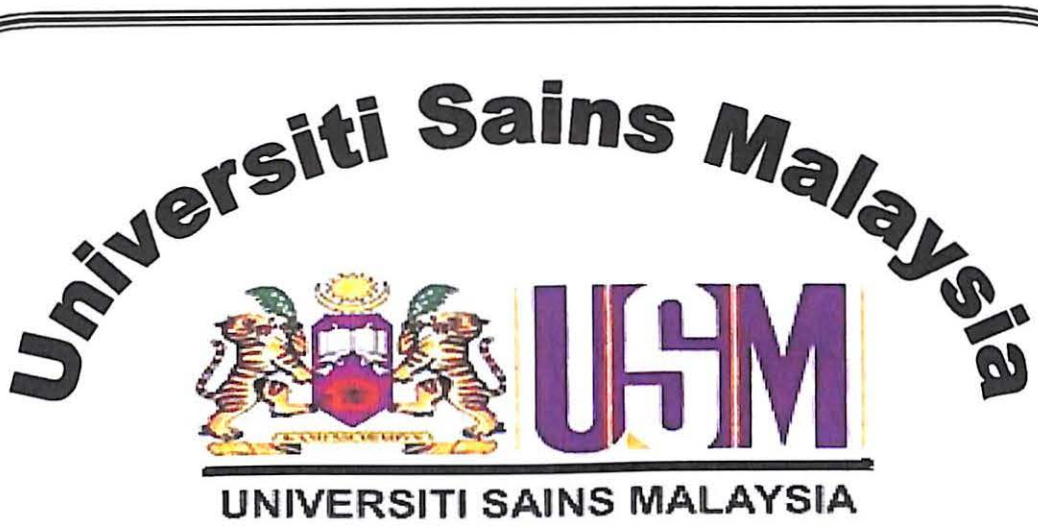

Expression studies of malarial antigen

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Science (Health) in Biomedicine

Rita Ting Ling

School of Health Sciences
Universiti Sains Malaysia
Health Campus
16150 Kubang Kerian, Kelantan
Malaysia

2004

CERTIFICATE

This is to certify that the dissertation entitled

"**Expression studies of malarial antigen**"

is the bonafide record of research work done by

Mr/Mrs/Ms **Rita Ting Ling** during the period of **August**

to **February** under my supervision.

.....

Professor Dr. Norazmi Mohd. Nor
Deputy Dean
School of Health Sciences
Universiti Sains Malaysia
16150 Kubang Kerian
Kelantan, Malaysia

ACKNOWLEDGEMENTS

This study is a dissertation submitted in partial fulfillment for the degree of Bachelor of Science (Health) in Biomedicine at Universiti Sains Malaysia. It was accomplished during the period from August 2003 to February 2004.

I would like to thank my supervisor, Professor Dr. Norazmi Mohd. Nor, for help and support throughout the research. Thanks for the ceaseless effort in his guidance and advice in my thesis write-up.

Thanks to Teo Wan Huai and Maryam for welcoming me in the laboratories and teaching me experimental routines. Again special thanks to Teo for guiding me through the writing process and providing various sources and information. Special appreciation to Kak Anis for critical and thorough reading of this thesis. I am also grateful to Nurul Asma for providing me with her thesis for reference and for giving me guidance and endless support.

Thanks to Corliss, Yong Kim Far and Jenny for support and valuable comments. I would also like to show my gratitude to Kiu Siik Hung and Wendy Yeow for lending me a hand when things are critical.

Thanks to fellow students and staff in the laboratories for welcoming, encouraging and taking care of a new student.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

ABSTRACT

Serine repeat antigen (SERA) of *Plasmodium falciparum* is believed to be an excellent candidate for the development of a malaria vaccine. In this study, a 22-kDa synthetic DNA fragment (SE22) from the N-terminal domain of SERA which was previously constructed by assembly polymerase chain reaction (PCR) was subcloned from the cloning vector, pCR®2.1-TOPO® into an expression vector, pPROEX™HTa for its expression in BCG. Prior to this study, the SE22 gene was constructed in favour of mycobacterium codon usage and cloned into pCR®2.1-TOPO® to produce pNMN009. In order to express the protein, the SE22 gene was extracted from pNMN009 and subcloned into pPROEX™HTa to produce pNMN019. The expression of the fusion protein was induced with isopropylthiogalactoside (IPTG). The fusion protein was isolated and used to immunize two New Zealand white rabbits to produce polyclonal antibody against the candidate antigen. This antibody will be used to detect the expression of the synthetic SE22 gene in *Mycobacterium bovis* bacille Calmette Guerin.

