate 3]

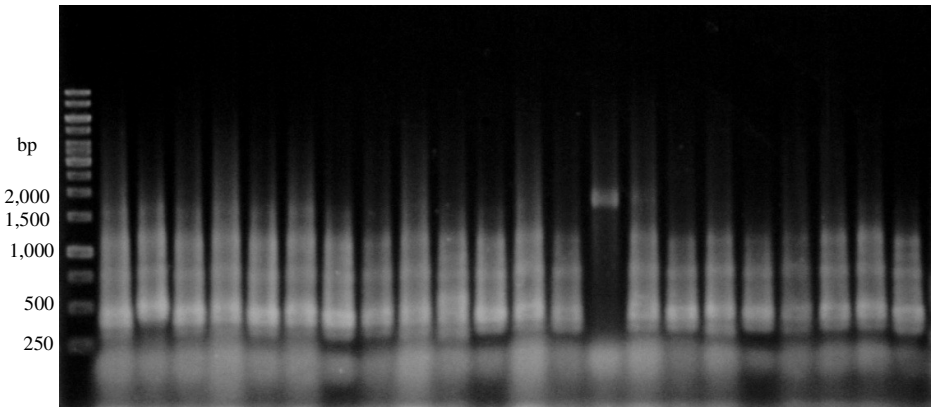


Figure 4.12: Agarose gel electrophoresis results on colony PCR conducted on colonies of Plate 3 using primers F-M13 and R-M13

Lane A : 1 kb ladder

Lane 1-22 : Colony PCR products on colonies 1-22 of Plate 3

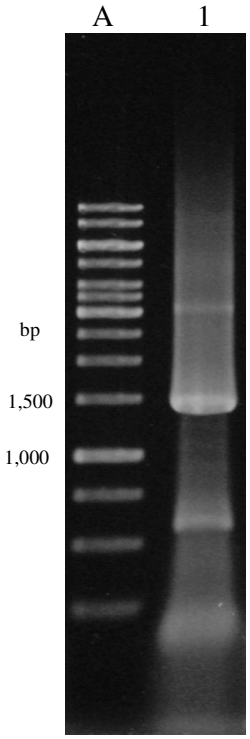


Figure 4.13: Agarose gel electrophoresis result on colony PCR conducted on colonies from plate 3 using primers F-norR-NLS and R-M13

Lane A : 1 kb ladder

Lane 1 : Colony PCR products on colony 14 of Plate 3

TOPO® cloning of *norR(SACT-NLS)* into pENTR™/D-TOPO® was performed and the gene-plasmid construct was transformed into Mach1 competent cells with heat shock transformation and positively selected on Kanamycin plate. As shown in Figure 4.11, 126 colonies had grown on the selective plate. Next, colony PCR was carried out to select positive recombinants with the correct orientation.

As the same with the other gene fragments, colony PCR was performed twice to select the positive clones from plate 3. Colony PCR was conducted on 22 randomly selected colonies with primers F-M13 and R-M13. The second round was performed on colony 14 for having the desired band around 1.5 kb in the first round.

From the results as shown in Figure 4.6 and 4.7, the desired band at approximately 1.4 kb was found in both rounds for colony 14. Thus, the colony successfully transformed with *norR(SACT-NLS)* recombinant into pENTR™/D-TOPO® was colony 14 and was designated as N14.

4.4 Recombinant pENTR™/D-TOPO® Plasmid Extraction & Purification

4.4.1 pENTR™/D-TOPO® and *norR(full-NLS)* Construct

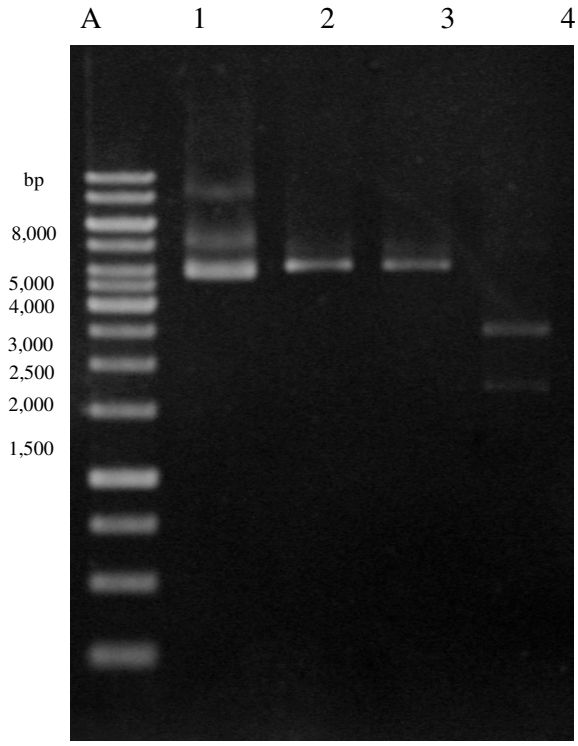


Figure 4.14: Agarose gel electrophoresis results on extracted and restriction digested plasmid from colony B8

