UNIVERSITY SCIENCE MALAYSIA

Construction of the *Aequorea victoria* Green Fluorescent Protein (GFP) for Expression in *Mycobacterium sp.*

Dissertation submitted in partial fulfilment for the Degree Of Bachelor Of Health Science (Biomedicine)

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CERTIFICATE

This is to certify that the dissertation entitled;

"Construction Of The *Aequorea victoria* Green Fluorescent Protein (GFP) For Expression In *Mycobacterium Spp*".

is the bonafide record of research work done by,

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ABSTRACT

The development of sensitive methods for observing individual bacterial cells in a population in experiment and natural environments is very crucial for rapid development of anti-mycobacterial drugs. This is because more pathogenic Mycobacteria are slow growing organisms, and therefore the screening compounds for anti-mycobacterial activity is slow and inefficient. Previously, studies have been done using fluorescent bacteria that expressing β-galactosidase (21), and luciferase (3) as a high screening format for anti-microbial activity. However, one drawback of these systems is that substrates such as luciferin have to be added at the required time points to induce fluorescence.

Recently Green Fluorescent Protein (GFP) has become a valuable and favourite tool as a marker of growth, which could be used for screening of anti-microbial activity. This is because the marker can be visualised without interruption or termination of an experiment as is required with the detection of other commonly used markers such as ß-galactosidase and luciferase.

In this study a synthetic gene of GFP was constructed with mycobacteria codon bias using assembly PCR. A strong mycobacterial promoter from 65 kD mycobacterial heat shock protein was added to derive the expression. The constructed gene was cloned into vector and transformed into *Escherichia coli*. The ultimate aim is to use the recombinant plasmid carrying the synthetic gene with mycobacterial codon bias to create a recombinant fluorescing BCG, which

can be used as a tool for screening compound libraries for anti-mycobacterial activity.

CHAPTER 1

1.0 INTRODUCTION

1.1 REVIEW OF TUBERCULOSIS

Tuberculosis has been present in the human population as old as human history. A fragment of the spinal column of on Egyptian mummy from 2400 BC showed definite pathological of tubercular decay. An ancient Greece philosopher, Hippocrates described a common illness that was known as pthisis. (33) It is believed that, pthisis is the same disease that nowadays called is tuberculosis. Over the centuries since Hippocrates, tuberculosis has been known as a major scourge of the human species, and other species as well. (33) In the first half of the 20th century, tuberculosis was known as 'consumption' or 'white plaque' and became the number one killer in many populations (35).

In 1720, the English physician Benjamin Marten was the first to conjecture, in his publication, *A New Theory of Consumption*, that TB could be caused by "wonderfully minute living creatures", which, once they had gained a foothold in the body, could generate the lesions and symptoms of the disease. (35). And, because of its infectious nature and usually long chronic course, tuberculosis had a great impact on the health and lives of many families. Worst still, until 1800s, there was no treatment for tuberculosis and the disease approved often cause death to the infected persons. (33)

In 1865, the French military doctor, Jean-Antoine Villemin, single-handedly demonstrated that consumption could be passed from humans to cattle and from cattle to rabbits (35). On the basis of this revolutionary evidence, he postulated a specific microorganism as the cause of the disease, finally laid to rest the centuries-old belief that consumption arose spontaneously in each affected organism. In 1882, Robert Koch discovered a staining technique that enabled him to discover the causative agent of tuberculosis and named it as *M. tuberculosis* (35).

In 1940, Selman A. Waksman and his team were able to isolate an effective anti-TB antibiotic, actinomycin, but this proved to be too toxic for use in humans or animals. However, in 1943, streptomycin was purified from *Streptomyces griseus*, which exerted maximal inhibition of *Mycobacterium tuberculosis* with relatively low toxicity. Following streptomycin, *p*-aminosalicylic acid (1949), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), ethambutol (1962) and rifampin (rifampicin; 1963) were introduced as anti-TB agents (35). Aminoglycosides such as capreomycin, vancomycin, kanamycin and amikacin, and the newer quinolones (e.g. ofloxacin and ciprofloxacin) are also used, but only in drug resistant situations. Combinations of a β-lactam antibiotic with a β-lactamase inhibitor enhance treatment effectiveness, but the newer drugs, including the macrolides, have not had much of the clinical testing (35).

