Construction of vector for *iclR* knockout in P solubilizing *Klebsiella pneumoniae* SM6 and SM11

A

Dissertation Thesis Submitted to

Nirma University

In Partial fulfilment of requirement for

The Degree of

Master of Science

in

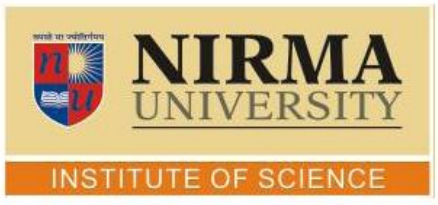
Biochemistry

Submitted by

Shivang Kapadia (12MBC012)

Bhupen Pandya (11MBC003)

Under the guidance of
Dr. Shalini Rajkumar
Associate Professor



Institute of Science, Nirma University,
Ahmedabad-382481
Gujarat, India

ACKNOWLEDGEMENT:

First and foremost we would like to thank **God** with our pure heart and soul for his divine guidance and internal support. We personally feel that he is the one behind every success. Words will always fall short to thank our **parents** for making us eligible to reach to this platform and perform something on our own. Their constant care and support was the driving force for us throughout the project.

We would like to express our deepest gratitude to our guide Dr. Shalini Rajkumar for showing her confidence in us and being immensely supportive throughout our project work. During the entire span of the project work she has always motivated us and shown her immense patience. We would also like to acknowledge Prof. Sarat Dalai, Director, Institute of Science, Nirma University for providing all the infrastructure and resources which were required for our project.

We would also like to thank Dr. Sriram Seshadri, Dr. Mili Das, Dr. Nasreen Munshi, Dr. Amee Nair, Dr. Sonal Bakshi and Dr. Vijay Kothari for their support.

We would also like to thank PhD Scholars, Mr. Mahendrapal Singh Rajput, Miss Bhagya Iyer, Mr. Rahul Jog, Mr. Maharshi Pandya, Mr. Manoj Patidar, Mr. Rajesh Parmar, Ms. Aditi Mathur, Mr. Hardik Patel, Mr. Naveen Yadav, Mrs. Kaveri Purandhar, Mr. Prasant Jena, Ms. Bhumika Prajapati and Mr. Parth Rajput, Ms. Suhani Palkhivala, Ms. Nazia Khan, Ms. Fulesh Kunwar, for their valuable advices and continuous motivation and encouragement during the entire span of this project. We are very thankful to all the technical and non-technical staff of Institute of science Mr. Sachin Prajapati, Mr. Hasit Trivedi and Ms. Shweta Patel for their valuable cooperation and support.

Last but not the least we would also like to thank our classmates for extending their helping hand always and making this dissertation project a cheerful journey.

Shivang Kapadia

Bhupen Pandya

CONTENTS

LIST OF TABLES:

LIST OF FIGURES:

Abstract

In the present study, *GFP* gene was incorporated in pUC18 cloning vector for the selection and tagging of fluorescent protein. The isolated plasmids pUC18 and pRV85 were digested with the help of *EcoRI*. The *GFP* gene was present in between the *EcoRI* site in pRV85. Thus, pRV85 was digested with *EcoRI*. Digestion yielded a *GFP* fragment of 1000 base pairs which was observed on agarose gel. Subsequently, the *GFP* gene fragment was extracted from pRV85 and was ligated into the cloning vector, pUC18 (2.7 kb). The resulting pUC18-*GFP* was around a fragment of 3.7kb. For the confirmation of cloning, the pUC18-*GFP* was digested with *EcoRI*, where we observed two bands (2.7 kb and 1000 base pairs). Further, transformation of *GFP* into transformation host *E. coli* DH5α was performed. The *GFP* gene was successfully cloned and transformed into *E. coli* DH5α and fluorescent colonies were selected. From this study, it was concluded that the green florescent protein tagging was useful for detecting the bacteria in labelled fluorescent *in vitro* as well *in vivo.*

LIST OF ABBREVIATIONS:

1. *mcs:* multiple cloning site
2. *GFP:* Green Fluorescent Protein
3. DNA: Deoxyribonucleic acid
4. pUC: Plasmid University of California
5. HGT: Horizontal Gene Transfer
6. RE: Restriction Endonuclease/ Restriction Enzyme
7. LDH: Lactate Dehydrogenase
8. RNA: ribonucleic acid
9. PCR: Polymerase Chain Reaction
10. ORI: Origin of Replication
11. kDa: Kilo Dalton
12. FRET: Fluorescence Resonance Energy Transfer
13. HeLa: **He**nrietta **La**cks cells
14. LB: Luria Bertani
15. BFP: Blue Fluorescence Protein
16. m- RNA: messenger RNA
17. PM: Plasma Membrane
18. ALS: Alkaline Lysis Solution
19. P:C : Phenol: Chloroform

20. rpm: rotations per minute
21. BSA: Bovine Serum Albumin
22. bp: base pairs

1. Introduction

Plasmids are small, circular DNA molecules, part of the main bacterial genome. However, they can be said to be distinct from the genome in the sense that they replicate their DNA independently of the bacterial chromosome (Griffiths AJF, *et al*.1999.) The term "plasmid" was coined by Joshua Lederberg in 1952. Quite often, the plasmids which carry the genes prove advantageous to the bacteria. One of the major advantages is antibiotic resistance. The length of plasmids is highly variable, ranging from one thousand base pairs to several hundreds of thousands of base pairs. (Griffiths AJF, *et al*.1999). Plasmids can be transferred from one bacterium to another by a process known as horizontal gene transfer. HGT involves three methods, namely transformation, transduction and conjugation.

Plasmids have numerous functions. One of the main functions is to carry genes for antibiotic resistance and to spread them in the human or animal body. This aids in treatment of diseases. Plasmids also help in carrying genes involved in metabolic activities, thereby digesting pollutants from the environment. (Rao, 2010). Another function of plasmids is producing antibacterial proteins. Genes concerned with increasing the pathogenicity of bacteria which cause diseases like anthrax and tetanus are also carried by plasmids.

Plasmids are of 5 types which are used for different purposes. They are as follows:

- **Resistance plasmids:** these are involved in bacterial conjugation. Antibiotic resistant genes and those which code for poisons are usually carried by them. (Rao, 2009)
- **Degradative plasmids:** this plasmid is capable of digesting dead organic matter from dead animals or plants. This organic matter is used in the process of biosynthesis, production of energy and it's recycling. (Rao, 2009)
- **Fertility plasmids:** these plasmids carry the *tra*- genes. These *tra* genes are used in the process of conjugation. They are helpful in the transfer of genetic material between bacteria. (Rao, 2009)
- **Col plasmids:** the plasmids of this type produce antibiotics which work against other harmful strains of bacteria by staying in the host bacterial cell. The antibiotics are known as colicin. (Rao, 2009)

- **Virulence plasmids:** these plasmids can transform bacteria into pathogens. Thus, they carry genes which are responsible for causing diseases (Rao, 2009)

❖ pUC18

The pUC18 vector is a small, high copy number, plasmid found in *E. coli.* Plasmid pUC18 contains pMB1 replicon. This replicon is responsible for the replication of the plasmid. This plasmid is derived from plasmid pBR322. (Yanisch-Perron, *et al.*, 1985)

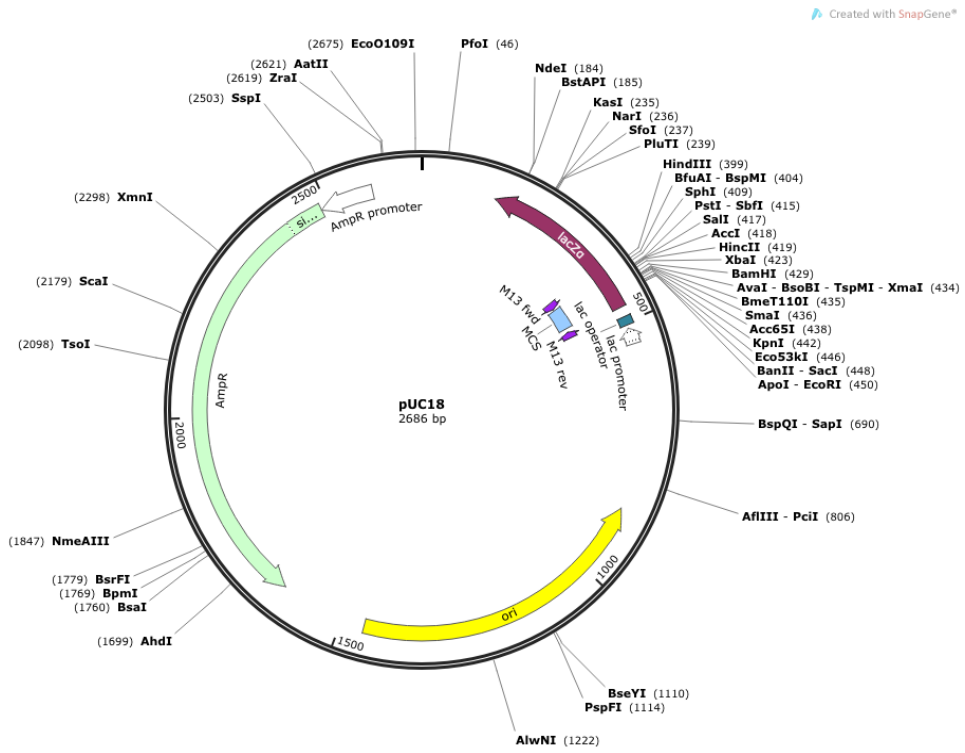


Figure 1: Diagrammatic representation of plasmid pUC18 (www.snapgene.com)

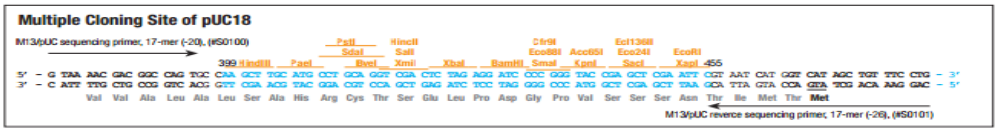


Figure 2: Multiple cloning site of plasmid pUC18 (Yanisch-Perron, *et al.*, 1985)

❖ pRV85

The plasmid pRV85 is a type of shuttle expression vector of 6.2kb size. It contains a strong constitutive promoter LDH, *GFP* as a marker gene. It also

provides resistance against erythromycin.