Lane A : 1 kb ladder

Lane 1 : Extracted and purified plasmid

Lane 2, 3 : Single digestion with *Not*I and *Eco*RV on extracted plasmid, respectively

Lane 4 : Double digestion with *Not*I and *Eco*RV on extracted plasmid

Colony B8 was cultured in 2x YT broth supplemented with 40 µg/ml Kanamycin overnight and subsequent plasmid extraction was carried out as shown in Lane 1 of Figure 4.14. The appearance of three bands was suspected to be the different conformation of the plasmid which are open circular, linear and covalently close circular from top to bottom, respectively.

Thus, in proving that the extracted plasmid was *norR(full-NLS)* and pENTR™/D-TOPO® construct, restriction digestion was performed to linearize the plasmid. Single digestion and double digestion with *Not*I and *Eco*RV were performed and the results are shown in Lane 2 , 3 and 4 of Figure 4.14. The results showed the desired bands at the expected size which was summarized in Table 4.1.

Table 4.1: Summary of the size of the bands obtained for restriction digestion of extracted plasmid of colony B8

Lane	Restriction Enzyme	Expected size (kb)
2	*Not*I	~4.0
3	*Eco*RV	~4.0
4	*Not*I and *Eco*RV	~2.5 and ~1.6

An additional level of confirmation was obtained through PCR with three sets of primers on the extracted plasmid. Besids, the purpose of conducting this step was to ensure the gene fragment was recombinant into the plasmid in the correct orientation. The results were shown in Figure 4.15.

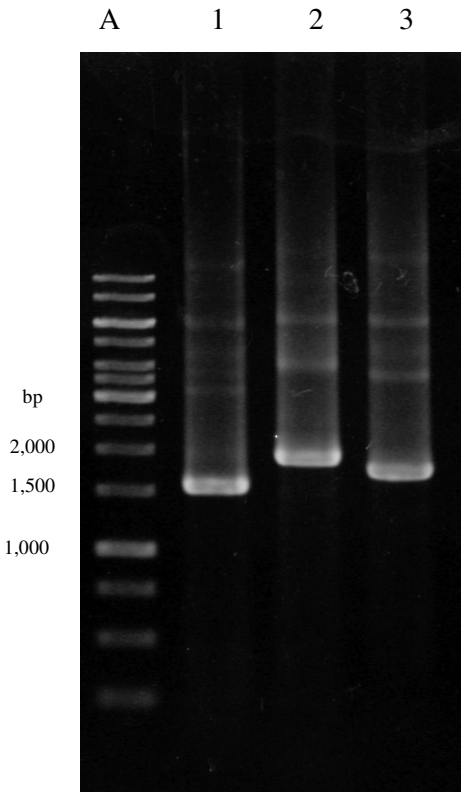


Figure 4.15: Agarose gel electrophoresis results on PCR amplification on extracted plasmid of colony B8

Lane A : 1 kb Ladder

Lane 1 : PCR product with F-norR-NLS and R-norR

Lane 2 : PCR product with F-M13 and R-M13

Lane 3 : PCR product with F-M13 and R-norR

From the results, the desired bands at the expected sizes were obtained for the three sets of primers as mentioned which were approximately 1.5 kb, 1.8 kb and 1.7 kb respectively. In conclusion, the colony B8 contained the desired gene-plasmid construct and the extracted plasmid from this clone was proven to be *norR(full-NLS)* recombinant into pENTR™/D-TOPO® and the sample was sent for sequencing.

4.4.2 pENTR™/D-TOPO® and *norR(SACT-NLS)*

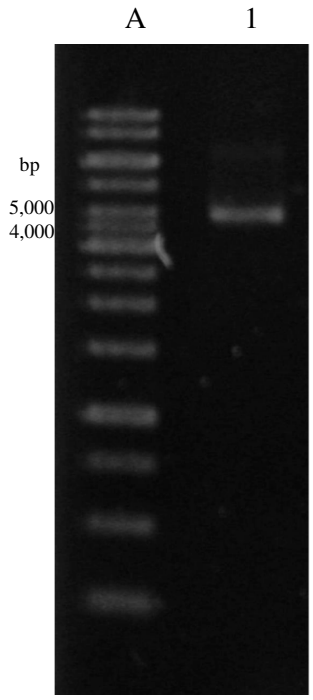


Figure 4.16: Agarose gel electrophoresis results on extracted plasmid from colony N14

Lane A : 1 kb ladder

Lane 1 : Extracted and purified plasmid

Colony N14 was cultured in 2x YT broth supplemented with 40 µg/ml Kanamycin overnight and subsequent plasmid extraction was carried out as shown in Figure 4.16. Additional level of conformation was carried out with restriction digestion and PCR.