Unfortunately, 50 years after the introduction of effective chemotherapy, TB remains, along with AIDS, the leading infectious cause of adult mortality in

the world, causing up to two million of deaths in every single year (30). The number of new TB cases each year climbed 6% between 1997 and 1999 to 8.4 million worldwide (31). And, nowadays, it is estimated that if the trend continued, there will be a total of 36 million deaths by 2020 each year (30).

In 1993, World Health Organization declared tuberculosis as a global emergency, responsible for causing death of more youth and adults than any other pathogens. During 2001, a standard form for reporting surveillance data was sent to 210 countries requested the information about policy and practice in TB control, the number and types of TB cases notified in 2000, and the outcomes of treatment and retreatment for smear-positive cases registered in 1999 (29).

Tuberculosis is a disease that caused by a grouped microorganism, which is known as *Mycobacterium tuberculosis* complex. The disease remains one of the world's most difficult problems, affecting one-third of the world population. And, among these, eight to ten million develop new active disease and caused more death per year worldwide than any other bacterial pathogens. Moreover, evidence showed tuberculosis is the greatest caused of death among women of reproductive age and it is estimated that 900 million women are currently infected and 2.5 million will develop active disease and one million will die (30).

According to World Health Organization, more than 1.5 million tuberculosis cases occur annually in Sub-Saharan Africa, nearly three million cases occur in Southeast Asia and over 250000 cases in Eastern Europe (31). In

the United States the rate of tuberculosis infection had been declining steadily until 1984, when there was an increase (31). Numerous factors have been identified to account for the resurgence of tuberculosis in United States as well as in Europe and all over the world. These includes reactivation of the disease in the elderly, the prevalence of Human Immunodeficiency Virus (HIV) infection and AIDS, immigration of infected persons from countries where tuberculosis is prevalent, socio-economic decline in urban areas, erosion of the system for diagnosis and treatment of the disease, and the emergence of multidrug-resistant strains of *Mycobacterium tuberculosis*. (30)

Because of these reasons, WHO estimates that eight million people get tuberculosis every year, of which 95% live in the developing country. Furthermore, AIDS or Autoimmune Deficiency Syndrome coexisting with of mycobaterial infection is bringing back tuberculosis into Western cities and seriously threatens health services in the developing country (35). In addition, it is believed that among HIV patients one in ten per year will develop active tuberculosis, while one in two or three tuberculin positive AIDS patients will develop active TB. (35)

The situation is worsening because there has been no new class of antimycobacterial drugs introduced over the last 30 years (28). Furthermore, the treatment regimen consists of a combination of drugs, which must be taken over a period of 6 to 12 months (4). The length of the treatment often results in non-

compliance when patients do not undertake the treatment properly. Improper treatments often result in bacteria to develop resistance to antibiotics.

The emergence of multi-drug resistance occurs as a result of mutation. The mutation is not only dependent upon the presence of the drug. For example, when exposed to a single effective anti-tuberculosis medication, the predominant bacilli, sensitive bacilli are killed, while a few drug resistant mutants, likely to be a present if the bacterial population are large, will multiply freely (35). Because of these reasons, it is crucially important that support be given to research efforts devoted to developing an effective TB vaccine, shortening the amount of time required to ascertain drug sensitivities, improving the diagnosis of TB, and creating new, highly effective anti-TB medications (36).