INTRODUCTION

Malaria

Malaria is one of the commonest diseases in the world affecting more than 200 million people and killing more than 3 million annually (Vegupalan, 2002). World Health Organization (WHO) estimates that approximately 40% of the world's population, mostly those living in the world's poorest countries especially in tropical and subtropical part of the world, is at risk of malaria. Countries without the administrative, financial and human resources necessary for disease control usually have high number of case fatalities. It is a major public health problem and should not be overlooked.

Etiology

Malaria is caused by a parasite known as Plasmodium and is transmitted by the Anopheles mosquito. Plasmodium is a small, single-cell organism (protozoan), which lives as a parasite in various animal hosts including man and a specific species of mosquito (Anopheles) (Easmon, 2002).

There are four different species of malaria parasite that infect humans namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. *P. falciparum* is the cause of malignant malaria and by far the most important plasmodium parasite in Africa. On the other hand, *P. vivax*, *P. ovale* and *P. malariae* cause benign types of malaria.

The disease is passed on when the mosquito bites a person who has the parasite in their blood. Development of parasites takes place in the intestine and salivary glands of the mosquito and can later be passed to another person the next time the mosquito takes a meal. Apart from that, malaria can also be passed on through blood transfusion and needle sharing.

Clinical features

Malaria begins as a flu-like illness. Normally it takes 10 to 15 days before the first symptoms appear. Typically, malaria causes fever, chills, headache, vomiting and without intervention, the disease can be fatal. Anemia also arises from the destruction of erythrocytes.

A typical attack of malaria begins with a feeling of intense cold as the hypothalamus is activated and body temperature rises rapidly to 41^0C (Roberts and Janovy, 1996). The victim will shiver and teeth chattering can take place. Nausea and vomiting are common. An hour later, hot stage begins with headache and intense heat. This is followed by the delirium stage, which may last for a few hours. Perspiration marks the end of the hot stage.

Anemia occurs when erythrocytes are destroyed and there is insufficient replacement. Huge quantities of erythrocyte destruction leads to increase in blood bilirubin, which might result in jaundice.

Falciparum malaria may cause some complications such as celebral malaria, pulmonary edema and black-water fever (Roberts and Janovy, 1996).

Control and prevention

Highlighting the seriousness of the disease, treatment as well as prevention is important. Throughout the world, use of insecticides to kill disease-carrying mosquito is favored. Insecticide spraying may be the most satisfying means of control but lack of financial and human resources prevent thorough destruction of the mosquito and its breeding habitat.

Prophylactic drug treatment for travelers is another method to prevent transmission of malaria. A number of drugs are used for this purpose such as quinine, mefloquine, proguanil, tetracycline and pyrimethamine. Due to multidrug resistance in various strains of Plasmodium, the drugs no longer provide the necessary protection. Therefore, the construction of a vaccine would help complement the control of this disease.

Life cycle

Two types of hosts are required for the life cycle of Plasmodium to be completed (Figure 1). The invertebrate host (mosquito) is where sexual reproduction takes place and the vertebrate host (e.g. human) is where asexual reproduction occurs.

When an infected mosquito takes a blood meal, sporozoites are injected into the bloodstream from the mosquito's salivary glands. Within an hour, the sporozoites travel within the blood to the liver and enter the liver cells. In the liver some of the sporozoites undergo asexual multiplication and become thousands of merozoites. The infected liver cells begin to burst releasing the merozoites into the peripheral circulation and these merozoites are taken up by the erythrocytes. Some of these

turn into trophozoites, which split again to form schizonts. Schizonts burst the erythrocytes releasing the merozoites. The trophozoites that are left over during division can develop into the sexual form, the gametocytes, which can be taken up by a mosquito and start another cycle (Easmon, 2003).

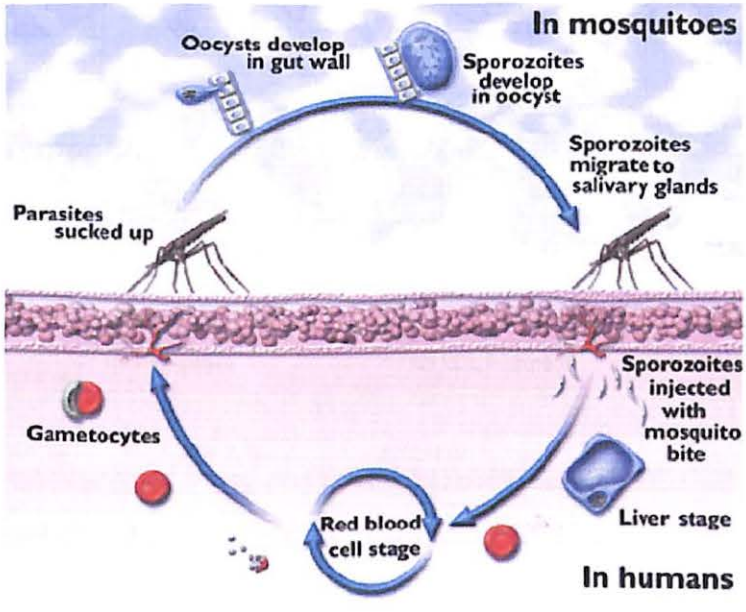

Figure 1: Life cycle of *P. falciparum* (Forsdyke, 2003)