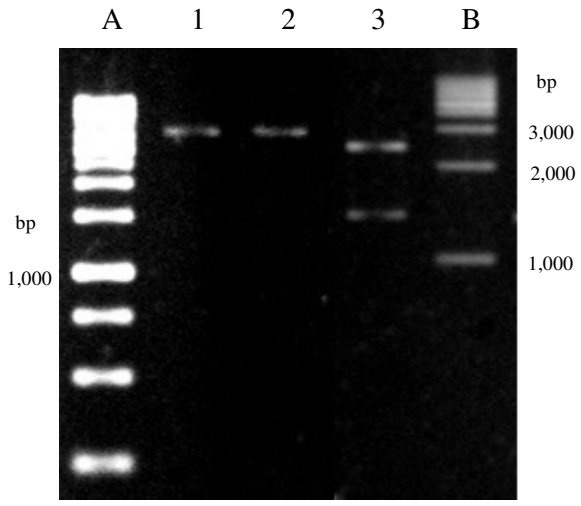


Figure 4.17: Agarose gel electrophoresis results on restriction digested extracted plasmid from colony N14

Lane A : 1 kb ladder

Lane 1, 2 : Single digestion with *Not*I and *Eco*RV on extracted plasmid, respectively

Lane 3 : Double digestion with *Not*I and *Eco*RV on extracted plasmid

Lane B : DNAM-M1000bp ladder

Similar with the *norR(full-NLS)* construct, the extracted plasmid from colony N14 was restriction digested with *Not*I and *Eco*RV and summarized in Table 4.2.

Table 4.2: Summary of the size of the bands obtained for restriction digestion of extracted plasmid from colony N14

Lane	Restriction Enzyme	Expected size (kb)
1	*Not*I	~3.8
2	*Eco*RV	~3.8
3	*Not*I and *Eco*RV	~2.5 and ~1.4

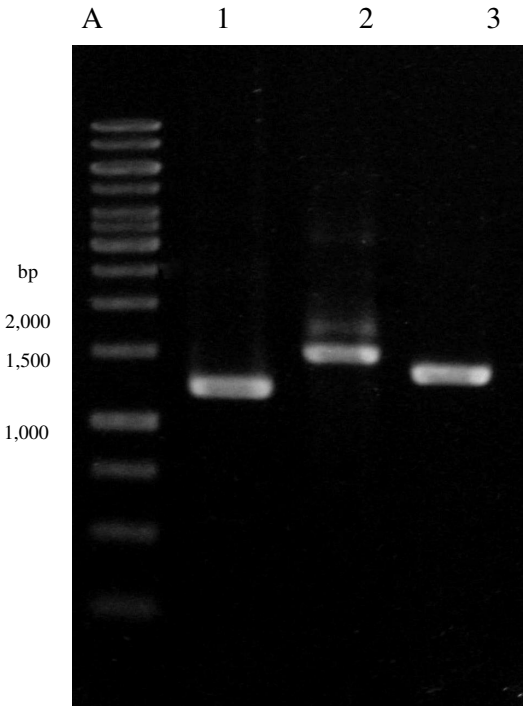


Figure 4.18: Agarose gel electrophoresis results on PCR amplification on extracted plasmid from colony N14

Lane A : 1 kb Ladder

Lane 1 : PCR products with F-norR-NLS and R-norR- SACT1224

Lane 2 : PCR products with F-M13 and R-M13

Lane 3 : PCR products with F-M13 and R-norR-SACT1224

The PCR showed that the extracted plasmid from N14 showed the desired bands at approximately 1.2 kb, 1.6 kb and 1.4 kb respectively. In conclusion, the extracted plasmid from colony N14 was transformed with *norR(SACT-NLS)* recombinant into pENTR™/D-TOPO® at the correct orientation and the sample can be sent for sequencing.

CHAPTER 5

DISCUSSION

In this project, the construction of the NO biosensor was at its preliminary stage focusing on amplifying *norR* with polymerase chain reaction and cloning into TOPO®

5.1 Evaluation of Results

5.1.1 Total DNA Extraction of *Escherichia coli*

Extraction of *E. coli* genomic DNA from DH5α served as a template for the amplification of *norR(full).* From the results shown in Figure 4.1, RNA contamination indicated by bands smaller than 250 bp and smearing of the band which indicated degradation of the genomic DNA was not present. Thus, the subsequent steps could be carried out.