CHAPTER 2

2.0 REVIEW OF LITERATURE

Mycobacterium is a slow growing bacterium, which may takes anything between 2 to 8 weeks in order to grow. This will cause the screening of compounds for antimycobacterial activity by conventional microbiological methods to be extremely slow and inefficient (4). Because of that, new methods with high echnology need to be developed to allow high throughput screening of compound for antimycobacteria activity. Previously, fluorescent bacteria expressing β-galactosidase (21) and luciferase (3) have been used in a high throughout screening format for anti-tuberculosis drug.

The use of fluorescent recombinant mycobacterium expressing luciferase gene have been reported, but this method has certain disadvantages. This is because the substrate, luciferin, has to be added at the require time points to induce fluorescent (10). Thus this detection method require the distruption of cells, which therefore do not allow a single culture to be followed through from the beginning until the end (4). β-galactosidase on the other hand is enzymatically active only when retained in the cytoplasm (13).

In addition to in vitro assays, the screening system must include assays that can detect antimycobacterial activity of the test samples against mycobaterium inside the macrophages. The luciferase expressing recombinant mycobacteria system has been used in a macrophage based on the assay

system (3). Furthermore, a system utilizing mycobacteria expressing the *Escherichia coli* beta-galactosidase genes have also been used in the macrophage format (17). However these methods require extracts to be prepared and the addition of substrate, which increase, time and labour. While, Green Fluorescent Protein expressing recombinant mycobacteria have been successfully used in in-vitro assay for anti-mycobacterial assay.

Green Fluorescent Protein (GFP) was discovered as a convenient reporter of cell viability (23), that have the ability to overcome these problems. The fluorescent protein has been shown to be useful in the development of recombinant mycobacteria for screening assay (9). However, the level of fluorescence emitted by these recombinant mycobacteria was found to be low and therefore decreasing sensitivity. This could be due to the fact that mycobacteria are GC rich organism, which may not express low GC rich genes.

2.1 PROPERTIES OF *Mycobacterium tuberculosis*

M. tuberculosis is a member of the closely related group of species, known as the *Mycobacterium tuberculosis* complex. Apart from *M. tuberculosis* this complex includes *Mycobacterium bovis, Mycobacterium africanum* and *Mycobacterium microti.* Apart from *Mycobacterium microti,* which affects voles and is of limited human pathogenicity, the other three can all cause human tuberculosis. The mycobacterial genome has been estimated to be around 3-5.5 x 10^9 daltons. (8). Unlike the fast growing mycobacteria and organisms like *Escherichia coli, Mycobacterium tuberculosis* has only one rRNA operon.

On the basis of growth rate, Mycobacterium can be divided into two groups of species, known as the Slow Growers and the Fast Growers. The Slow Growers tend to be associated with human or animal disease, while the Fast Growers tend to be non-pathogenic. They are widely distributed in soil and some marine environments (34). Over 300 species have been named. The complete genome sequence of the best-characterized strain of *M. tuberculosis*, H37Rv, has been determined and analysed in order to improve the understanding of the biology of this slow-growing pathogen and to help the development of new prophylactic and therapeutic interventions. (8).

The genome comprises 4,411,529 base pairs, contains around 4,000 genes, and has a very high guanine and cytosine content. (8). The GC content of the DNA of those mycobacterial species studied varies from 66-71 of molecule

percentage. (34). *M. tuberculosis* differs radically from other bacteria in that a very large portion of its coding capacity is devoted to the production of enzymes involved in lipogenesis and lipolysis, and to two new families of glycine-rich proteins with a repetitive structure that may represent a source of antigenic variation (8).

Mycobacteria are Gram-positive non-motile, pleomorphic rods, related to the *Actinomyces*. Most mycobacteria are found in habitats such as water or soil. However, a few are intracellular pathogens of animals and humans. *Mycobacterium tuberculosis* is a highly contagious, airborne, rod-shaped organism (bacillus) that thrives on oxygen, grows slowly and possesses a 'waxy' cell wall. It also an obligate aerobes that grow most successfully in tissues with high oxygen content. Mycobacteriums are facultative intracellular pathogens that usually multiply in the mononuclear phagocytes.