Serine repeat antigen (SERA)

The serine repeat antigen (SERA) of *P. falciparum* is an asexual eryhtrocyte-stage antigen. Upon schizont rupture, SERA is processed into a 47-kDa N-terminal (SE47), a 50-kDa central (SE50), an 18-kDa C-terminal (SE18) and a 6-kDa domain (Figure 2) (Debrabant *et al.*, 1992). The covalently associated N- and C-terminal fragments appear to be associated with the merozoite surface, whereas the central 50-kDa and 6-kDa species are shed upon schizont rupture (Pang *et al.*, 1999) The overall

structure of SERA is remarkably conserved; nearly all nucleotide replacements are found in exon II of the gene, which corresponds approximately to the 47-kDa domain of the protein (Horii *et al.*, 1988; Bzik *et al.*, 1988; Li *et al.,* 1989; Knapp *et al.*, 1989; Knapp *et al.*, 1991; Fox and Bzik, 1994; Morimatsu *et al.*, 1994). Exon II comprises two repetitive domains, the octamer repeats (OR) and the serine repeats (SR).

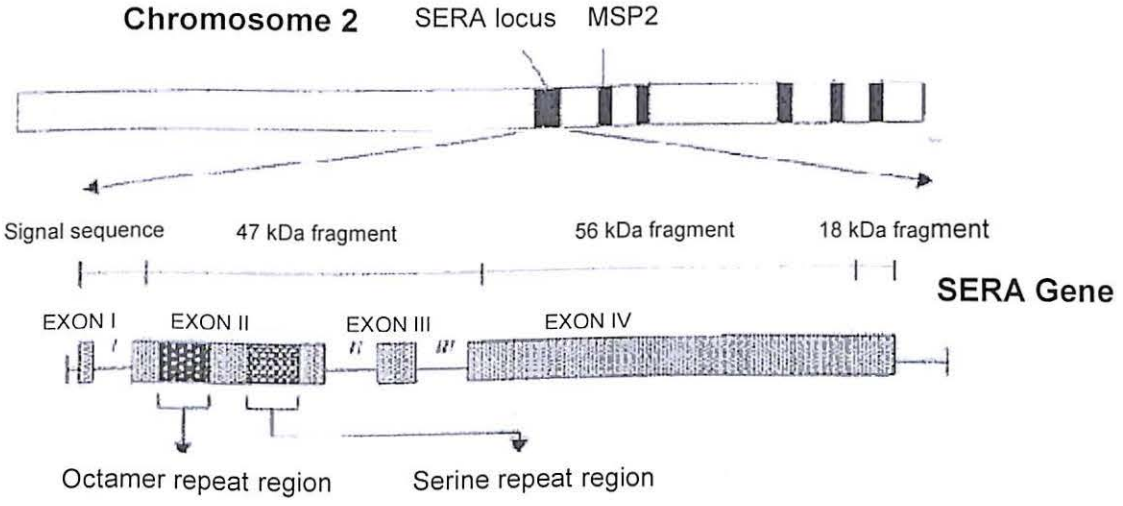

Figure 2: Schematic representation of *P. falciparum* SERA (Pang *et al.*, 1999)

SERA contains genes that play important roles in the blood stage. The genes are in a cluster of 8 homologues in Chromosome 2 and a ninth one in Chromosome 9 (Figure 3). Whereas all the SERA proteins are predicted to possess a central domain with strong homology with the papain family of cysteine proteases, some, including SERA itself, were found to contain a highly unusual cysteine to serine substitution in the active site position (Eakin *et al.*, 1989; Higgins *et al.*, 1989). All SERA homologues encode a centrally located "papain-like" protease domain, although in SERA1-SERA5 and in SERA9 the active site cysteine residue is present as a serine

(Miller *et al.*, 2002). The conserved genes in the central region of Chromosome 2 are believed to be responsible for the maintenance of blood cycle.

Monoclonal and polyclonal antibodies raised against SERA inhibit parasite growth and a major parasite-inhibitory epitope has been recently mapped to its 47 kDa N-terminal domain (Fox *et al.*, 2002). As a result, dispersion of merozoites is inhibited and parasite multiplication does not take place.

Partial protection may be induced by immunizing *Saimiri* and *Aotus* monkeys with different fractions of SERA (Perrin *et al.*, 1984; Inselburg *et al.*, 1991) including the 47 kDa N-terminal domain (Sugiyama *et al.*, 1996). Therefore, SERA is one of malaria antigens that have been proven to induce a certain degree of immunity.