5.1.2 PCR Amplification

5.1.2.1 *norR(full), norR(full-NLS), norR(HTH-NLS)* and *norR(SACT-NLS)*

Initially, amplifying *norR(full-NLS), norR(HTH-NLS)* and *norR(SACT-NLS)* directly from genomic DNA of *E. coli* had proved a challenge where a single band at the desired size could not be obtained. The reason for this occurrence could be due to the PCR primers used that did not anneal 100% to the template as it contained additional sequences, NLS and 5'-CACC-3'.

Thus, *norR(full)* was amplified by using minimal primers, F-norR and R-norR. Besides, the use of Montage™ life science kit to purify the PCR product of *norR(full)* was based on the principle that the kit was able to remove any nucleotide fragment with sizes below 200 bp. Thus, primer dimmers and RNA from the PCR product that were smaller than 200 bp were able to be removed by the kit.

However, the DNA fragment of *norR(full)* was lacking its extra sequence which was essential for cloning, therefore, nested PCR was carried out by using primers F-norR-NLS and its respective reverse primers to amplify *norR(full-NLS), norR(HTH-NLS)* and *norR(SACT-NLS).*

On the other hand, the procedure was optimized by diluting the template and running a gradient PCR. We discovered that the optimized temperature was 55.4°C. Besides, using a PCR product as a template for another PCR would give very high specificity therefore the concentration of the template should be reduced so that non-specific bands were not present. Thus, a serial dilution up to 1000x was performed for the purified PCR product of *norR(full)*.

To prepare the PCR products for subsequent TOPO® cloning, ethanol precipitation and RNase treatment were carried out. The purpose of this step was to increase the concentration of the DNA fragments where 50 µl of PCR product mixture was combined to give 30 µl of purified PCR product besides removing impurities such as PCR primers, enzymes, dNTPs, salt, RNA, etc.

5.1.3 TOPO® Cloning

Selection for *E. coli,* DH5α transformed with pENTR™/D-TOPO® utilized Kanamycin selective plate because the plasmid contained the Kanamycin resistance gene, Kan^R while the cells do not (Invitrogen®, 2006a). Thus, if DH5α was able to survive on a plate containing the antibiotic, it was assumed that acquired resistance was due to the presence of the plasmid in the cells while untransformed cells died off.

Meanwhile, selection for the positive recombinant of the desired genes into pENTR™/D-TOPO® was carried out with colony PCR. The purpose of using two sets of primers was to confirm the presence of the desired gene and recombinant with the correct orientation into pENTR™/D-TOPO®.

The first set of primers encompassed both forward and reverse of M13 which binds to the plasmid. The presence of desired band at a total of 306 bp plus the size of gene could only mean that there was a gene insert in the recombinant site (Invitrogen®, 2006a). For example, if *norR(full-NLS)* was inserted into the plasmid, a total of 1,843 bp would be expected.

While, bands at 306 bp proved that the plasmid had re-annealed and no gene insert was present. The absence of any bands could be due to transformation inefficiency where selection alone with Kanamycin was not selective enough as Kan^R mutants were also selected even if pENTR™/D-TOPO® was not present in the cell.

The second set would include a forward primer of the desired gene and reverse primer of M13. Thus, the primers would anneal to both the plasmid and the gene insert only if the insert was recombinant in the correct orientation.

After plasmid extraction was carried out to obtain the gene-plasmid construct, an additional level of confirmation with restriction digestion and PCR was performed. The purpose of this step was to avoid the risk of losing the gene-plasmid construct during the culturing and extraction process.

*Not*I and *Eco*RV were utilized as pENTR™/D-TOPO® contained only one of the restriction sites for each (Invitrogen®, 2006a). Thus, the restriction enzymes were only able to cleave at one site to give a linear plasmid at the expected size (2,580 bp plus with insert size) of that if single digestion was carried out. Besides, if double digestion was carried out, positive results would show two bands at expected size 2435 bp and a smaller fragment (145 bp plus with insert size).

PCR on the extracted plasmids, with three sets of primers consisting of the combination of the forward and reverse primers of both M13 and the gene insert, was also carried out. The expected size of the band is shown in Figure 5.1.

Table 5.1: Expected size of PCR products of extracted plasmid, pENTR™/D-TOPO®

Primer		**Expected size (bp)**		
Forward	**Reverse**	***norR(full-NLS)***	***norR(HTH-NLS)***	***norR(SACT-NLS)***
F-M13	R-M13	1,843	1,355	1,569
F-NorR-NLS	R-M13	1,690	1,202	1,416
F-NorR-NLS	R-NorR	1,537	1,049	1,263

The results showed that the successful TOPO® cloning for the recombinant of gene fragments into pENTR™/D-TOPO® only applied to *norR(full-NLS)* and *norR(SACT-NLS)*. Whereas, *norR(HTH-NLS)* was not successful.