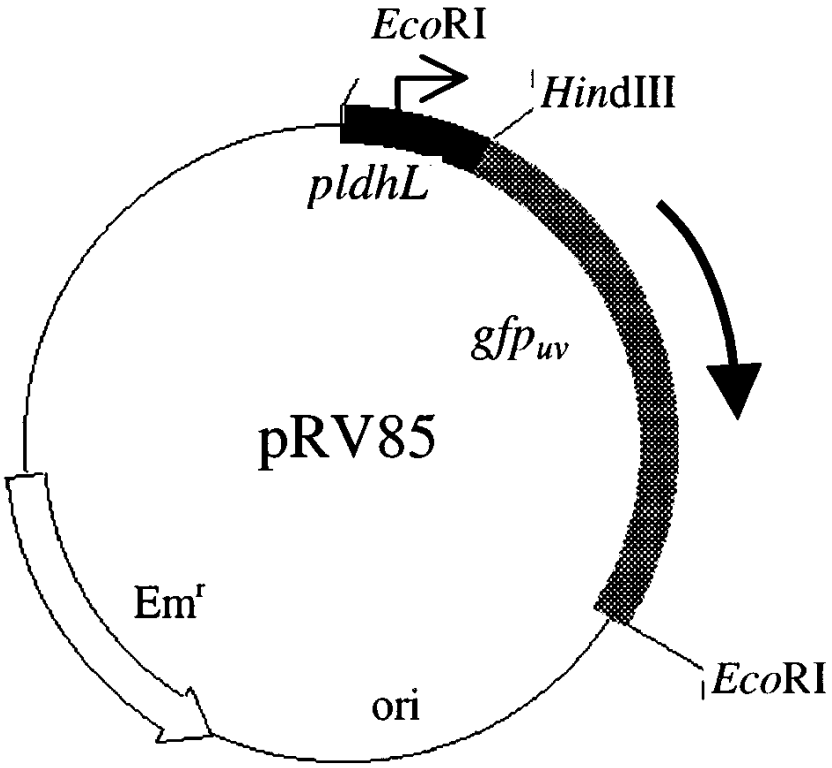


Figure 3: Diagrammatic representation of plasmid pRV85 (Gory *et al.*, 2001)

❖ pRV86

The plasmid pRV86 is a type of chromosomal integrated shuttle expression vector of L. sakei of size 6.3kb, containing a strong constitutive promoter LDH, *GFP* as a marker gene. It also provides resistance against erythromycin and ampicillin.

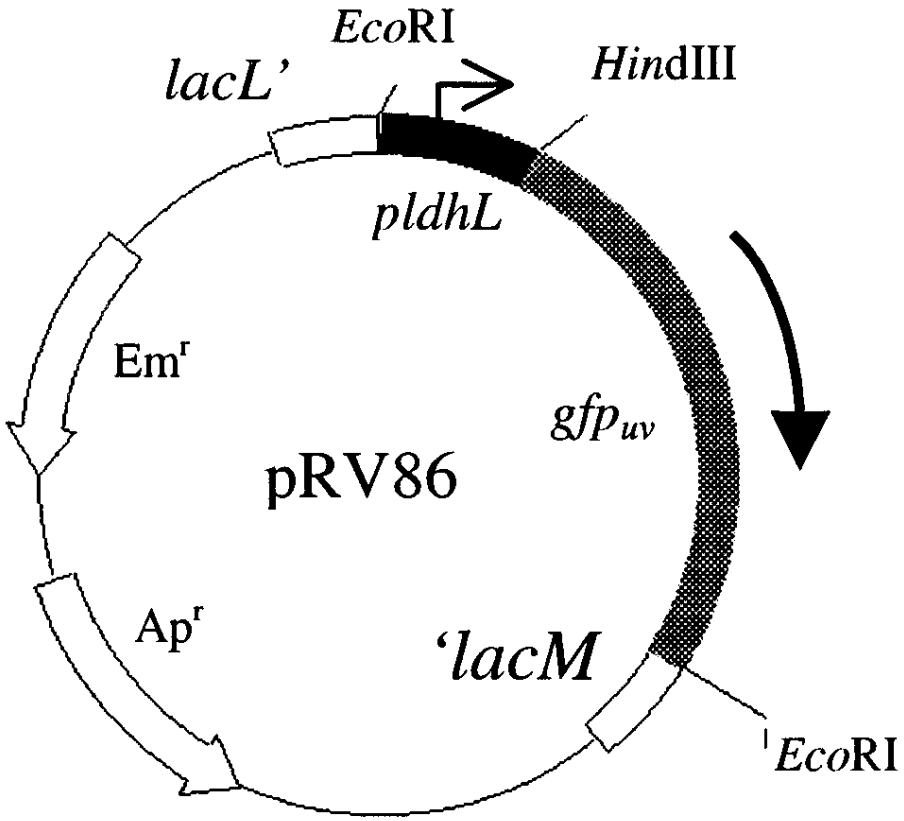


Figure 4: Diagrammatic representation of pRV86 (Gory *et al.*, 2001)

1.1 Cloning

Cloning is the process of creating an exact copy of a biological unit from which it was derived. Identical recombinant DNA molecules are produced because of molecular cloning. These are useful for certain applications like gene expression and mutational analysis. The entire cloning process is divided into several steps, the first of which is nucleic acid isolation. The nucleic acid may include plasmid DNA, genomic DNA or total RNA. (Winfrey *et, al.* 1997) After DNA isolation, if the yield of DNA obtained is low, then PCR amplification of the gene of interest is preferred. The next step with regard to genomic or plasmid DNA is restriction digestion of the sample. Restriction digestion and gel analysis are carried out to detect the loci of interest. (Lopez, 2004) The next step in this method of cloning is the ligation step. There is a possibility that the free ends of the DNA obtained after digestion anneal with each other. If this happens, the sites won't be accessible to the plasmid and ligation won't occur. To prevent this, the DNA is extracted from the gel, purified and modified. The DNA gets inserted into the bacterial cell with the

help of the plasmid acting as a vector. (Winfrey *et, al.* 1997)

There are three main types of cloning. They are as follows:

- **DNA cloning:** DNA cloning has a number of names like molecular cloning, gene cloning, recombinant DNA technology, etc. This type of cloning is used mainly with an aim of generating replicas of the same gene. DNA cloning helps in identifying the bad genes and replacing it with a good one. Scientists are able to know different genes which might be responsible for different diseases. (Lopez, 2004)

- **Reproductive cloning**

The process of reproductive cloning goes as follows:

a. Cell is removed from an organism which will be replicated. This is then extracted by an incision in the skin.
b. The female organism is the donor of the female reproductive organ.
c. Moreover, an entire deoxyribonucleic enzyme is also removed from the donor cell.
d. Another process known as the **fusion** comes into play in the process of reproductive cloning: the donor DNA is then forced into the second cell nucleus.

The first mammal ever to be cloned was in 1996, a sheep by the name of Dolly. A Scottish scientist named Professor Ian Wilmut was responsible in producing the first clone ever. (Lopez, 2004)

- **Therapeutic cloning:** this type of cloning is also known as embryonic cloning. It is used for studying human stem cells in order to study human development and diseases. (Lopez, 2004)

1.2 *GFP* (Green Fluorescence Protein)

The green fluorescence protein (*GFP*) is found in a jellyfish, *Aequorea victoria*, inhabiting the North Pacific. This fish contains a bioluminescent protein by the name of Aequorea, which gives blue fluorescence. (Ormo, *et, al.* 1996) Now, the green fluorescence protein has a property of converting the blue light to green light, which is seen when the jellyfish lights up. This protein absorbs the UV radiation from the sun and emits it as green light, which has much lower energy than the yellow light. (Ormo, *et al. 1996*) The machinery of *GFP* is quite simple, consisting only of amino acid side chains, where there is a special sequence of three amino acids: serine-tyrosine-glycine (Ormo, *et, al.* 1996). The *GFP* gene is isolated from pRV85 andpRV86.

Dynamic processes in cells or organisms can be monitored effectively with the help of *GFP*. Some other applications of *GFP* are localization of proteins, monitoring the dynamics of the subcellular compartments to which these proteins are targeted and following their movements across the cell (Gerdes and Kaether, 1996).

2 Review of literature

2.1 Plasmids:

DNA molecules found in bacteria which are different from the bacterial genome are known as plasmids. Plasmids are small and circular. They contain only a single origin of replication and usually carry one or a few genes. During replication, plasmids are replicated either at the same rate or at a rate higher than that of the replication of the chromosome. These plasmids are known as high copy number plasmids and are more preferred for cloning. Genes present on plasmids with high copy number are usually expressed more. These genes encode proteins (e.g., enzymes) and thereby protect the bacterium or confer resistance to heavy metals.

Plasmids are widely used as cloning vectors. An ideal cloning vector is a small high copy number vector and contains a plasmid origin of replication (ORI). A selectable marker should always be present. This marker allows cells containing the plasmid to be isolated. Moreover, a second selectable gene which is inactivated by insertion of the passenger should also be present. (Butterworth and Heinemann, 1991). A multiple cloning site (*mcs*) or polylinker region is always present. This *mcs* provides numerous restriction enzyme cleavage sites. Plasmid vectors have one or more markers. These markers help the researcher to keep track of the vector's condition in the cell.