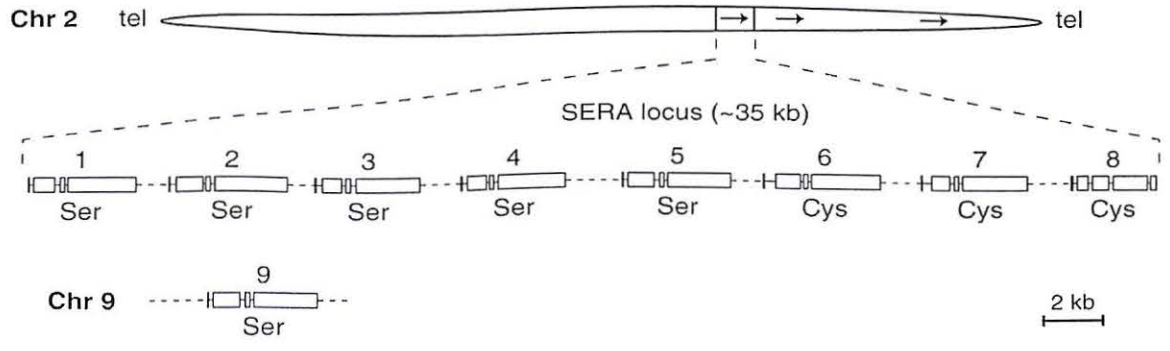

Figure 3: SERA genes in a cluster of 8 homologues in chromosome 2 and a ninth one in chromosome 9 (Miller *et al.*, 2002)

REVIEW OF LITERATURE

Safitri *et al.* (2002) reported the sequence diversity in exon II of SERA gene. Exon II comprises two repetitive domains and the variation in exon II is essentially dimorphic. All known alleles may be grouped into two basic types, named FCR3 and K1 (Morimatsu *et al.*, 1997). A third type, Honduras1, was the product of homologous recombination between FCR3-type and K1-type alleles (Liu *et al.*, 2000).

The patterns of sequence variation in SR are reminiscent of those found in the repeat arrays in the central region of the circumsporozoite protein (CSP) of *Plasmodium falciparum*. Two major mechanism may generate new SERA alleles; one is insertion or deletion of repeat units and the second mechanism is homologues recombinant between alleles of different dimorphic families.

Miller *et al.* (2002) reported that in SERA, the fifth genes in a cluster of eight homologues in chromosome 2 and a ninth homologue in chromosome 9 are essential to blood stage growth. In chromosome 2, genes at both ends were dispensable whereas those in central region could not be disrupted. This implies that they are consistent with dominant expression and play the role in maintenance of erythrocytic cycle.

SERA processing is dependent on schizont rupture and at least three proteases (Li *et al.*, 2002). SERA processing was mediated by trans-proteases from the parasite itself and not due to autocatalytic activity. The proteases are membrane associated,

correlating with the secretion and accumulation of SERA within the parasitophorous vacuole membrane (PVM). Schizont rupture and merozoite release can be inhibited by serine protease inhibitor, DFP (responsible for inhibiting cleavage of SERA to P47 and P73). Cysteine protease inhibitors E-64, leupeptin and iodoacetoamide (each inhibiting conversion of P56 to P50) also play a role.

Following studies on roles of proteases in processing of SERA, Li *et al.* (2002) studied the localization of its processed fragments. The processing took place in the erythrocytes just before schizont ruptures. Cleavage in between the N-terminal and central domain takes place first. SE47 is further processed into 2 fragments and the cleavage site is located near the centre of the amino acid sequence depending on the allelic site.

Sugiyama *et al.* (1996) showed that antisera against SE47 and another epitope SE50A inhibited malaria parasite growth. Anti-SE47 serum was more inhibitory than anti-SE50A. This suggests that the N-terminal domain of SERA is more accessible than the central domain to the antibodies or the binding of the antibodies is more toxic to the parasite than the binding to the central domain.

Puentes *et al.* (2000) reported that SERA peptides bind specifically to red blood cells. Seven peptides with high erythrocyte binding activity were found from a total of 49 non-overlapping 20-residue-long peptides encompassing the whole SERA protein of the FCR3 strain. Six of the seven high binding peptides were found in protein

conserved regions including in the SE47. Conserved peptides 6725, 6733 and the polymorphic peptide 6727 were found in the 47-kDa fragment (Figure 4). Since Puentes published the paper, many researchers have carried out research on the high binding peptides.