The cell walls are hydrophobic with high lipid content, and are often known as acid-fast bacilli. The cell wall's structure and function are not well understood but appear to allow the bacteria to survive within immune cells called macrophages, specialized cells that destroy bacteria and viruses. It is also provides the organism with a resistant barrier to many common drugs.

2.2 GREEN FLUORESCENT PROTEIN

Green fluorescent protein, GFP, is a spontaneously fluorescent protein isolated from the Pacific jellyfish, *Aequoria victoria* (19). Its role is to transduce, by energy transfer, the blue chemiluminescence of another protein, aequorin, into green fluorescent light (25). The light is produced when energy is transferred from the Ca^{2+}- activated photoprotein aequorin to green fluorescent protein (26).

The molecular cloning of GFP cDNA (20) and the evidence that GFP can be expressed as a functional transgene (7) has opened exciting new avenues of investigation in cell, developmental and molecular biology. Fluorescent GFP has been expressed in bacteria (7), yeast (14), slime mold (18), plants (6), drosophila (12), zebrafish (24), and in mammalian cells (2). This protein function as a protein tag, as it tolerates N- and C-terminal fusion to a broad variety of proteins many of which have been shown to retain native function (18).

Green Fluorescent Protein had become an important expression marker for monitoring gene expression and protein localization *in vivo, in situ*, and real time (15), and is useful for monitoring of bacteria in laboratory model systems and in actual environment (27). It is also attracting wide spread interest because of its tremendous potential for cell lineage determination and *in-situ* monitoring of cell growth and development. GFP expressing recombinant Mycobacterium have been used in vitro assays for screening compound for antimycobacterial activity (16).

Furthermore, these proteins are stable, species independent, do not require a substrate or cofactor and can be easily detected. The GFP marker can be visualized by using standard microscope equipped with commonly available fluorescent filter sets. In addition, green fluorescent protein has been proved to be a valuable tool for studying a variety of biological questions with living systems (7).

GFP may be useful as a growth marker for mycobacteria as it may be detected at a very early stage of growth. This will lead to detection of antimycobacterial activity at an early stage without requiring colonies to form, which may take up 8 weeks. In addition, because of its cloning and commercial availability, the applications of GFP as a reporter gene have become prevalent in many studies (1). For example, some research demonstrated that the green recombinant *Mycobacterium bovis* BCG were clearly visualized by UV microscopy (27). This study proposed new method for monitoring BCG bacteria trafficking with recombinant BCG expressing Green Fluorescent Protein (27). Meanwhile, plasmid pBEN, containing the gene encoding a red-shifted, high-intensity GFP mutant, was incorporated into *Mycobacterium terrae* (ATCC 15755), and the GFP expression was observed by epifluorescence microscopy .

In other studies, two green fluorescent protein (GFP) fusion vectors were constructed for use in *Mycobacterium spp.* The first plasmid facilitated quantification of mycobacterial promoter activity (11). The second vector