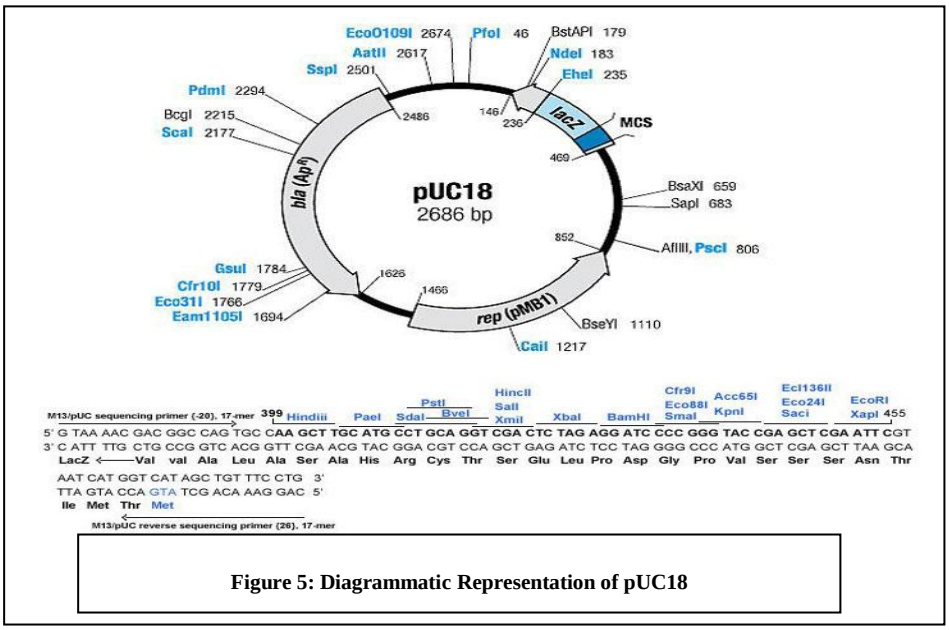


Figure 5: Diagrammatic Representation of pUC18

There are various components present in any vector. These genes are the Amp^R gene, the origin of replication (ORI) and promoters. There is a multiple cloning site as well which contains sites for different restriction enzymes.

2.2 Restriction Endonucleases:

Restriction endonucleases, more commonly known as restriction enzymes are used in restriction digestion, i.e., digestion of the DNA at specific sites in order to procure the gene of interest. Restriction enzymes have a property of recognizing specific sequences in DNA. They recognize the sequences and cleave the phosphodiester bonds at those sites. Irrespective of the source of DNA, these enzymes will cleave the sequence whenever it appears in the DNA. The operation of restriction enzymes is quite specific, cutting only at their unique recognition sequences. (Bloom, *et, al.*, 1996) This cutting at specific sites results in the formation of DNA fragments (restriction fragments from a DNA molecule having these recognition sites.

Restriction enzymes usually recognize palindromic sequences. For example, the enzyme BamHI recognizes the GAATTC sequence. (Raven and Johnson, 1996) This is a palindromic sequence as the complementary strand will have the same sequence in the reverse direction.

2.3 Blunt and Sticky Ends

During digestion, some enzymes cut in the center of the recognition sequence. This results in the formation of **blunt ends.** On the other hand, most restriction enzymes cut at sites one or two bases away from the axis of symmetry. This results in the formation of single stranded ends, two to four pairs long at the end of each fragment. (Raven and Johnson, 1996) These ends are complementary and are referred to as cohesive or sticky ends.

2.4 Naming of Restriction Enzymes

The name of any restriction enzyme is taken from the first letter of the bacteria genus and the first two letters of the species of that bacterium. This is how the enzymes are named. Sometimes, the enzyme names may have additional numbers or letters. These designate a particular strain, isolate or plasmid. (Raven and Johnson, 1996) Restriction digestions are carried out by adding the DNA to be digested and the enzyme to a system containing the buffer that is optimal for the enzyme. Digestion carried out with the help of two enzymes is

known as a **"double digestion"** or a **"double digest".** (Bloom *et, al.* 1996)

2.5 Green fluorescence protein (*GFP*)

GFP is a protein by the name of Green Fluorescent Protein, which fluoresces green in presence of UV light. The green fluorescence protein is natively found in the jellyfish *Aequorea victoria* which emits green fluorescent light from photocytes which are present at the margin of its umbrella. (Gerdes and Keather, 1996)

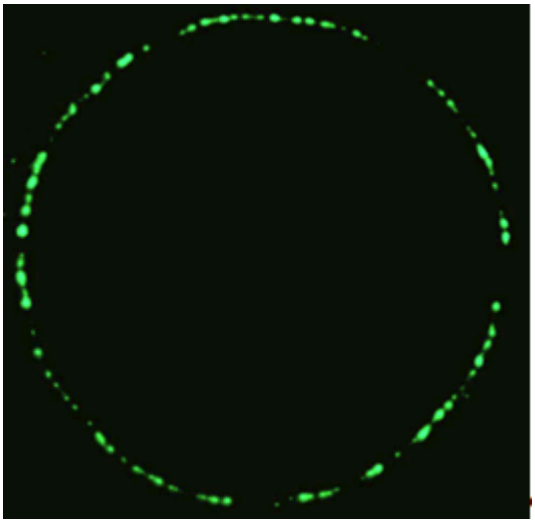


Figure 6: The green colored ring of fluorescence in Jellyfish *A. Victoria* (O. Shimomura, Nobel Prize Website)

The fluorescence produced by the jellyfish, *A. victoria* is produced because of the sequential activation of two photoproteins, namely Aequorea and green fluorescent protein. (Prasher, 1995) Aequorin has the property of emitting blue light which occurs as a result of calcium binding. This in turn excites the green fluorescent protein to fluoresce green. (Prasher, 1995) The function of the *GFP* is independent of any cofactors unlike other bioluminescent molecules. Moreover, it forms a chromophore of three amino acids in its primary structure. The gene for *GFP* was first cloned in 1992 into *E. coli*. *GFP* can produce green light in diverse organisms such as bacteria, slime molds, plants and mammals. (Cubitt *et, al.*, 1995) *GFP* has various applications, some of which are given under:

- As a reporter for gene expression (Chalfie, *et, al.* 1994)
- As a marker to study cell lineage during development (Tannahill, *et, al.* 1995)
- As a tag to localize proteins in living cells (Wang and Hazelrigg,

1994)

2.6 Structure of *GFP*

GFP is a 238 amino acid protein (Hein and Tsien, 1996) with a molecular weight of 27 kDa(Shimomura, 1979). Cyclization and oxidation of three amino acids namely Ser65, Tyr66 and Gly67 is responsible for formation of its chromophore. Even though the structure of *GFP* includes 238 amino acids, (Hein and Tsien, 1996), only 4 of them are directly involved in producing any fluorescence effect.(Cubitt *et, al* 1995). This is the primary structure. The secondary structure involves a series of helices and pleated sheets caused as a result of H- bonding within the chain. The tertiary structure is a barrel made from 11 of the sheets, capped with the helices. The part of the structure of *GFP* responsible for generating fluorescence is the chromophore, a short chain of altered amino acids, which lies at the center of the barrel. (Cubitt *et, al*,1995).

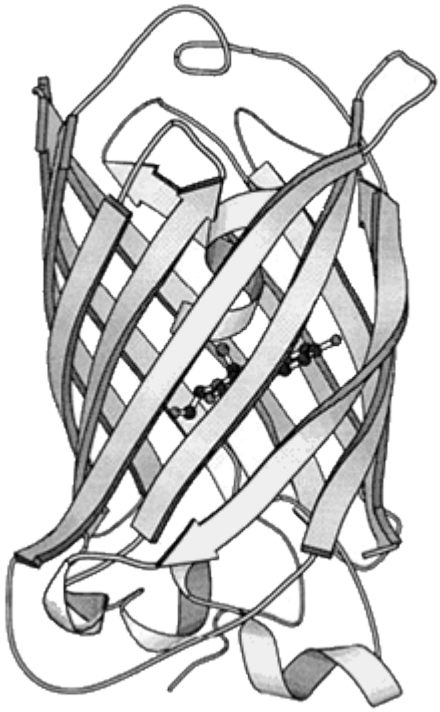


Figure 7: Tertiary Structure of *GFP* (M. Chalfie, 1994)

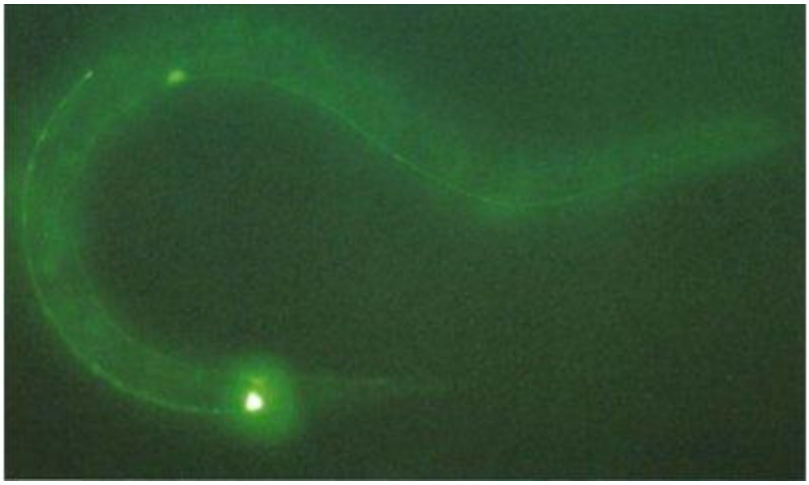


Figure 8: *C. elegans* with *GFP* replacing a touch receptor protein (A. Miyawaki, 2004)

The above figure shows the role of *GFP* in the round worm, *C. elegans.* M. Chalfie first replaced one of the genes of *C. elegans* with the genetic code for *GFP*. (M. Chalfie, 1994).This resulted in *GFP* being expressed in the worm in the places where the original gene would have been expressed. No kind of toxic effects were observed because of the protein, and it came to be known that almost all organisms could be made to express *GFP*. (M. Chalfie, 1994). Since then, it has been used in organisms diverse as fruit flies, mice, rabbits, tobacco plants and human cells. (Gerdes and Kaether,1996). *GFP* is relatively small for a protein molecule. This enables it to be used as a tag or a reporter gene. The genetic code for *GFP* can be added to the end of the gene for the protein that needs to be tagged and growing the organism. Thus, the protein is produced with a small tag, which doesn't affect the organism or the function of the protein at all. The protein can then be seen because of the green fluorescence imparted by *GFP* (Gerdes and Kaether, 1996).