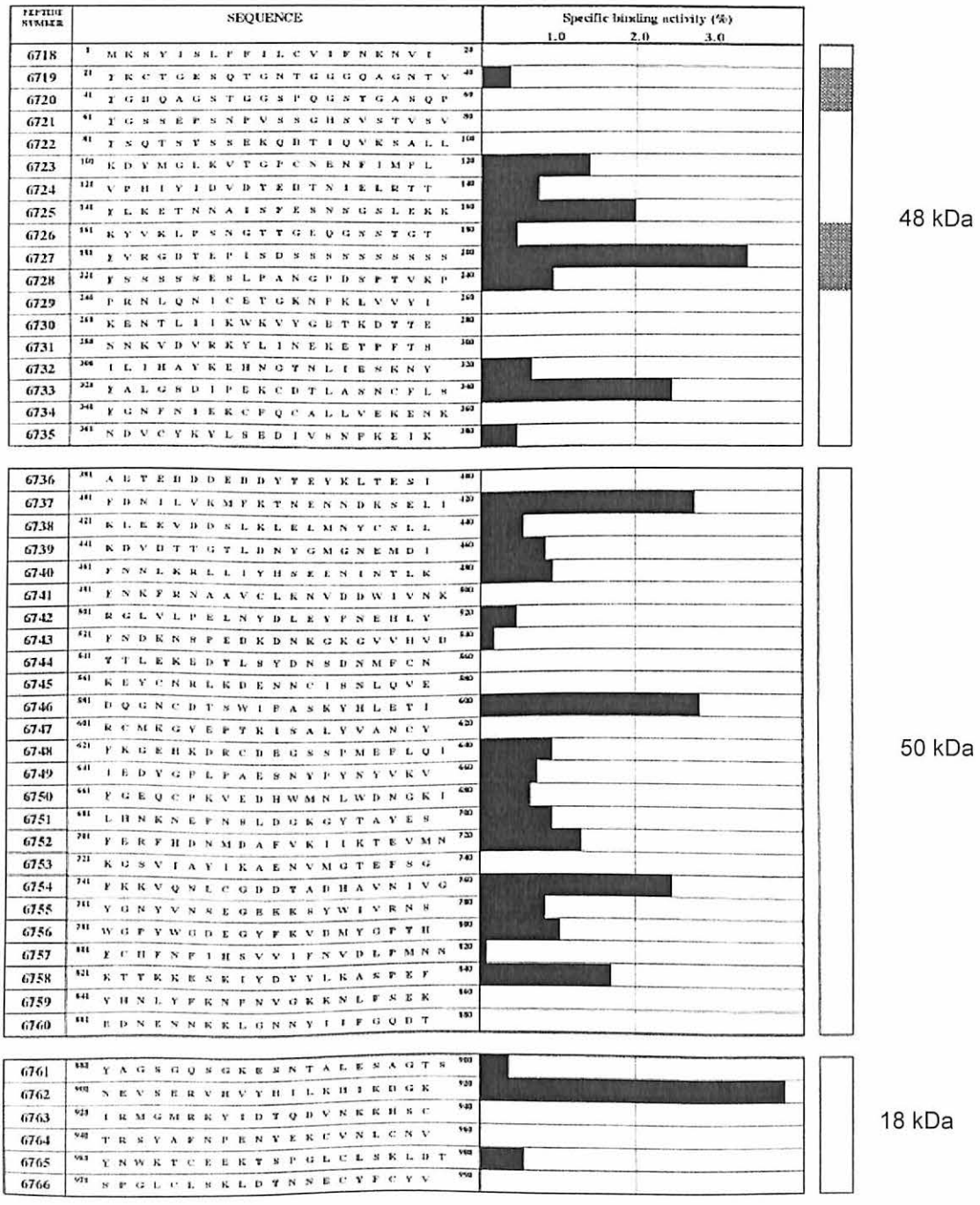

Figure 4: Erythrocyte binding assays using SERA peptides (Puentes *et al.*, 2000)

LACUNA IN THE LITERATURE

From the 49 non-overlapping 20-residue-long peptides encompassing the whole of SERA protein of the *P. falciparum* FCR3 strain, it is found that each peptide from 6723-6728 has specific binding activity with 2 of the peptides being classified as high binding peptides.

A 22-kDa DNA fragment (SE22) encodes peptides spanning the 6723 –6728 region (Puentes *et al.*, 2000). Puentes *et al.* (2000) has shown that considering the high-binding peptides are very hydrophilic, there is a high possibility that these peptides are exposed on SERA surfaces and that it may induce strong antibody responses which can inhibit merozoite invasion and contribute towards antimalarial immunity.

The importance of recognizing SERA protein fragments, which binds specifically to erythrocyte membranes, implies a possible role for protein or fragments cleaved from it in interactions with the erythrocyte membrane in schizont rupture, merozoite liberation and merozoite reinvasion of erythrocytes.