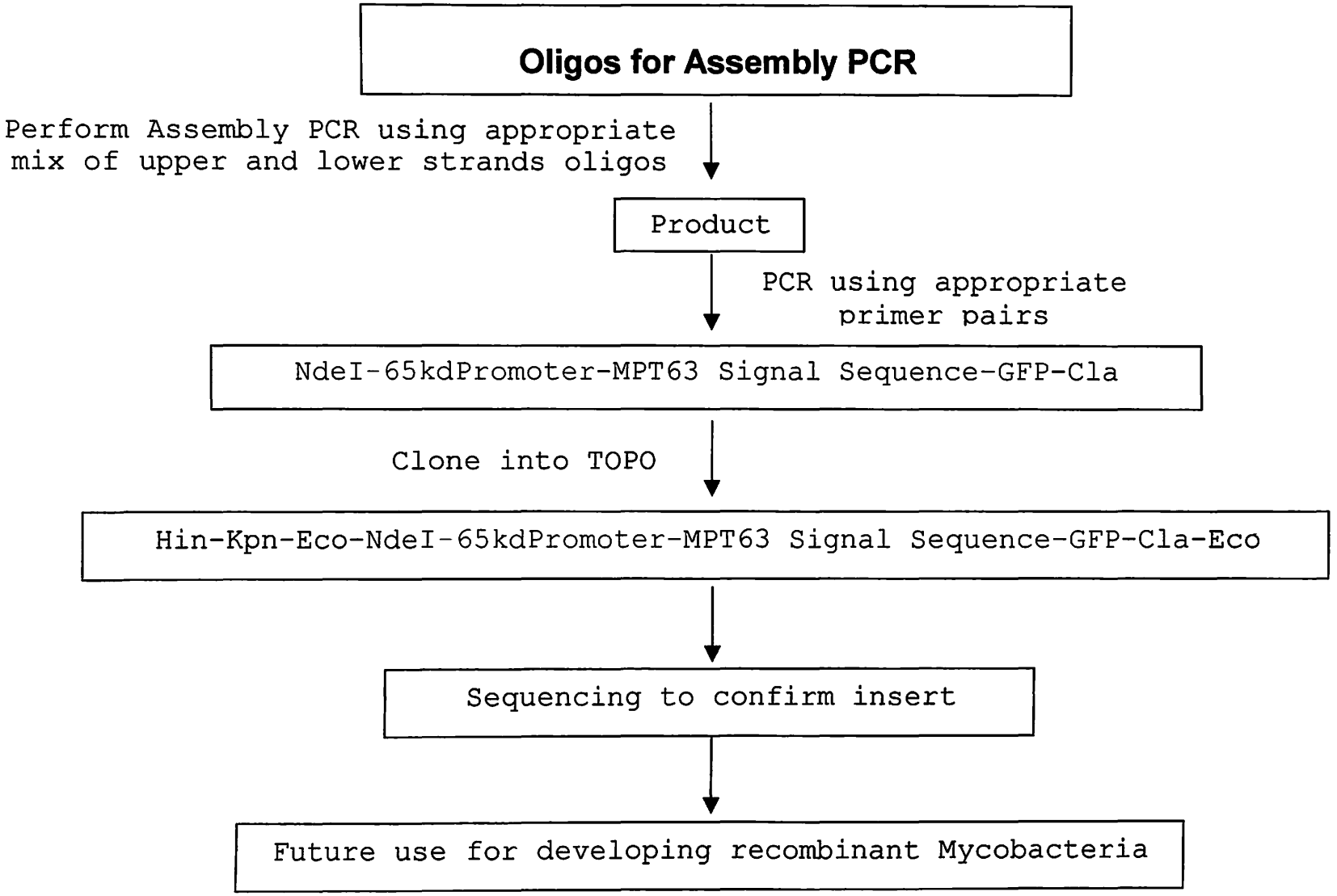
permited construction of translational fusions of mycobacterial proteins to GFP in order to study subcellular localization including protein secretion (11). Using this translational fusion construct, it was possible to show that a GFP fusion to the putative secreted *M. tuberculosis* protein ChoD was translocated to the extracellular milieu when cloned and expressed in *Mycobacterium smegmatis* (11).

CHAPTER 3

3.0 OBJECTIVE OF THE STUDY

In this research, further enhancement of fluorescence was attempted through the construction of a synthetic gene of GFP that will be developed with mycobacterial codon bias and fused to a mycobacterial signal sequence to enable secretion of the protein into the medium. The ultimate aim in this research is to develop a recombinant plasmid that can be used to express GFP at an optimum level in BCG. This fluorescent recombinant BCG can then be used as a tool for rapid screening of compounds for antimycobacterial activity. The overall research strategy will be shown in the Figure 1.