GFP can also be used in techniques such as FRET (Fluorescence Resonance Energy Transfer), which can be used to image real time events in a cell. Two variants of *GFP* are bound to interacting proteins. (A. Miyawaki, 2004).These two variants absorb and emit light at different wavelengths to each other. In a case where these two proteins are far apart, and the system is illuminated by a light that excites only one of the *GFP* variants, then only that protein will fluoresce. In another case where two proteins interact, and the same light is used, the two *GFP* variants are brought close together for the energy from the absorbed light to be transferred to between the *GFP* molecules. (A. Miyawaki, 2004)

This result in change of the colour emitted to that of the second *GFP* (Cubitt, *et, al.,* 1995). *GFP* has a significant role in various cell organelles like cytoskeleton, plasma membrane, nucleus, the secretory pathway, mitochondria and peroxisomes. (Gerdes and Keather, 1996)

- **Cytoskeleton:** it is essential for intracellular transport processes and for cell growth, division, migration and differentiation. With *GFP*, it is possible to tag actin and microtubule binding proteins, or to label organelles interacting with the cytoskeleton. The *exu* protein of drosophila was the first one to be tagged with *GFP*. The function of this protein is m-RNA localization during oogenesis.(Gerdes and Kaether, 1996)
- **Plasma Membrane:** Endocytic and Exocytic vesicles, proteins like hormones, zymogens or transporters can be recruited by regulated vesicular transport. GLUT-4 is the insulin glucose transporter, capable of being regulated. It is temporarily exposed at the site of PM. The exposure to the surface stimulated by insulin and its retrieval could be analysed in single living cells using GLUT4-*GFP* chimeras. In other studies, the PM was labelled with a GPI anchored *GFP* or with the membrane associated *GFP*. (Gerdes and Kaether, 1996)
- **Nucleus:** the nuclear pore regulates bidirectional transport of proteins and RNAs across the nuclear envelope. A good example is glucocorticoid receptors. These are translocated into the nucleus upon steroid hormone binding. (Gerdes and Kaether, 1996)
- **Mitochondria and peroxisomes:** most peroxisomal and mitochondrial proteins are synthesized in the cytoplasm. These are imported post translationally with the help of well defined targeting signals. The simultaneous expression of tagged *GFP* and BFP fusion proteins in HeLa cells made it possible to tag the nucleus and mitochondria in green and blue respectively. Interactions between organelles or assortment of differentially tagged proteins could also be studied. (Gerdes and Kaether, 1996).

3. Hypothesis

It has been reported that gfp can be used as an effective reporter gene because of its relatively small size with respect to other proteins, as an important marker to study cell lineage during development and also as a tag to localize proteins. Therefore, we hypothesize that it can be used as a reporter gene and a marker in pUC18 also.

4. Objectives

1. To clone the gfp gene in pUC18
2. To transform the pUC-*gfp* clone in *E. coli* DH5α

5. Materials and Methods

5.1 Bacterial Strains, Growth Conditions and Media

The expression vector, plasmids and bacteria used in this experiment were shown in Table 1. Ampicillin, Erythromycin, Agar and Luria bertani (LB) medium were purchased from Himedia. The electrophoresis reagents were purchased from Merck Bioscience. Plasmid isolation kits and Rapid ligation kits were purchased (Fermentas). Restriction endonucleases (GeneI) were used in this study.

Table 1: Plasmids and strains used in this study

Strains/Plasmids	Description	Source
E. coli DH5α	Transformation host	MTCC
pRV85, pRV86	EmR, Shuttle vector, *gfp* gene, *ldh* promoter	Gory et al, 2001
pUC18	Cloning vector	Invitrogen, USA

5.2 Plasmid Isolation (Miniprep Alkaline Lysis Method) (Sambrook and Russell, 2001)

Two ml of culture was taken in a microfuge tube and centrifuged at 8000 rpm for 10 minutes. The supernatant was removed and the bacterial pellet was resuspended in 100µl ice cold ALS I by vigorous vortexing and the mixture was incubated for 5 minutes at room temperature. After that, 200µl of freshly prepared ALS II was added, and the contents were mixed by tapping/inverting the tubes. These tubes were kept on ice for 20 minutes. After that, 150µl of ice cold ALS III was added and the tubes were inverted several times. This was followed by storage on ice for 5 minutes. After centrifugation, the supernatant was transferred to a new tube and the plasmid was precipitated by adding $1/10^{th}$ the volume of sodium acetate and double the volume of chilled ethanol. The DNA pellet was collected by centrifugation. The supernatant was discarded and 1 ml of 70% ethanol was added. The tubes were centrifuged and were kept at room temperature for the ethanol to dry away. Finally, the plasmid pellet was resuspended in 50µl TE buffer (pH: 8.0).

5.3 Plasmid Isolation (Midiprep Alkaline Lysis) (Sambrook and Russell, 2001)

10 ml of a bacterial culture was transferred to a 15ml tube and the bacteria were recovered by centrifugation at 7000 rpm for 5 minutes. The pellet was resuspended in 200µl of ice cold alkaline lysis solution I. This suspension was vortexed and transferred into a microfuge tube. Freshly prepared 400µl of alkaline lysis solution II was further added; the tubes were inverted to ensure proper mixing, and were stocked on ice. This was further followed by addition of 300µl of alkaline lysis solution III and the tubes were inverted several times. Centrifugation of the suspension was carried out at 12000 rpm for 10 minutes. 600µl of the supernatant was transferred to a new tube. This was followed by addition of equal amount of a P:C mixture. The inorganic and aqueous phases were mixed by vigorous vortexing and the emulsion was centrifuged at 12000 rpm for 5 minutes. The upper layer was transferred to a new tube and 600µl of isopropanol was added to the tube. The precipitated nucleic acids were collected by a spin at 12000 rpm for 5 minutes. The tubes were then kept in an inverted position for all the isopropanol to dry away. This was followed by addition of 1ml of 70% ethanol to the obtained pellet and centrifugation in turn. The tubes were kept in a dry bath for 10-15 mins so that traces of ethanol could be removed. The final suspension was eluted in 50µl of nucleus-free milliQ water and the tubes were stored at -20˚C.

5.4 Plasmid Isolation (HiPurA Plasmid Purification Kit) (HiMedia)

An overnight bacterial culture grown in LB medium containing Ampicillin as the antibiotic was used. 2 ml of culture was taken in a microfuge tube and the cells were centrifuged at 12000 rpm for 1 minute. The supernatant was discarded and the bacterial pellet was then resuspended in 250 µl Resuspension Solution (HP1) and mixed. After that, 250 µl of Lysis solution (HP2) was added and the cells were mixed thoroughly by inverting the tubes several times. this was followed by addition of 350 µl of Neutralization Solution (HN3), and the suspension was mixed immediately by inverting the tubes. This reaction mixture was centrifuged at 12000 rpm for 10 minutes to obtain a compact white pellet. The supernatant was removed and transferred to a HiElute Miniprep Spin Column and was centrifuged at 12000 rpm for a minute; the flow through liquid was discarded. The column was then washed by adding 500 µl of wash solution(HPB) and was centrifuged at 12000 rpm for one minute; the flow through liquid was discarded. After the first wash, the

column was then washed by adding 700 µl of diluted wash solution (HPE) and was centrifuged at 12000 rpm for 1 minute. The flow through liquid was discarded and the empty tube was centrifuged once again to remove all traces of the wash solution. The column was then transferred to a collection tube. The tubes were kept for incubation at 55˚C to remove all traces of ethanol. After that, the sample was eluted in 50 µl of milliQ water. This was allowed to stand for 1 minute, which was followed by a final centrifugation at 12000 rpm for 1 minute.

5.5Restriction Endonuclease Digestion of DNA

RE digestion was carried out with following reaction and requirements as follows:

- DNA
- Restriction Enzymes
- RE buffer
- Agarose Gel electrophoresis apparatus
- UV Transilluminator
- Ice box
- Water bath

Different components like water, buffer, DNA and RE were added in a microfuge tube, and were mixed by vortexing or finger tapping. The reaction mixture was incubated in a water bath at the recommended temperature for 90 minutes to 2 hours, and it was kept at -20˚C until further analysis. The digested DNA fragments were separated in 0.8-1% agarose gel using 1X TAE buffer. Around 2µl of 6X DNA loading dye was added in the digested sample and it was mixed well by finger tapping. The DNA marker (O' gene ruler DNA ladder mix) was loaded in the first and the last lane and the DNA samples were loaded in the remaining lanes. Finally, the gel was viewed on the UV Transilluminator and photographed.

Table 2: RE digestion system of pUC18 (*Hind*III)

Materials	Volume
10X buffer D	2.0µl
Restriction Enzyme (*Hind*III)	0.5µl
BSA	0.5µl
DNA template (pUC18)	5.0µl
Nuclease free water	12.0µl
Total Reaction system	20.0µl

5.5.1RE digestion system of pUC-*gfp* (HindIII)

Table 3: RE digestion system of pUC-*gfp* (HindIII)

Materials	Volume
10X buffer D	2.0 µl
Restriction Enzyme (*Hind*III)	0.5 µl
BSA	0.5 µl
DNA template (pUC-*gfp*)	10.0 µl
Nuclease free water	7.0 µl
Total Reaction system	20.0µl

5.5.2RE Digestion of pUC-*gfp* (*EcoR*I)

Materials	**Volume**
10X buffer D	2.5 µl
Restriction Enzyme (*EcoR*I)	1.0 µl
BSA	1.0 µl
DNA template (pUC18*GFP*)	5.0 µl
Nuclease free water	15.5 µl
Total Reaction System	**25.0 µl**

5.6Agarose gel Electrophoresis:

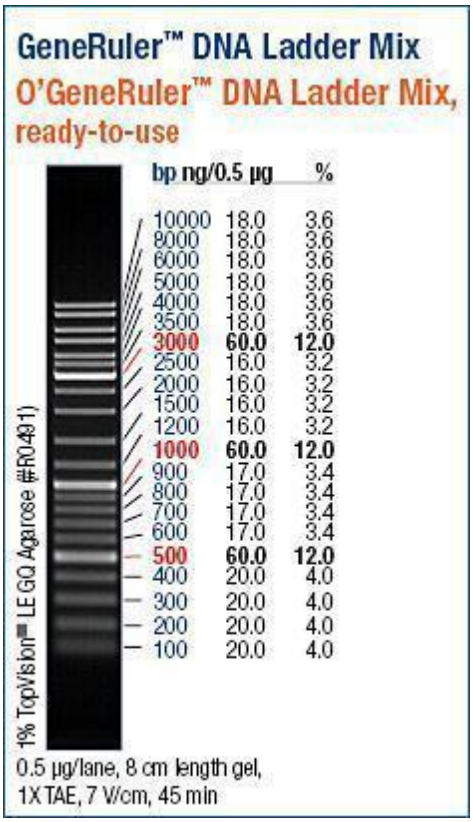


Figure 9: O' gene ruler DNA ladder mix

The DNA ladder contains 21 discrete fragments from 100 bp to 10000 bp.