However, several attempts were needed before positive clones could be selected. The possible reasons could be that fresh PCR product of *norR(full-NLS)* used for TOPO® cloning reaction as recommended from the instruction manual provided by Invitrogen® (2006a) was not abided to. As for *norR(HTH-NLS)* and *norR (SACT-NLS),* the PCR products were a few weeks old since it was carried out after the *norR(full-NLS)* transformed clone was positively identified.

However, TOPO® cloning and transformation for *norR(SACT-NLS)* were successful in the sense that positive clones transformed with the pENTR™/D-TOPO® and *norR (SACT-NLS)* were positively identified at the second trial.

On the other hand, the successful cloning and transformation of *norR(HTH-NLS)* into pENTR™/D-TOPO® could be achieved by a few more trials by optimizing the concentration and purity of the PCR product used as recommended by Invitrogen® to optimize the molar ratio of vector: PCR product to 0.5:1 to 2:1 (2006a). Besides, obtaining sterile and highly competent cells for transformation could improve the transformation efficiency.

5.2 Other NO Sensing Tools

NO determining tools has not been new in this century; scientists have claimed that "the determination of NO has received much attention as an essential requirement" (Abdelwahab, Koh, Noh, & Shim, 2010). Thus, a variety of approaches for NO sensing and quantification tools have been developed encompassing chemiluminescence, UV-vis spectroscopy, flurorescence, electron paramagnetic/spin resonance spectroscopy (EPR/ESR) and electrochemical techniques (Xu, Hu, & Hu, 2010).

Development of NO sensors using electrochemical based techniques has grabbed the attention of scientists lately. A few of the examples include a voltammetric NO biosensor based on haemoglobin-affinity (Fan, Liu, Pang, Li, & Scheer, 2004), a DNA modified glassy carbon electrode (Liu, Zhao, Wu, & Yang, 2007) and a sodium dodecyl sulfate modified electrode (Chen, Long, Wu, & Yang, 2009) using cytochrome *C* based NO biosensor, a mircroperoxidase-based nanocomposite biosensor (Abdelwahab et al., 2010) and a haemoglobin-based gold colloids modified carbon paste electrode biosensor (Xu et al., 2010).

In general, electrochemical techniques are widely used because they give a higher sensitivity, and fast and stable quantification of NO which can be used *in vivo*. The detection can be carried out through the electrooxidation and electroreduction of NO due to the NO redox behaviour, interconvering between nitroxyl anion, itself and nitrosonium cation (Abdelwahab et al., 2010). This oxidation and reduction of NO can be detected through the use of electrodes, whether basic solid or chemically modified (Fan et al., 2004).

The techniques that have been mentioned are limited to biochemical approaches. However, there are a few different ways of determining NO which are through microarrays, western blotting, assays and cell cultures by exposing the target cells with NO (Clegg, 1998).

Thus, the molecular recombinant technology is still new when used for detecting NO. Students at Nanyang Technology University in Singapore (iGEm, 2009) have developed a NO biosensor which was very similar to NO biosensor construct of this project whereby they also made use of the sensing activity of NorR and undergo real-time NO sensing through the reporter protein, GFP. However, the problem with this NO biosensor was it was only catered for prokaryotic NO sensing. Comparatively, the NO biosensor developed in this project has an advantage of providing a wider range of target organisms inclusive of eukaryotes.

CHAPTER 6

CONCLUSION

Molecular cloning of *norR(SACT-NLS)* into pENTR™/D-TOPO® was also accomplished and a sample of recombinant plasmid, named N14 was sent for sequencing. However, *norR(HTH-NLS)* insertion into pENTR™/D-TOPO® was not successful.

The orientation and accurate insertion of the gene fragment into the vectors were confirmed through sequencing and the pairwise alignment with reference sequence of *norR* (Accession number: NC_00913.2) using blastn from NCBI. The results showed up to 91% to 99% identity for all three samples. Besides, forward and reverse sequences flanked the ends of the gene fragments at the correct positions. Thus, the gene fragments inserted into the vectors were confirmed of their identity.

REFERENCES

Abdelwahab, A. A., Koh, A. W., Noh, H. B., & Shim, Y. B. (2010). A selective nitric oxide nanocomposite biosensor based on direct electron transfer of microperoxidase: Removal of interferences by co-immobilized enzymes. *Biosensors and Bioelectronics*, *26*, 1080-1086.

