CHAPTER 4: TRANSFORMATION, ISOLATION & PURIFICATION OF PLASMID DNA

4.1 Transformation of Plasmid DNA:

4.1.1. Preparation and Transformation of competent E.Coli using TransformAid™ Bacterial Transformation Kit (Fermentas):

The TransformAid™ Bacterial Transformation Kit provides a new method for rapid preparation of chemically competent *E.coli* cells from overnight bacterial culture or bacterial colonies. The chemically competent cells can be easily transformed by introduction of plasmid DNA (pDNA) into bacterial cells by simple incubation. This technique is simple, rapid, and less cumbersome than Calcium Chloride – Heat Shock Method and used frequently to prepare batches of competent bacteria with high yield of transformation.

The key component of this system is the T-solution, which produces competent cells in a few easy steps. The quick and convenient procedure provides transformation efficiencies in a range of 106-107 transformants per μg of plasmid DNA. The TransformAid™ Bacterial Transformation Kit can be applied to most *E.coli* strains commonly used for cloning.

Materials:

Bacterial Strain: **Escherichia Coli strain (E Coli DH 5 α)**

Solutions: **TransformAid™ Bacterial Transformation Kit containing C medium, T solution-A and T solution-B.**

Media:

Sterile Luria broth (LB) for initial growth of culture (LB -2% w/v in water).

Sterile Luria broth (2% w/v in water) with appropriate antibiotic (Ampicillin- 100 μg/ml).

Sterile Luria broth agar plates (LB 2% w/v and Agarose 1.5% w/v in water).

Sterile Luria broth agar plates (LB 2% w/v and Agarose 1.5% w/v in water) with appropriate antibiotic (Ampicillin- 100 μg /ml).

Nucleic Acid:

Recombinant Plasmid DNA (pDNA) constructs

1. pCB6+ ARG p53 – Tumor Supressor Plasmid - Provided by Dr. Karen Voudson, Beatson Institute of cancer research, Glasgow, UK (8.3 kb)
2. pCMV·SPORT-bgal – β Galactgosidase Plasmid – Provided by Dr. Rajkumar Banarjee, Indian Institute of Chemical Technology, Hydrabad, India (7.8 kb)

Centrifuge and special equipments: Sigma Centrifuge 3K30, Germany, Water bath, Laminar Air Flow, Shaker, Incubator.

Method: Transformation of pDNA in bacterial strain E. Coli DH 5 α:

1. To the lyophilized powder containing bacterial strain, 0.5 ml of autoclaved sterile LB is added aseptically in laminar air flow, mixed thoroughly and spread the loopful of solution on LB agar plate.
2. Seed a LB plate with a single bacterial colony using the streak plate method and incubate the plate overnight at 37°C.
3. Before the transformation pre-warm culture tubes containing 1.5 ml of C medium (for each 2 transformations) at 37°C for at least 20 min.
4. Pre-warm LB antibiotic agar plates in a 37°C incubator for 20 min before plating.
5. Prepare T-solution by thawing T-solution (A) and T-solution (B), mix contents thoroughly. Combine 250 µl of T-solution (A) and 250 µl of T-solution (B) in a separate tube and keep on ice.
6. Transfer a portion of freshly streaked bacterial culture (4 x 4 mm size) to 1.5 ml of pre-warmed C-medium using an inoculating loop. Suspend the cells by gently mixing and incubate the tubes at 37°C for 2 hours in a shaker.
7. Pellet bacterial cells by 1 min centrifugation in a sigma centrifuge at 10000 RPM, 4^0 C and discard the supernatant.
8. Resuspend cells in 300 µl of T-solution. Incubate on ice for 5 min.
9. Centrifuge for 1min in a sigma centrifuge at 10000 RPM, 4^0 C, discard the supernatant.
10. Resuspend pelleted cells in 120 µl of T-solution. Incubate 5 min on ice. **(Note: After addition of T solution, in step 10, the cells become competent.)**
11. Add up to 1 µl of supercoiled DNA (10-100 pg) into new microcentrifuge tubes. Chill on ice for 2 min.

12. Add 50 µl of the prepared cells to each tube containing DNA, mix and incubate on ice for 5 min.

13. Add 10 µl of the prepared cells each to a LB agar plate as a positive and negative control.

14. Plate immediately on pre-warmed LB antibiotic agar plates. Incubate overnight at 37°C.

15. Plates used are

- + Control : Prepare competent cells and grow on LB plate not containing antibiotic.
- – Control: Prepare competent cells and grow on LB plate containing antibiotic.
- Plasmid Transformation Plate: Prepare competent cells, transform with plasmid and grow on LB plate containing antibiotic.

4.1.2 Result and Discussion:

TransformAid™ Bacterial Transformation Kit provides a rapid means of transformation of plasmid DNA into E. Coli bacterial strain (devoid of any other plasmid) for future multiplication and DNA isolation for further experiments. The kit prepares the competent E. Coli cells (Cells ready to receive plasmid) for easy transformation with significantly higher efficiency than conventional Calcium Chloride Heat Shock method.