The edges of a dry, clean glass plate were sealed with a tape to form a mould. The mould was set on a horizontal section of the bench. The electrophoretic tank was filled with 1X TAE buffer. A solution of agarose in the electrophoresis buffer was prepared at a certain concentration appropriate for separating the particular size of the fragments expected in the DNA sample. Agarose gels were casted by melting the agarose in presence of the desired buffer until a clear, transparent solution was achieved. The flask/bottle was transferred to a water bath at 55˚C. When the molten gel cooled down, ethidium bromide was added to a final concentration of 0.5 µg/ml, and the contents were mixed thoroughly. Meanwhile, the comb was placed 0.5-1.0mm above the plate so that a complete well formed when the agarose was added into the mold. The warm agarose solution was poured into the mould and the gel was allowed to set completely. After complete solidification, the comb was removed carefully and the gel was mounted in the electrophoresis tank. The DNA samples were mixed with 6X gel loading buffer. The sample mixture was slowly loaded into the lane of the gel using a micropipette. The ladder was loaded into slots on both, the right and left sides of the gel. The lid of the gel tank was closed and the electrical leads were attached so that the DNA would migrate towards the positive anode (red). A voltage of 1-5 V/cm was applied. After a few minutes, the bromophenol blue migrated from the wells into the gel. The gel was run until the bromophenol blue and xylene cyanol FF had migrated an appropriate distance through the gel.

5.7 Competent Cell Preparation and Transformation:

A single bacterial colony was picked from a plate that had been incubated for 16-20 hours at 37˚C. This colony was transferred into a 100 ml LB broth in a flask. The culture was incubated for 3 hours at 37˚C with vigorous agitation, monitoring the growth of the culture. The bacterial cells were transferred to sterile, disposable, ice cold 50 ml polypropylene tubes and they were cooled to 0˚C by storing them on ice for 10 minutes. The cells were recovered by centrifugation at 2700 g for 10 minutes at 4˚C. The medium from the cell pellets was decanted and the tubes were made to stand in an inverted position on paper towels for 1 minute to allow the last traces of the media to drain away. Each pellet was resuspended by gentle vortexing in 30 ml ice cold $MgCl_2$- $CaCl_2$ solution. The cells were then recovered by centrifugation at 2700 g for 10 minutes at 4˚C. The medium from the cell pellets was decanted

and the tubes were made to stand in an inverted position on paper towels for 1 minute to allow the last traces of media to drain away. The pellet was resuspended by swirling or gentle vortexing in 2ml of ice cold 0.1 M $CaCl_2$ for each 50 ml of original culture. This was followed by transformation. Around 200µl of the suspension of the competent cells was transferred to a sterile, chilled 17X100mm polypropylene tube using a chilled micropipette tip. The DNA was added to each tube. The contents of the tubes were mixed by swirling gently and the tubes were stored on ice for 30 minutes. The tubes were then transferred to a rack placed in a preheated 42˚C circulating water bath and stored for 90 seconds. The tubes were transferred directly to an ice bath and the cells were allowed to chill for 1-2 minutes. Around 800µl of LB medium was added to each tube. The cultures were incubated for 45 minutes in a water bath set at 37˚C to allow the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid. An appropriate volume of transformed competent cells was transferred onto LB agar medium containing 20mM $MgSO_4$ and the appropriate antibiotic. The plates were stored at room temperature until the liquid was absorbed. The plates were inverted and incubated at 37˚C. Transformed colonies were observed in around 12-16 hours. (Sambrook and Russell, 2001)

6. RESULTS:

6.1 Isolation of pUC18

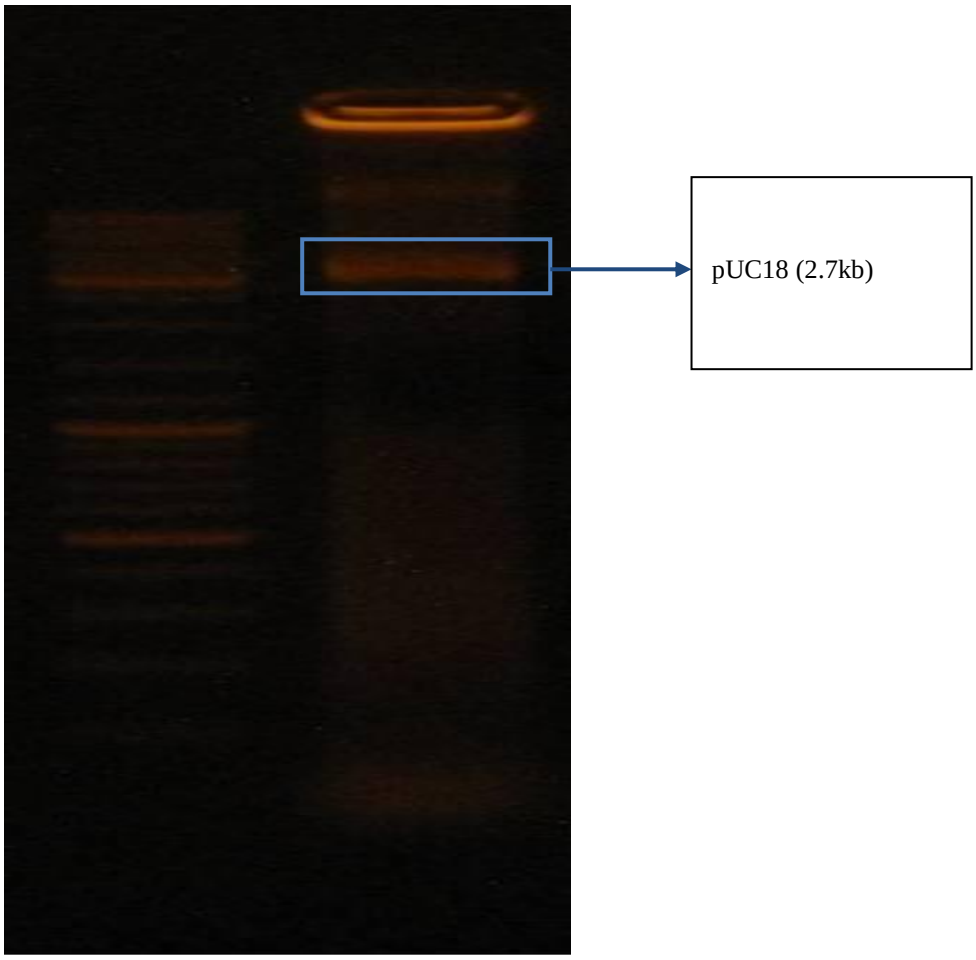


Figure 10: Plasmid Isolation: pUC18 (midiprep alkaline lysis method). Lane 1: O' gene ruler DNA ladder mix (100 bp to 10 kb). Lane 2: pUC18 (2.7 kb).

The figure above shows the agarose gel image of isolation of pUC18. This isolation was done by following the Midiprep alkaline lysis method (Sambrook and Russell, 2001). Lane 1 contains the O' gene ruler DNA ladder mix. When compared to the O' gene ruler the band of pUC18 is seen to be at 2.7 kb. Some amount of DNA shearing is also seen.

6.2 Isolation of pRV85 and pRV86

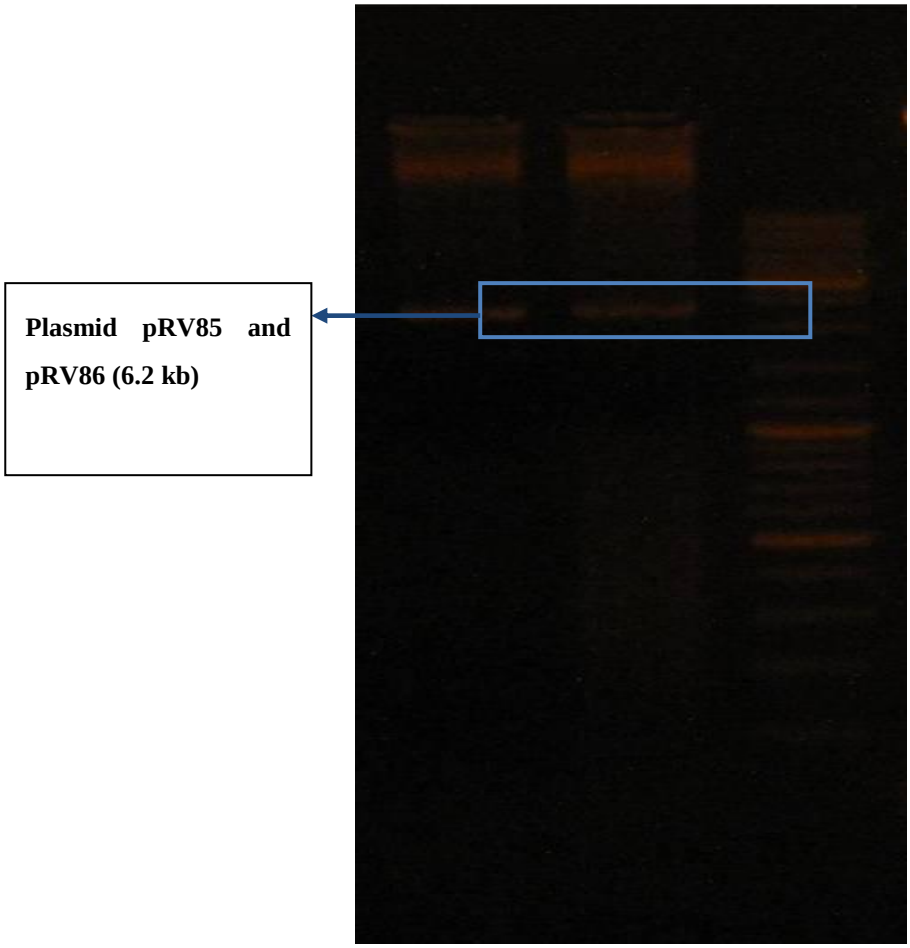


Figure 11: Plasmid Isolation: pRV85 and pRV86 (miniprep alkaline lysis method). Lane 1: pRV85 (6.2 kb). Lane 2: pRV86 (6.2 kb). Lane 3: O' gene ruler DNA ladder mix.