Fig. 1.0 Overall Research Strategy



CHAPTER 4

4.0 MATERIALS AND METHOD

4.1 MATERIALS

The materials and reagents that were used in this project were listed as below, categorized by the product company.

BIO-RAD

The power supply for Agarose Gel electrophoresis.

FERMENTAS

Taq DNA Polymerase, deoxynucleotides triphosphates (dNTPs), $MgCl_2$, (Concentration-10x) PCR Reaction Buffer, PCR reaction buffer with ammonium sulphate, restriction enzyme and their respective 10x buffers.

INVITROGEN

100bp DNA Ladder, TOPO TA Cloning ® Kit

PROMEGA

Agarose, X-Gal, Tris Base, dATPs, 100 bp marker

SIGMA

Disodium EDTA $2H_2O$ (Ethylene Diaminotetraacetic Acid), Bromophenol Blue

AMNESCO

N', N-Dimethylformamide (DMF)

PRONADISA

Tryptone, Yeast Extract

MERCK

Agar-agar

SYSTEM

Boric Acid

BDH Chemicals Ltd.

Ethanol (absolute), MgCl2-6 hydrate

BOEHRINGER MANNHEIM

Expand™ High Fidelity PCR System

ROCHE

10x concentration PCR Reaction Buffer

MILLIPORE

DNA Gel Extraction Kit

4.1.1 Tris-borate-EDTA (TBE), pH 8, 10 X strength

Table 1: The ingredient of reagents used in the preparation of TBE.

Reagent	Volume/weight
Tris base	108 g
Boric Acid	54 g
$Na_2EDTA.2H_2O$	4.65 g
dH_2O is added up to	1000 ml

The solution was autoclaved and kept at room temperature. For agarose gel electrophoresis, the solution was diluted to 0.5 X strength. In order to obtain 1000

ml of 0.5 X, 50 ml of 10 X TBE solutions will be diluted using 950 ml of distilled water. The solution had been reused twice. It is because reused of running buffer lead to increasing of pH and therefore reduce yields of DNA. In addition, in the absence of ions, electrical conduction is minimal and the DNA migrates very slowly or not at all. In the high ion concentration the current conductance is so efficient, that a lot of heat is generated and the resulting the gel melt and DNA is denatured.

4.1.2 Loading Buffer

Table 2: The ingredient of reagents used in the preparation of loading buffer

Reagent	Volume/weight
Gliserol (BDH chemicals)	3 ml
Bromophenol Blue (Sigma, USA)	0.03 g

The deionised-distilled water is being added to make up the end volume to 10 ml. The solution is being kept at 4°C.

4.1.3 Preparation Of Ethidium Bromide

10 μg/μ1 of stock or working solution of 0.5 to 1.0 μg/μl prepared by dilution of 100 μ1 of stock solution in the 500 μl of distilled water. The prepared solution was stored in the dark bottle because it is sensitive to the light.

4.1.4 Preparation of 100 bp marker (PROMEGA)

Table 3: The ingredient of reagents used in the preparation of 100bp marker.

Reagent	Volume (μl)
DNA ladder	20
Loading buffer	20
1X TRIS/EDTA (pH 7.6)	60

The DNA ladder that provided by OMEGA was prepared by adding particular volume of loading buffer and 1XTRIS/EDTA (pH 7.6). The marker solution is kept at 4°C. The marker is being used to determine the size of double stranded DNA. The ladder is consisting of 11 double-stranded DNA fragments with the size of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500 bp. The 500 bp band is present at triple the intensity of the other fragments and served as a reference indicator while other fragment appear equal intensity on the gel. The loading volume used is 5 μl.

4.1.5 Preparation Of λ HindIII marker (500 mg/μ1)

Table 4: The ingredient of reagents used in the preparation of λ HindIII marker.