Adhya, S., & Garges, S. (2003). *RNA polymerase and associated factors*. London: Elsevier Inc.

Ahmed, K., Allende, J. E., & Issinger, O. (Eds.). *Protein kinase CK2: from structure to regulation.* Massachusetts: Kluwer Academic Publishers.

Akira, S., Gossen, M., Kaufmann, J., & Triezenberg, S. J. (Eds.). (2004). *Transcription factors.* New York: Spinger.

Arai, H., Hayashi, M., Kuroi, A., Ishii, M., & Igarashi, Y. (2005). Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of *Pseudomonas aeruginosa*. *The Journal of Bacteriology*, *187* (12), 3960-3968.

Autreaux, B. D., Tucker, N. P., Dixon, R., & Spiro, S. (2005). A non-haem iron centre in the transcription factor NorR senses nitric oxide. *Nature*, *437* (29), 769-772.

Baumberg, S. (Ed.). (1999). *Prokaryotic gene expression*. Oxford: Oxford University Press.

Bonavida, B. (Ed.). (2010). *Nitric oxide, NO, and cancer*. New York: Springer Science+Business Media.

Brakmann, S., & Johnsson, K. (Eds.). (2002). *Directed molecular evolution of proteins: or how to improve enzymes for biocatalyts*. Germany: Wiley-VCH.

Butler, A., & Nicholson, R. (2003). *Life, death and nitric oxide*. Cambridge: The Royal Society of Chemistry.

Chen, X., Long, H. Y., Wu, W. L., & Yang, Z. S. (2009). Direct electrochemical behavior of cytochrome c on sodium dodecyl sulfate modified electrode and its application to nitric oxide biosensor. *Thin Solid Films*, *517*, 2787-2791.

Clark, V. L. & Bavoil, P. M. (Eds.). (2002). *Bacterial pathogenesis, Part C: identification, regulation and function of virulence factors.* USA: Elsevier Science.

Clegg, R. A. (Ed.). (1998). *Protein targeting protocols.* New Jersey, NJ: Humana Press Inc.

Cold Spring Harbor Laboratory. (1998). *Cold spring harbor symposia on quantitative biology*. New York: CSHL Press.

Courey, A. J. (2008). *Mechanisms in transcriptional regulation*. Los Angeles, LA: Blackwell Publishing.

Cragg, P. J. (2010). *Supramolecular chemistry: from biological inspiration to biomedical applications.* New York: Spinger Science+Business Media.

Fan, C., Liu, X., Pang, J., Li, G., & Scheer, H. (2004). Highly sensitive voltammetric biosensor for nitric oxide based on its high affinity with hemoglobin. *Analytica Chimica Acta*, *523*, 225-228.

Friedberg, E. C., Walker, G. C., & Siede, W. (1995). *DNA repair and mutagenesis*. Washington: American Society for Microbiology.

Glass, R. E. (1982). *Gene function: E. coli and its heritable elements.* Great Britain: University of California Press.

Groban, E. S., Johnson, M. B., Banky, P., Burnett, P. G. G., Calderon, G. L., Dwyer, E. C., et al. (2005). Binding of the *Bacillus subtilis* LexA protein to the SOS operator. *Nucleic Acid Research*, *33*, 6287-6295.

Gryglewski, R. J. & Minuz, P. (Eds.). (2001). *Nitric oxide: basic research and clinical applications.* Netherlands: IOS Press.

Gudi, T., Casteel, D. E., Vinson, C., Boss, G. R., & Pilz, R. B. (2000). NO activation of FOS promoter elements requires nuclear translocation of G-kinase 1 and CREB phosphorylation but is independent of MAP kinase activation. *Oncogene*, *19*, 6324-6333.

Hurtley, S. M. (Ed.). (1996). *Protein targeting.* New York: Oxford University Press.

iGEM. (2009). *Sensing device.* Retrieved December 1, 2010 from http://2009.igem.org/Team:NTU-Singapore/Project/Prototype/Sense

Ignarro, L., & Murad, F. (1995). *Nitric oxide: biochemical, molecular biology, and therapeutic implifications.* California: Academic Press.

Invitrogen®. (2006a). *pENTR™ directional Topo® cloning kits.* Author.

Invitrogen®. (2006b). *Subcloning efficiency™ DH5α™ competent cells.* Author.

Invitrogen®. (2010). *Gateway® technology with Clonase II: A universal technology to clone DNA sequences for functional analysis and expression in multiple systems.* Author.

Jugdutt, B. I. (2004). *The role of nitric oxide in heart failure*. USA: Kluwer Academic Publishers.

Justino, M. C., Goncalves, M. M., & Saraiva, L. M. (2005). Binding of NorR to three DNA sites is essential for promoter activation of the flavorubredoxin gene, the nitric oxide reductase of Escherichia coli. *Biochemical and Biophysical Reseach Communications*, *328*, 540-544.