The figure above shows the isolation of plasmids pRV85 and pRV86. This isolation was done by following the Miniprep alkaline lysis method (Sambrook and Russell, 2001). Lane 1 contains the isolated plasmid pRV85; lane 2 shows the isolated plasmid pRV86 and lane 3 has the O' gene ruler DNA ladder mix. When compared to the O' gene ruler, the band of pRV85 and pRV86 is seen to be of 6.2 kb. A faint band of sheared genomic DNA is also seen on the gel.

6.3 Isolation of pRV85 and pRV86 (Midiprep Alkaline Lysis method)

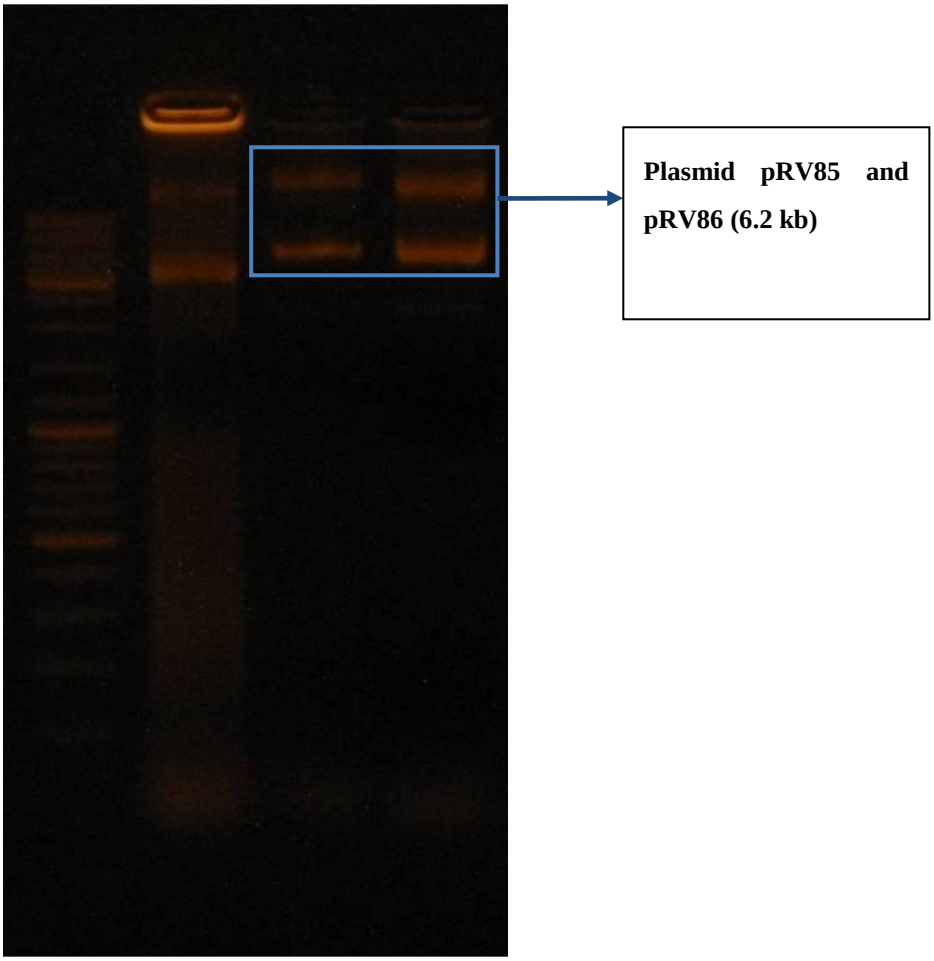


Figure 12: Plasmid Isolation: pRV85 and pRV86 (midiprep alkaline lysis method). Lane 1: O' gene ruler DNA ladder mix (100 bp to 10 kb). Lane 3: pRV85 (6.2 kb). Lane 4: pRV86 (100 bp to 10 kb).

The figure above shows the isolation of plasmids pRV85 and pRV86. This isolation was done by following the Midiprep alkaline lysis method (Sambrook and Russell, 2001). Lane 1 contains the O' gene ruler DNA ladder mix; lane 3 shows the isolated plasmid pRV85 and lane 4 shows the isolated plasmid pRV86. Two distinct bands can be seen on agarose gel, which confirm the linearization and circularization of the plasmid DNA. When compared to the O' gene ruler, the band of pRV85 and pRV86 is seen to be of 6.2 kb.

6.4 Isolation of pUC18 (miniprep alkaline lysis method):

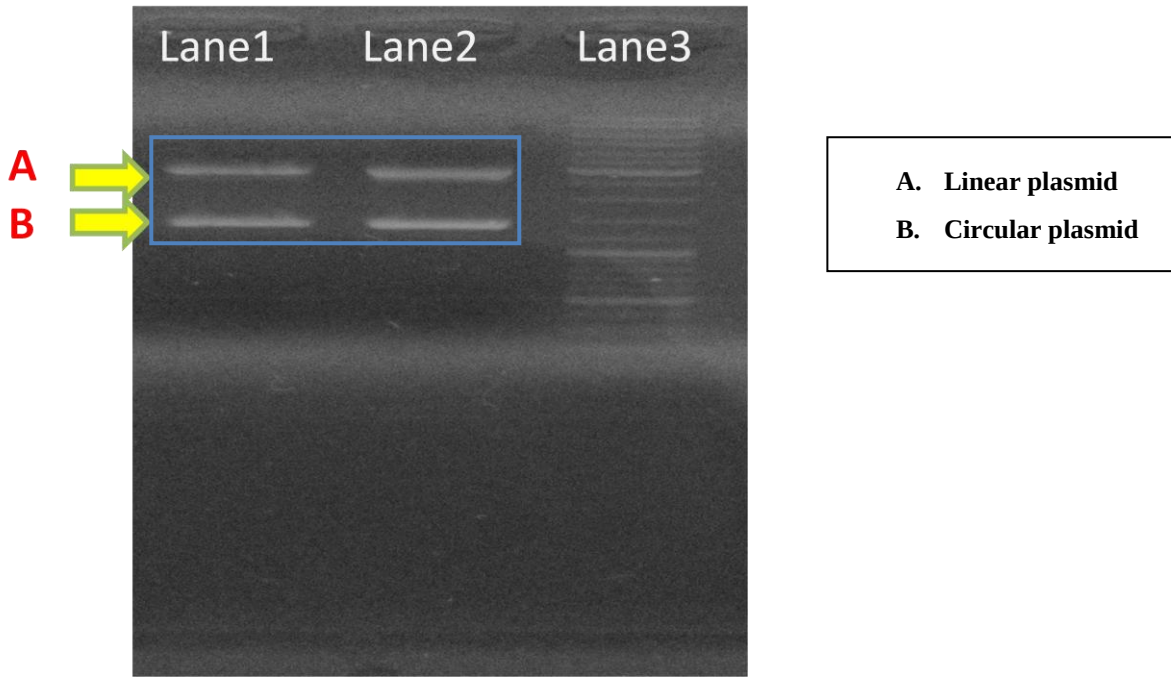


Figure 13: Plasmid Isolation: pUC18 (Miniprep Alkaline lysis method). Lane 1: pUC18 (2.7 kb). Lane 2: pUC18 (2.7 kb). Lane 3: O' gene ruler DNA ladder mix (100 bp to 10 kb)

The figure shown above depicts the isolation of pUC18 which was done by the **miniprep alkaline lysis** method. Two distinct bands are observed denoted by **"A"** and **"B"** in the figure. The first band denoted by **"A"** is the **linear plasmid** and that denoted by **"B"** is the **circular plasmid**. The size of **pUC18** is **2.7 kb**. Both the lanes, namely lane 1 and lane 2 show the pUC18 plasmid and lane 3 has the O' gene ruler DNA ladder mix.

6.5 Plasmid Isolation: pUC18 (HiPurA plasmid DNA miniprep purification kit):

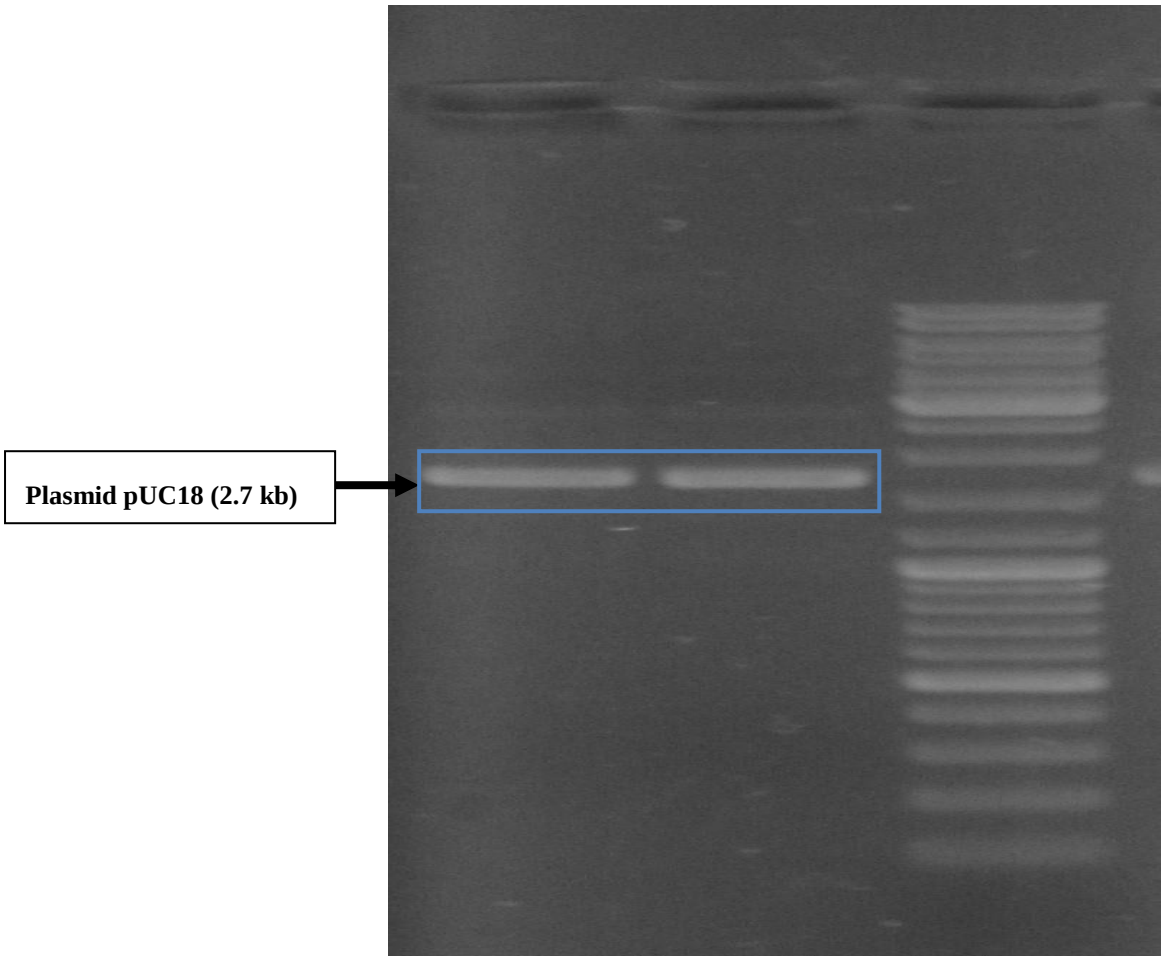


Figure 14: Plasmid Isolation: pUC18 (HiPurA plasmid DNA miniprep purification kit). Lane 1: pUC18 (2.7 kb). Lane 2: pUC18(2.7 kb). Lane 3: O' gene ruler DNA ladder mix (100 bp to 10 kb).