Reagent	Volume (μl)
λ Hind III marker	20
Loading buffer	20
1X TRIS/EDTA (pH 7.6)	60

The final concentration for the marker was 50 mg/μ1. λ HindIII marker consist of 564, 2027, 2322, 4361, 6557, 9416, 23130 base pair in size. The marker must be incubated at 65°C for 10 minutes before used for first time usage.

4.1.6 Blue Orange 6X Loading Dye (G190A)

The blue orange 6X dye was supplied with the commercial markers. It has a composition of 15% Ficoll® 400, 0.03% Bromophenol Blue, 0.03% Xylene cyanol, 0.4% Orange G, 10 mM Tris-HCl (pH 7.5) and 50 mm EDTA.

4.1.7 10 X TE buffer (Tris 100 mM, EDTA 10 mM)

Table 5: The ingredient of reagents used in the preparation of 10 X TE buffer.

Reagent	Weight (g)
Tris, pH 8.0	12.1
EDTA	3.7

Distilled water was added to 100 ml and the solution were autoclaved at 121°C for 15 minutes. The solution was kept at room temperature. 1 X TAE was prepared, by diluting the solution with sterile water at particular volume.

4.1.8 Preparation of Ethanol 70 % (BDH Chemicals)

Table 6: The ingredient of reagents used in the preparation of Ethanol 70%.

Reagent	Volume (ml)
Ethanol (Analar Grade)	70
Sterile water	30

To prepare 70 % ethanol concentration, 70 ml ethanol was diluted with 30 ml of sterile water. The solution was kept in the room temperature for not longer than 1 year.

4.1.9 Preparation Of 0.1M $MgCl_2$ (BDH Chemicals)

Table 7: The ingredient of reagents used in the preparation Of 0.1 M $MgCl_2$

Reagent	Volume/weight
$MgCl_2$	2.03 g
Sterile water	100 ml

2.03 g of $MgCl_2$ powder was added to 100 ml of distilled water before autoclaving. The solutions were kept at room temperature.

4.1.10 Preparation Of 0.1M $CaCl_2$ (BDH Chemicals)

Table 8: The ingredient of reagents used in the preparation Of 0.1 M $CaCl_2$

Reagent	Volume/ weight
$CaCl_2$	2.03 g
Sterile water	100 ml

2.03 g of $CaCl_2$ dehydrate was added to 100 ml of distilled water before autoclaving. The solution was kept at room temperature.

4.1.11 Preparation of Culture Media

4.1.11.1 Luria-Bertani Broth

Table 9: The ingredient of reagents used in the preparation Of LB Broth.

Reagent	Volume/weight
Bactotryptone	15 g
BactoYeast extract	5 g
$NaCl_2$	10 g
dH_2O make up to	1000 ml

Ingredients were dissolved in 800 ml of distilled water and pH was adjusted to 7.5. The volume was made up to 1000 ml with distilled water. The solutions were aliquoted into 5 or 10 ml per bottle. All media were autoclaved at 121°C for 15 minutes, and allowed to cool to room temperature before addition of antibiotic or other substances if required. The broth was kept at room temperature.

4.1.11.2 Luria-Bertani Agar

Table 10: The ingredient of reagents used in the preparation Of LB Agar

Reagent	Volume/weight
Bactotryptone	15 g
Yeast extract	5 g
$NaCl_2$	10 g
dH_2O make up to	1000 ml
Agar	7.5 g

The ingredients were dissolved into 800 ml of water and pH was adjusted to 7.2. The volume was then made up to 1000 ml with water. The media were autoclaved at 121°C for 15 minutes, and allowed to cool to 50-55°C before addition of antibiotic or other substances if required. Ampicillin is usually added to a final concentration of 50 μg/ml. Then, the medium was plated out onto petri dish. The plates were incubated overnight as control for contamination or incomplete sterilization process. The plates were then kept in the cold room before used.