Karp, G. (2009). *Cell and molecular biology: concepts and experiments.* UK: John Wiley and Sons.

Kim, S. J., Hwang, S. G., Shin, D. Y., Kang, S. S., & Chun, J. S. (2002). p38 kinase regulates nitric oxide-induced apoptosis of anticular chondrocytes by accumulating p53 via NF-κB-dependent transcription and stabilization by serine 15 phosphorylation. *The Journal of Biology and Chemistry, 277*, 33501-33508.

Lamas, S., & Cadenas, E. (Eds.). (2006). *Nitric oxide, cell signalling, and gene expression.* USA: Taylor & Francis Group.

Lengeler, J. W., Drews, G., & Schlegel, H. G. (1999). *Biology of prokaryotes*. Germany: Library of Congress.

Liu, Y. C., Zhao, J., Wu, W. L., & Yang, Z. S. (2007). Direct electrochemical behavior of cytochrome c on DNA modified glassy carbon electrode and its application to nitric oxide biosensor. *Electrochimica Acta*, *52*, 4848-4852.

MacDonald, P. N. (Ed.). (2001). *Two-hybrid systems: methods and protocols*. New Jersey, NJ: Humana Press.

MacMicking, J., Xie, Q. W., & Nathan, C. (1997). Nitric oxide and macrophage function. *Annual Reviews Immunology*, *15*, 323-350.

Maloy, S. R., Cronan, J. E., & Freifelder, D. (1994). *Microbial genetics*. Mississauga: Jones and Bartlett Publishers.

Mayer, B. (Ed.). (2000). *Nitric oxide*. New York: Springer.

Metzen, E., Zhou, J., Jelkmann, W., Fandrey, J., & Brune, B. (2003). Nitric oxide impairs normoxic degradation of HIF-1α by inhibition of prolyl hydroxylases. *Molecular Biology of the Cell*, *14*, 3470-3481.

Minarovits, J., Gonczol, E., & Valyi-Nagy, T. (Eds.). (2007). *Latency strategies of herpesviruses.* New York: Springer Science+Business Media.

Morgan, J. R. (Ed.). (2002). *Gene therapy protocols.* (2nd ed.). New Jersey, NJ: Humana Press.

Mukhopadhyay, P., Zheng, M., Bedzyk, L. A., LaRossa, R. A., & Storz, G. (2004). Prominent roles of the NorR and Fur regulators in the Escherochia coli transcriptional responses to reactive nitrogen species. *PNAS*, *101*, 745-750.

Nacalai Tesque. (n. d.). *DNA ladder markers*. Retrieved March 20, 2011 from http://www.nacalai.co.jp/global/reagent/biology/02.html

Nunoshiba, T., deRojas-Walker, T., Wishnok, J. S., Tannenbaum, S. R., & Demple, B. (1993). Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. *Proceedings of the National Academy of Sciences USA*, *90*, 9993-9997.

Ottosen, S., Herrera, F. J., Doroghazi, J. R., Hull, A., Mittal, S., Lane, W. S., et al. (2006). Phosphorylation of the VP16 transcriptional activator protein during herpes simplex virus infection and mutational analysis of putative phosphorylation sites. *Virology*, *345*, 468-481.

Patterson-Fortin, L. M., & Owttrim, G. W. (2008). A Synechocystis LexA-orthologue binds direct repeats in target genes. *FEBS Letters*, *582*, 2424-2430.

Pohlmann, A., Cramm, R., Schnetz, K., & Friedrich, B. (2000). A novel NO-responding regulator controls the reduction of nitric oxide in *Ralstonia eutropha*. *Molecular Mirocbiology*, *38*, 626-638.

Pullan, S. T., Monk, C. E., Lee, L., & Poole, R. K. (2008). Microbical responses to nitric oxide and nitrosative stress: growth, "omic," and physiological methods. *Methods in Enzymology*, *437*, 499-519.

Rappas, M., Bose, D., & Zhang, X. (2007). Bacterial enhancer-binding proteins: unlocking 45-dependent gene transciption. *Current Opinion in Structural Biology*, *17*, 110-116.

Ratledge, C., & Kristiansen, B. (2006). *Basic biotechnology*. Cambridge: Cambridge University Press.

Robinson, J. W. (Ed.). (2005). *Frontier in protein research*. New York: Nova Science Publishers.

Schaechter, M., & Lederberg, J. (Eds.). (2004). *The desk encyclopedia of microbiology*. Oxford: Elsevier Ltd.