The figure above shows the gel image of the isolation of **plasmid DNA pUC18** with the help of the **HiPura plasmid DNA miniprep purification kit.** The bands are seen distinctly and the size is found to be **2.7 kb** when compared with the O' gene ruler DNA ladder mix.

6.6 Plasmid Isolation: pRV85 and pRV86 (HiPurA Plasmid DNA miniprep purification kit):

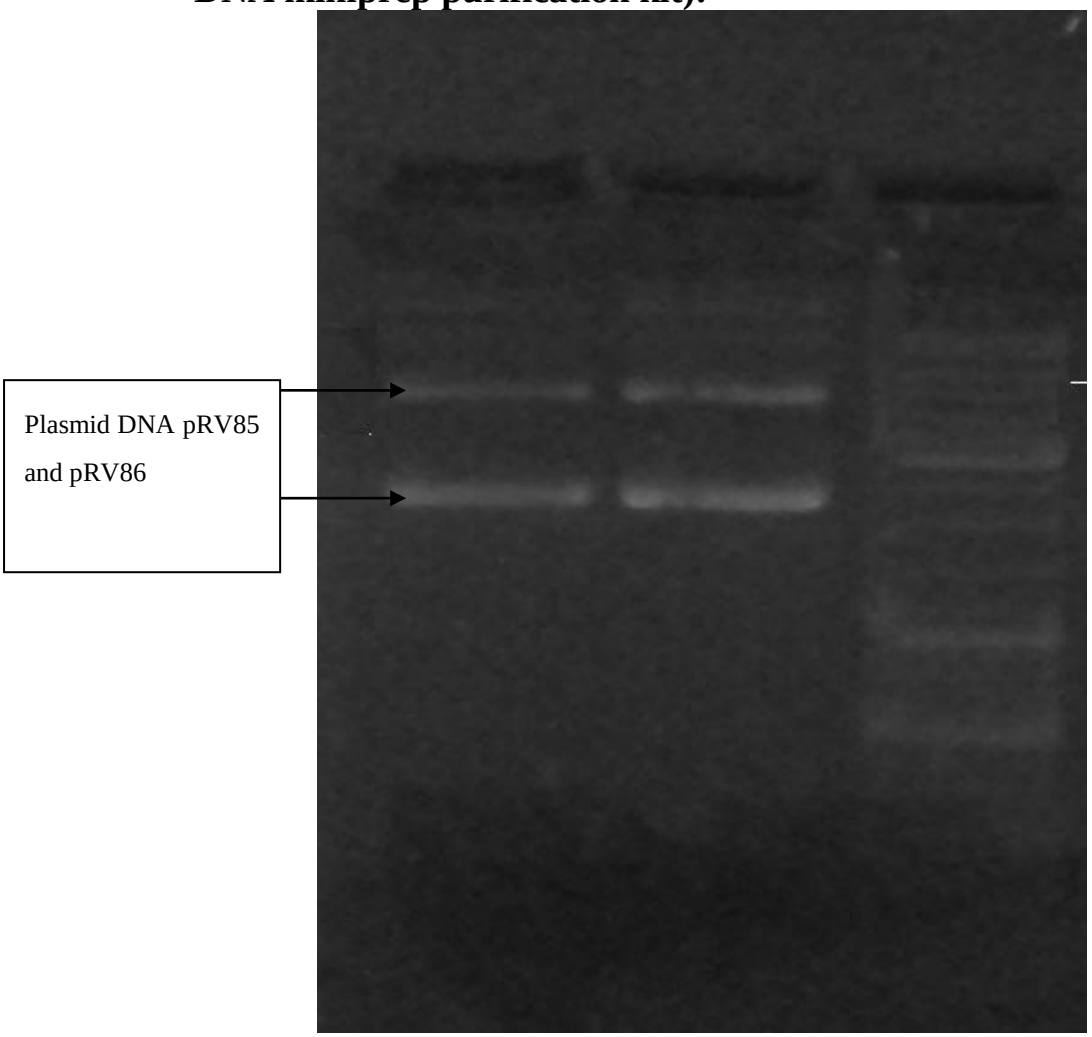


Figure 15: Plasmid Isolation of pRV85 and pRV86 (HiPurA Plasmid DNA miniprep Purification kit). Lane 1: pRV85 (6.2 kb). Lane 2: pRV86 (6.2 kb). Lane 3: O' gene ruler DNA ladder mix.

The figure shown above depicts the agarose gel image of the isolation of plasmid DNA pRV85 and pRV86. Two bands are seen in the image above. The band denoted by "A" is the linear plasmid and that denoted by "B" is the circular plasmid. Plasmids pRV85 and pRV86 are seen in lanes 1 and 2 respectively, and lane 3 contains the O' gene ruler DNA ladder mix. When viewed on agarose gel, the size of pRV85 and pRV86 was found to be 6.2 kb.

6.7 pUC18*GFP* Digestion:

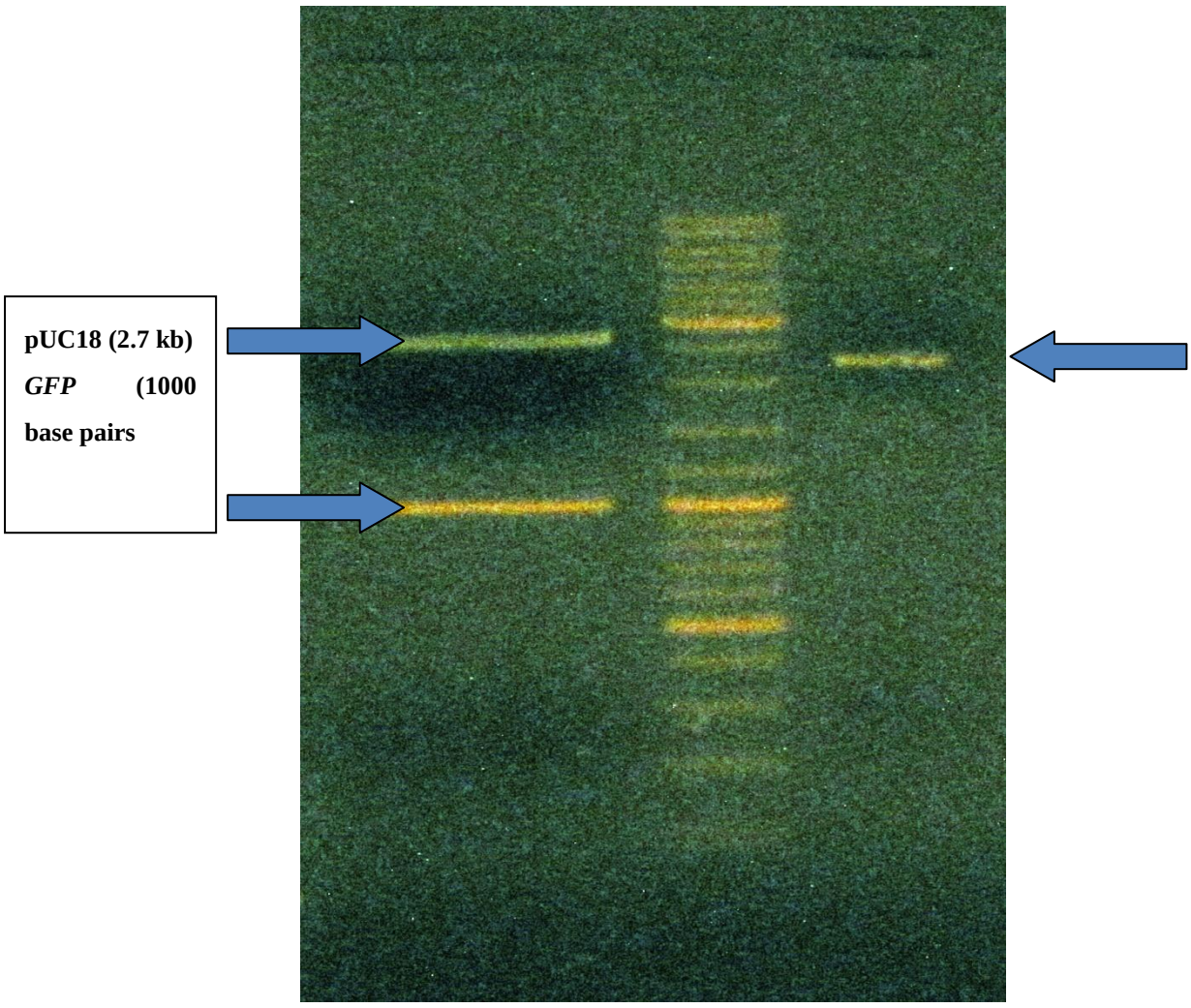


Figure 16: RE Digestion of pUC18*GFP* (*EcoRI*). Lane 1: pUC18*GFP* (digested with *EcoRI*). Lane 2: O' gene ruler DNA ladder mix. Lane 3: pUC18*GFP* uncut

The figure above shows the gel image of pUC18*GFP* digested with *EcoRI*. Lane 1 contains the digested pUC18*GFP* fragments. Lane 2 contains the O' gene ruler DNA ladder mix and lane 3 contains the uncut pUC18*GFP* (3.7 kb). Two bands can be seen in lane 1, those of pUC18 (2.7 kb) and that of *GFP* (1000 base pairs).