So far, there have been no studies on the 22-kDa DNA fragment (SE22) encoding peptides spanning the 6723 –6728 region in the effort to create a vaccine for malaria.

OBJECTIVE OF THE STUDY

The aim of this project is to express a synthetic DNA fragment of the SE22 epitope of *P. faciparum* in the expression vector, pPROEX™HTa. The fusion protein produced will be isolated and used to immunize rabbits to produce specific antibodies against the antigen in a future project. The antibody produced will subsequently be used to detect the expression of a synthetic SE22 gene to be cloned into *Mycobacterium bovis* bacille Calmette Guerin.

MATERIALS AND METHODS

Materials

1. Reagents, chemicals and kits

All the reagents, chemicals and kits used are tabulated below (Table 1).

Table 1: Reagents, chemicals and kits

No	Reagents, chemicals and kits	Supplier
1.	QIAprep Spin Miniprep kit, QIA quick gel extraction, QIA quick PCR purification kit, QIAexpressionist kit.	Qiagen, USA
2.	Restriction enzymes (*Eco* RI, *Eco* RV, *Xba* I) and buffer, Buffer Y^+ Tango 2X, 100bp marker, λ *Hind* III solution, λ *Hind* III fragments, Protein molecular weight marker.	Fermentas, USA
3.	PCR solution (10X buffer, Taq polymerase, dNTPs), T_4 ligase and buffer.	Roche, Germany
4.	PPROEX™HTa, Phosphate Buffer Saline.	Invitrogen, USA
5.	Tris base, Ethidium bromide, 0.5 Ethylene-diamine tetraacetate pH 8.0, Orange G, amplicilin, kanamycin, 4-chloro-1-napthol, Ponceau S.	Sigma, USA
6.	Agarose.	Promega, USA
7.	Tryptone, yeast extract.	Pronadisa, Madrid

8.	Agar base, acetic glacial acid, sodium hydroxide, Tween-20, β-mercaptoethanol, hydrogen peroxide.	Merck, Germany
9.	Sodium chloride, ammonium persulfate, TEMED.	Gibco-BRL, USA
10.	Sucrose.	Univar, England
11.	Coomasie blue, N,N methylene-bis-acrylamide, Sodium-dodecyl-sulphate, 0.8%Bis-acrylamide.	Bio-Rad, USA
12.	Glysine.	Amresco, USA
13.	Glycerol.	JT Baker, USA
14.	Di-sodium hydrogen orthophosphate anhydrous, Sodium di-hydrogen orthophosphate.	BDH, England
15.	Anti-6xHis monoclonal antibody.	Boehringer Mannheim, Germany
16.	Nitrocellulose filter Hybond membrane.	Amersham, USA
17.	Filter membrane (0.2μm).	Schleicher & Schuell, Germany

2. Equipment

Equipment used is tabulated below (Table 2).

Table 2: Equipment

No.	Equipment	Supplier
1.	Innova 4080 Rotary Shaker	New Brunswick Scientific Co. Inc., USA
2.	Labconco Purifier Class II Safety Cabinet	Labconco Corp., USA
3.	Cyberscan 1000 pH Meter	Eutech, UK
4.	Macrovue UV-25 Hoefer UV Transilluminator, Electrophoresis power supply	Amersham Pharmacia Biotechnology, USA
5.	Chemi System UVP Bioimaging Systems Image Analyser	Ultra Violet Products Ltd., USA
6.	Gene Amp PCR System 9700 Thermal Cycler	Applied Biosystems, USA
7.	Lambda EZ150 Spectrophotometer	Perkinelmer Life and Analytical Sciences, USA
8.	Eppendorf Fixed Angle Rotor Microcentrifuge, Pipettes	Eppendorf AG, Germany
9.	TBVX Hexomix Water Bath	Heto-High Technology of Scandinavia
10.	Agarose Gel Apparatus	CBS Scientific Co., USA
12.	Trans-blot semi-dry transfer cell, Power pac, Mini-protean 3 cell eletrophoresis system.	Bio-Rad Laboratories, USA.

Methods

1.0 Preparation of reagents and media

1.1 Sodium hydroxide (NaOH) 1M

A 1M NaOH was prepared by dissolving 12g of NaOH into 300ml deionised distilled H_2O. The solution was autoclaved at 121^0C for 15 minutes and stored at room temperature.

1.2 Sodium chloride (NaCl) 1M

A 1M NaCl was prepared by adding 29.2g of NaCl into 500ml deionised distilled H_2O. The solution was autoclaved at 121^0C for 15 minutes and stored at room temperature.

1.3 Hydrogen chloride (HCl) 1M

A 1M HCl was prepared by adding 8.28ml of HCl stock solution into 91.72ml deionised distilled H_2O. The solution was stored at room temperature.