Schoenfisch, M. H., Rothrick, A. R., Shin, J. H., Polizzi, M. A., Brinkley, M. F., & Dobmeier, K. P. (2006). Poly(vinylpyrrolidone)-doped nitric oxide-releasing xerogels as glucose biosensor membranes. *Biosensors & Bioelectornics*, *22,* 306-312.

Shors, T. (2009). *Understanding viruses*. Sudbury: Jones and Bartlett Learning.

Solomom, E. P., Berg, L. R., & Martin, D. W. (2008). *Biology*. (8th ed.). Belmont: Thomas Higher Education.

Sonenshein, A. L., Hoch, J. A., & Loisck, R. (Eds.). (2002). *Bacillus subtilis and its closest relatives: from genes to cells.* Washington: ASM Press.

Stagljar, I., Korostensky, C., Johnson, N., & Heesen, S. T. (1998). A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. *Proc. NatI.Acad.Scie. USA*, *95*, 5187-5192.

Thermo Fisher Scientific Inc. (2011). *Gene ruler^{TM} 1kb DNA ladder, 250-10,000 bp*. Retrieved March 20, 2011 from http://www.fermentas.com/en/products/all/dna-electrophoresis/generuler-dna-ladders/sm031-generuler-1kb

Tokada, H., Akihisa, T., Tanaka, R., Takayasu, J., & Nishino, H. (2006). Conventional screening method of short-term in vitro assay for nitric oxide scavenger reagents. *Nitric oxide*, *14*, A20.

Tucker, N. P., Autreaux, B. D., Spiro, S., & Dixon, R. (2005). DNA binding properties of the *Escherichia coli* nitric oxide sensor NorR: towards and understanding of the regulation of flavarubredoxin expression. *Biochemical Society Transactions*, *33* (1), 181-183.

Tucker, N. P., Autreaux, B. D., Spiro, S., & Dixon, R. (2006). Mechanism of transcriptional regulation by the *Escherichia coli* nitric oxide sensor NorR. *Biochemical Society Transactions*, *34* (1), 191-194.

Tucker, N. P., Autreaux, B. D., Studholme, D. J., & Spiro, S. (2004). DNA Binding Activity of the *Escherichia coli* nitric oxide sensor NorR Suggests a conserved target sequence in diverse proteobacteria. *Journal of Bacteriology*, *186*, 6656-6660.

Tucker, N. P., Ghosh, T., Bush, M., Zhang, X., & Dixon, R. (2010). Essential toles of three enhancer sites in σ^{45}-dependent transcription by the nitric oxide sensing regulatory protein NorR. *Nucleic Acids Research*, *38*, 1182-1194.

Turpaev, K., Bouton, C., Diet, A., Glatigny, A., & Drapier, J.-C. (2005). Analysis of differentially expressed genes in nitric oxide-exposed human monocytic cells. *Free Radical Biology & Medicine*, *38*, 1392-1400.

Valsecchi, V., Pignataro, G., Del Prete, A., Sirabella, R., Matrone, C., Boscia, F., et al. (2011). NCX1 is a novel target gene for hypoxia-inducible factor-1 in ischemic Brain preconditioning. *PubMed*, *42* (3), 754-763.

Wang, G., Rudland, R. S., White, M. R., & Barraclough, R. (2000). Interaction *in vivo* and *in vitro* of the metastasis-inducing S_{100} protein, $S_{100}A_4$ (p_9 Ka) with S_{100} A_1. *Journal of Biological Chemistry*, *275* (15), 11141-11146.

Watson, J. D., Baker, T. A., Bell, S. P., Gann, A., Levine, M., & Losick, R. (2008). *Molecular biology of the gene*. San Francisco: Pearson Education, Inc.

Weber, G. F. (2007). *Molecular mechanisms of cancer*. Netherlands: Springer.

White, R. J. (2001). *Gene transcription: mechanisms and control.* Oxford: Blackwell Science Ltd.

Wysocka, J., & Herr, W. (2003). The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. *TRENDS in Biochemical Science*, *28* (6), 294-304.

Xu, Y., Hu, C., & Hu, S. (2010). A reagentless nitric oxide biosensor based on the direct electrochemistry of hemoglobin adsorbed on the gold colloidds modified carbon paste electrode. *Sensors and Actuators B*, *148*, 253-258.

Zhang, X., Ju, H., & Wang, J. (Eds.). (2007). *Electrochemical sensors, biosensors and their biomedical applications.* USA: Elsevier Inc.

Zumft, W. (1997). Cell biology and molecular basis of denitrification. *Microbiology Molecular Biology Reviews*, *61*, 533-616.

Zuo, J., Niu, Q. W., & Chua, N. H. (2000). An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *The Plant Journal*, *24*, 265-273.