6.8 Transformation of *GFP* into *E. coli* DH5α:

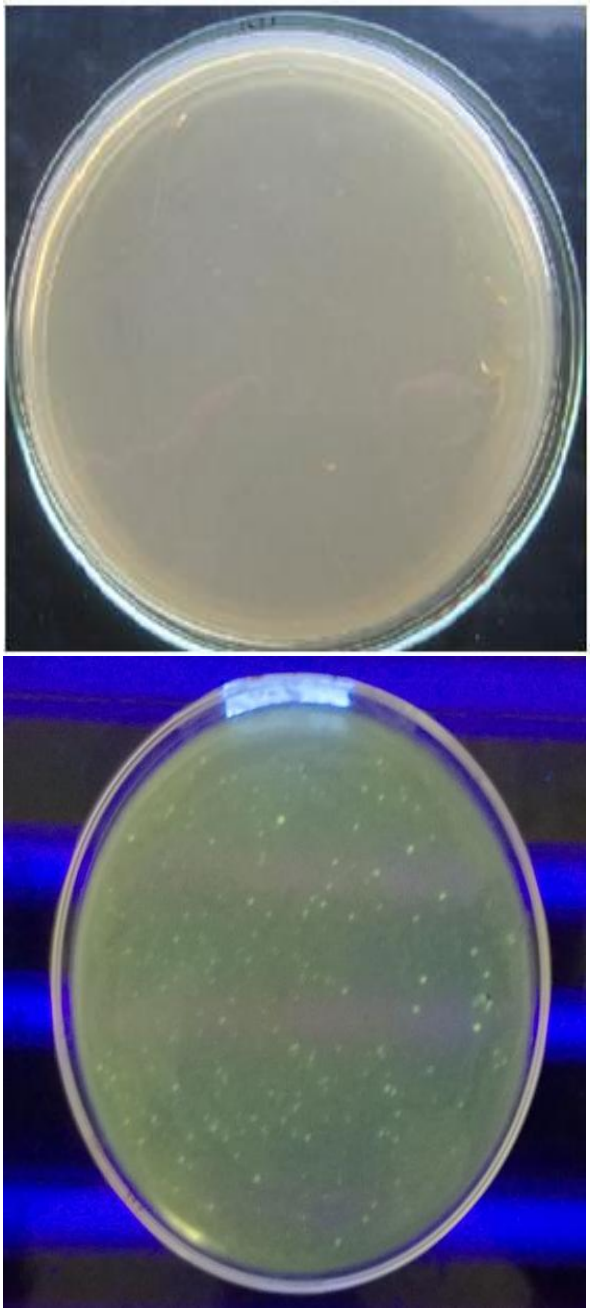


Figure 17: (a) Control Plate;
(b): *GFP* transformed plate

The above figures show the transformation of *GFP* gene into E. coliDH5α strains. The *GFP* gene was transformed successfully and various green coloured colonies could be seen. Transformation was possible and green colonies were selected.

7. DISCUSSION:

Upon isolation of pUC18, it was found to be 2.7kb in size. Plasmid pRV85 and pRV86 were also isolated with the help of the alkaline lysis method, and a 6.2 kb fragment was obtained. The *GFP* gene is located at the *EcoRI* site on pRV85. Further, extraction of *GFP* was done by digesting plasmid pRV85 with *EcoRI*. A 1000 base pairs long *GFP* fragment was obtained when pRV85 was digested with *EcoRI*. Restriction digestion of pUC18 was also done with the same enzyme. Thereafter, the *GFP* gene was ligated into pUC18 on the *EcoRI* site. When observed on agarose gel, the pUC18*GFP* fragment was found to be 3.7 kb in size. This was because *GFP* (1000 base pairs) was ligated into the cloning vector, pUC18 (2.7kb). This result was further consolidated by undertaking restriction digestion of the ligated pUC18*GFP* fragment with *EcoRI*. On doing this, two bands could be seen, one of 2.7kb (pUC18) and the other one of 1000 base pairs (*GFP*). Thus the results were consolidated and it proved that the *GFP* fragment was ligated at the *EcoRI* site in the cloning vector, pUC18. Further, transformation of *GFP* into E. coli DH5α was done and fluorescent colonies were selected.

8 SUMMARY:

1. In this study, *gfp* gene was cloned in transformation host *E. coli* DH5α.
2. This showed that *gfp* showed good fluorescent colonies upon visualization in the UV-transilluminator.
3. The cloning vector pUC18 was used and the *gfp* gene from pRV85 was successfully incorporated into it.
4. The molecular approaches to digest the vector and carry out ligation were at par to clone the gene of interest into the cloning vector.
5. In conclusion, the *gfp* fragment from pRV85 was successfully cloned in pUC18 cloning vector and the resultant pUC-*gfp* vector was transformed in the transformation host *e. cloi* DH5α for further applications.

9. APPENDIX

I. Media:

LB Medium (Luria-Bertani HiVeg Broth)

Ingredients	**(g/L)**
HiVeg hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	10.00

II. Buffers and Solutions:

1. Alkaline Lysis Solution 1 (plasmid preparation)

- 50mM glucose
- 25mM Tris-Cl (pH: 8.0)
- 10mM EDTA (pH: 8.0)

The solution was prepared from standards and was used after autoclaving it. The solution was stored in the refrigerator.

2. Alkaline Lysis Solution 2 (plasmid preparation)

- 0.2 N NaOH
- 1% (w/v) SDS

The ALS 2 was freshly prepared and used at room temperature.

3. Alkaline Lysis Solution 3 (plasmid preparation)

- 5 M potassium acetate: 60.0 ml
- Glacial acetic acid: 11.5 ml
- H_2O: 28.5 ml

4. TAE Buffer (50X):

- 242 g of Tris base
- 57.1ml of Glacial Acetic Acid
- 100 ml of 0.5M EDTA (pH: 8.0)

The concentrated stock buffer was diluted to 1X before use.

III. Chemicals and Reagents:

1. Ethidium Bromide (10 mg/ml)
2. Antibiotics:
a. Ampicillin
b. Erythromycin

3. Chemicals:
a. Agarose
b. Ethylene Diamine Tetra Acetic acid (EDTA)

4. Sodium Acetate

5. SDS

6. Magnesium Chloride

7. Calcium Chloride

IV. Protocols:

1. Plasmid Isolation by Miniprep Alkaline Lysis Method (Sambrook and Russell, Molecular Cloning, Third Edition, Volume 1, 2001)

2. Plasmid isolation by Midiprep Alkaline Lysis Method (Sambrook and Russell, Molecular Cloning, Third Edition, Volume 1, 2001)

3. Plasmid isolation by HiPurA plasmid purification kit

4. Plasmid isolation by GeneJet plasmid purification kit

5. Restrcition Digestion of plasmid DNA by Restriction Enzymes

6. Competent cell preparation and transformation (Sambrook and Russell, Molecular Cloning, Third Edition, Volume 1, 2001)

10. REFERENCES

1. Griffiths AJF, Gelbart WM, Miller JH, 1999. New York: Modern Genetic Analysis; W. H. Freeman.
2. Sridhar Rao, 2010. Mobility of Plasmids. Microbiol. Mol. Biol. Rev. 74(3):434
3. Sridhar Rao, 2009. Pathogenomics of the Virulence Plasmids of Escherichia coli at Microbiology And Molecular Biology Reviews, 73(4):750-774.
4. Yanisch-Perron, C., J. Vieira, J. Messing, 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequencing of the M13mp18 and pUC9 vectors. Gene 33:103-119
5. http://www.snapgene.com/resources/plasmid_files/basic_cloning_vectors/pUC18/
6. Laure Gory, Marie-Christine Montel; 1, Monique Zagorec, 2001; Use of green £uorescent protein to monitor Lactobacillus sakei in fermented meat products. FEMS Microbiology Letters 194; 127-133
7. Emilia Lopez, 2004; Three types of Cloning and the Necessity to Regulate. University of California.
8. Gerdes and Kaether, 1996. Green Fluorescent Protein: applications in cell biology. Institute for Neurobiology, University of Heidelberg. FEBS Letters 389(1996)-44-47.
9. R.Y. Tsein, Ann. Rev. Biochem., 1998; 67:509-44
10. M. Chalfie, Current Biol, 1994; 4, 443-443.
11. A. Miyawaki, Cell Structure and Function, 2004, 29, 6
12. A. B. Cubitt, R. Heim, S. R. Adams, A. E. Boyd, L. A. Gross and R. Y. Tsien, 1995. Trends in Biochem. Sci., 20, 448-455.
13. Sambrook and Russell: Molecular Cloning, Third Edition, Volume 1, 2001.
14. HiPurA plasmid DNA miniprep purification kit (HiMedia)
15. Acquaah, G. 2004. Understanding Biotechnology. Prentice Hall, New Jersey. Pp. 1-2.
16. Bloom, M., Freyer, G., Micklos, D. 1996. Laboratory DNA Science. Benjamin/ Cummings, California. Pp. 31, 37.
17. Raven, P., Johnson, G., 1996. Biology, 4th Ed; Wm. C. Brown: Dubuque, IA. Chapter 19; p434.
18. Winfrey, M., Rott, M., Wortman, A., 1997. Unravelling DNA. Prentice Hall, New Jersey; 26-27.
19. Ormo, M., Cubitt, A., Kallio, K., Gross, L., Tsien, R., Remington, J., 1996. Crystal structure of the *Aequorea victoria* Green Fluorescent Protein. Science Magazine. 273 (5280) pp. 1392-1395.
20. R. Hein and R.Y.Tsien, 1996. Current Biol., 6:178-182
21. O. Shimomura, 1979. Febs Letters; 104: 220-222
22. Prasher, D.C., 1995. Trends Genet; 11: 320-323

23. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher D.C., 1994. Science 263,802-805.
24. Tannahill, D., Bray S., and Harris, W.A., 1995. Dev. Biol. 168; 694-697.
25. Wang,S., and Hazelrigg, T. 1994. Nature 369, 400-403.
26. O. Shimomura, Nobel Prize Website