1.4 Luria-Bertani agar (LB agar)

LB agar was prepared by dissolving 15g of tryptone, 5g of yeast extract, 10g of NaCl and 15g of agar into 750ml of deionised distilled H_2O. The pH was adjusted to 7.2 with 1M NaOH. Deionised distilled H_2O was added to make the final volume of 1000ml. The media was autoclaved at 121^0C for 15 minutes. The media was cooled down before appropriate antibiotic was added. The agar was stored in the cold room.

1.5 Luria-Bertani broth (LB broth)

LB broth was prepared by dissolving 15g of tryptone, 5g of yeast extract and 10g of NaCl into 750ml of deionised distilled H_2O. The pH was adjusted to 7.2 with 1M NaOH. Deionised distilled H_2O was added to make the final volume of 1000ml. The media was left to cool before being aliquoted into universal bottles. The media was autoclaved at 121^{0}C for 15 minutes and stored at room temperature.

1.6 Ampicilin stock solution (50mg/ml)

Ampicilin stock was prepared by adding 500mg of ampicilin into 10ml deionised distilled H_2O to a final concentration of 50mg/ml. The mixture was filtered using 0.2μm filter membrane prior to use and aliquoted (1ml) into microcentrifuge tubes. The antibiotic was stored at -20^{0}C.

1.7 Kanamycin stock solution (10mg/ml)

Kanamycin stock was prepared by adding 10mg of kanamycin sulfate into 10ml deionised distilled H_2O. The mixture was filtered using 0.2μm filter membrane prior to use and aliquoted (1ml) into microcentrifuge tubes. The antibiotic was stored at -20^{0}C.

1.8 Ethylene-diamine tetraacetate (EDTA) 0.5M

A 0.5M EDTA solution was prepared by dissolving 93.06g EDTA in 300ml sterile deionised distilled H_2O while adjusting the pH to 8.0 using 1M NaOH. The solution

was made up to 500ml with deionised distilled H_2O. The 0.5M EDTA solution was stored at room temperature.

1.9 Tris Acetate EDTA (TAE) 50X

A 50X stock solution of TAE was prepared by dissolving 121g of Tris base in deionised distilled H_2O and adding 50ml of 0.5M EDTA and 28.5ml glacial acetic acid. Deionised distilled H_2O was added to make a final volume of 500ml and stored at room temperature. A working solution (1X TAE) was prepared by diluting 20ml of stock solution with 980ml deionised distilled H_2O.

1.10 Stock solution of ethidium bromide (10mg/ml)

This was prepared by dissolving 1g of ethidium bromide in 100ml deionised distilled H_2O. The solution was stored in dark bottle at room temperature.

1.11 Loading buffer

Loading buffer was prepared by adding 20g of sucrose and 0.25g of Orange-G dye into a microcentrifuge tube containing 30µl of deionised distilled H_2O. After mixing, the volume was added to 50µl deionised distilled H_2O and stored at 4^0C.

1.12 Molecular size marker (100bp) (50ng/µl)

Molecular size marker (100bp) was by adding 20µl of stock preparation (250ng/µl) (Fermentas, USA), 20µl of 1X TAE and 60µl of Orange-G dye in a microcentrifuge tube. The marker was stored at 4^0C.

1.13 Lambda *Hind* III marker (50ng/μl)

Lambda *Hind* III marker was prepared by adding 20μl of stock preparation (250ng/μl) (Fermentas, USA), 20μl of 1X TAE and 60μl of Orange-G dye in a microcentrifuge tube. The marker was stored at 4^0C.

1.14 Magnesium chloride ($MgCl_2$) 100mM

A 100mM $MgCl_2$ solution was prepared by dissolving 2.033g of $MgCl_2$ in 70ml deionised distilled H_2O. The solution was made up to 100ml with deionised distilled H_2O. The solution was autoclaved at 121^0C for 15 minutes and stored at room temperature.

1.15 Calcium chloride ($CaCl_2$) 100mM

A 100mM $CaCl_2$ solution was prepared by dissolving 1.470g of $CaCl_2$ in 70ml deionised distilled H_2O. The solution was made up to 100ml with deionised distilled H_2O. The solution was autoclaved at 121^0C for 15 minutes and stored at room temperature.

1.16 Glycerol solution (80%)

A 80% glycerol solution was prepared by adding 80ml of 100% glycerol into 20ml deionised distilled H_2O. The solution was autoclaved at 121^0C for 15 minutes and stored at room temperature.

1.17 *E. coli* Competent cells

One colony of *E. coli* was selected and cultured overnight in 10ml of LB broth. Six hundred μl of the LB broth was aliquoted into a conical flask containing 60ml LB broth. The culture was incubated at 37^0C with vigorous shaking until the optical density at 600nm wavelength (OD_{600}) was 0.3. The tube was then centrifuged at 3000g for 10 minutes at 4^0C. The supernatant was discarded and pellet was resuspended in 6ml cold 10mM $MgCl_2$. The tube was then placed on ice for 45 minutes. The tube was recentrifuged at 3000g for 10 minutes at 4^0C and supernatant discarded. The pellet was finally resuspended in 400μl of cold 10mM $CaCl_2$. Sixty μl of cold 80% glycerol was then mixed into the cell suspension which were then aliquoted (30μl) into microcentrifuge tubes. The tubes were snap-frozen in ice-cold ethanol and stored at -80^0C.

1.18 Isopropyl-β-D-thiogalactosidase (IPTG) 1M

A 1M IPTG stock was prepared by adding 2.38g of IPTG into 100ml of deionised distilled H_2O to a final concentration of 238mg/ml. The mixture was filtered and aliquoted (1ml) into microcentrifuge tubes and stored at -20^0C.

1.19 Stacking gel buffer

Stacking gel buffer was prepared by dissolving 6.05g of Tris base and 0.4g of sodium-dodecyl-sulphate (SDS) into 40ml of deionised distilled H_2O. The pH was adjusted to 6.8 with 1M HCl. Deionised distilled H_2O was added to make the final volume of 100ml. The solution was filtered and stored at 4^0C.

1.20 Resolving gel buffer

Resolving gel buffer was prepared by dissolving 90.85g of Tris base and 2g of SDS into 40ml of deionised distilled H_2O. The pH was adjusted to 9.3 with 1M HCl. Deionised distilled H_2O was added to make the final volume of 500ml. The solution was filtered and stored at 4^0C.

1.21 Protein loading sample buffer

Protein loading sample buffer was prepared by dissolving 0.76g of Tris base, 1g of SDS and 10ml of glycerol into 40ml of deionised distilled H_2O. The pH was adjusted to 6.8 with 1M HCl. Deionised distilled H_2O was added to make the final volume of 50ml. The solution was filtered and stored at 4^0C.

1.22 Ammonium persulfate (20%)

A 20% ammonium persulfate solution was prepared by dissolving 0.1g ammonium persulfate in 500µl deionised distilled H_2O.

1.23 Protein running buffer

Protein running buffer was prepared by dissolving 3g of Tris base and 1g of SDS into 900ml of deionised distilled H_2O. The pH was adjusted to 8.3 with 1M HCl. Deionised distilled H_2O was added to make the final volume of 1000ml. The solution was stored at 4^0C.

1.24 Protein transfer buffer

Protein transfer buffer was prepared by dissolving 3.03g of Tris base, 14.4g of glysine and 200ml of methanol into 900ml of deionised distilled H_2O. Deionised distilled H_2O was added to make the final volume of 1000ml. The solution was stored at 4^0C.

1.25 Coomassie Blue solution

Coomassie Blue solution was prepared by dissolving 0.8g of Coomassie Blue and 100ml of isopropyl solution into 50ml of sterilized deionised distilled H_2O. Deionised distilled H_2O was added to make the final volume of 200ml and stored at room temperature. A 10% acetic acid was added prior to use.

1.26 Ponceau S solution

Ponceau S solution was prepared by dissolving 0.5g of Ponceau S in 1ml of acetic acid. Deionised distilled H_2O was added to make a final volume of 100ml and stored at room temperature.

1.27 Skimmed milk solution 3%(v/V)

Skimmed milk solution was prepared by dissolving 3g of skimmed milk powder into 50ml of sterilized deionised distilled H_2O. Deionised distilled H_2O was added to make the final volume of 100ml to give a 3% (v/v) solution.

1.28 Phosphate buffered saline (PBS)

Phosphate buffered saline (PBS) was prepared by dissolving 11.5g of Di-sodium hydrogen orthophosphate anhydrous, 2.96g of sodium di-hydrogen orthophosphate and 5.84g NaCl into 900ml of deionised distilled H_2O. The pH was adjusted to 7.5 with 1M HCl. Deionised distilled H_2O was added to make the final volume of 1000ml. The solution was stored at 4^0C.

1.29 PBS-Tween 20 (PBS-T20)

PBS-Tween 20 (PBS-T20) was prepared by adding 500µl of Tween-20 into 999.5ml of PBS to give a 0.1% (v/v) solution. The solution was stored at 4^0C.

1.30 Chromogenic substrate (4-chloro-1-napthol and hydrogen peroxide) solution

Reagent A was prepared by dissolving 0.3g of 4-chloro-1-napthol into 100ml of cold methanol. Reagent B was prepared by mixing 300ml of hydrogen peroxide with 500ml of PBS. Both reagents were stored at 4^0C.