The transformation of p53 [pCB6+ ARG p53 – Tumor Supressor Plasmid 8.3 kb] and β galactgosidase [pCMV·SPORT-bgal] plasmid onto prepared competent cells was confirmed by the growth of the competent cells on agar plates (with and without antibiotic), which acts as positive and negative control. During transformation of each plasmid, 3 agar plates were streaked with observations as below:

Table 4.1 – Results observed during transformation of pCB6+ ARG p53 and pCMV·SPORT-β-gal plasmid DNA:

Agar Plate (For pCB6+ ARG p53)	Observation	Inference
+ Control : Prepare competent cells and grow on LB plate not containing antibiotic	High cell growth observed through out plate.	Competent cells are not damaged and are capable of growing on agar plate.
– Control: Prepare competent cells and grow on LB plate containing antibiotic.	No Cell growth observed.	Competent cells are sensitive to the antibiotic used.
Test Plate: Plasmid Transformation Plate: Prepate competent cells, transform with p53 plasmid and grow on LB plate containing antibiotic (Amp).	Discrete colonies of cells are observed through out the plate (130 colonies)	Transformed cells acquiring plasmid DNA with antibiotic resistance are observed to be growing.
Agar Plate (For pCMV·SPORT-bgal)	**Observation**	**Inference**
+ Control : Prepare competent cells and grow on LB plate not containing antibiotic	High cell growth observed through out plate.	Competent cells are not damaged and are capable of growing on agar plate.
– Control: Prepare competent cells and grow on LB plate containing antibiotic.	No Cell growth observed.	Competent cells are sensitive to the antibiotic used.
Test Plate: Plasmid Transformation Plate: Prepare competent cells, transform with β gal plasmid and grow on LB plate containing antibiotic (Amp).	Discrete colonies of cells are observed through out the plate (100 colonies)	Transformed cells acquiring plasmid DNA with antibiotic resistance are observed to be growing.

The prepared competent cells were found to grow heavily on agar plate without Ampicillin; however, on Amp containing plates, no cell growth was observed indicating complete suppression of cells by Ampicillin. When transformed cells (i.e competent cells treated with plasmid containing Antibiotic resistance marker base pairs) were allowed to grow on Ampicillin containing plates, the actually transformed cells were found to grow because of the Amp resistance acquired by the cells. These transformed cells were developed as small colonies and were further sub-cultured and used or cell multiplication and DNA isolation.

4.2 Maintaince of Bacterial Cells Containing Plasmids:

1. Transformed cell colony are selected and cultured on LB agar plate with appropriate antibiotic (Ampicillin).
2. Glycerol stock solution of the bacterial cell [pellet of bacteria stored in sterile Water : Glycerol (1:1)] were stored in -70°C
3. Working cell culture plate containing transformed bacterial cell were further used for plasmid isolation with every 15 days subculturing.

Transformed cells were selected and isolated by culturing the cells on appropriate antibiotic plate as the transformed bacterial cells were resistant to antibiotic. Pure culture of E.coli containing pβ-gal or pCB6+ ARG p53 were stored as glycerol stock and working plates were used for the regular plasmid isolation experiments.

4.3 Plasmid Isolation and Purification: *ALKALINE LYSIS METHOD*

Alkaline lysis in combination with detergent as sodium lauryl sulphate (SLS) has been used for more than 20 years to isolate pDNA from E.coli. Bacterial suspension when exposed strong anionic detergent at high pH opens the cell wall, denatures chromosomal DNA and protein and releases the pDNA into the supernatant. Although the alkaline solution completely disrupts base paring, the strands of the closed circular pDNA are unable to separate from each other as they are topologically interwinded. As long as the intensity and duration of exposure of alkali is not too great, the two strands of pDNA fall once again into register when then pH is returned to neutral. During lysis, bacterial protein, broken cell walls, and the denatured chromosomal DNA becomes enmeshed in large complex that are coated with SLS. These complexes are efficiently precipitated from the solution when sodium ions are replaced with potassium ions. After the denatured material has been removed by centrifugation, native pDNA can be recovered from the supernatant by addition of isopropyl alcohol and ethanol. The closed circular pDNA recovered from the lysate can be further purified by using various techniques such as Phenol –Chloroform extraction and PEG-Lithium Chloride Extraction.

Materials:

1. Buffers and Solutions:

- **Tris-HCl (1 M):** 121.1 g of Tris base was dissolved in 800 ml of water. pH was adjusted to 8.0 by adding 42 ml of concentrated Hydrochloric acid. Final pH was adjusted and the volume was made up with water to 1 liter and sterilized by autoclaving.
- **EDTA 0.5 M (pH 8.0):** 186.1 g of disodium EDTA. $2H_2O$ was dissolved in 800 ml of water with vigorous stirring on a magnetic stirrer, pH was adjusted to 8.0 with sodium hydroxide and the volume was made up with water to 1 liter and sterilized by autoclaving.
- **1 M NaCl :** 58.4 g of NaCl was dissolved in 900 ml of water with vigorous shaking and the volume was made up with water to 1 liter and sterilized by autoclaving.
- **10X Tris-EDTA (TE):** 100 mM of Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0) in water was prepared and the solution was sterilized by autoclaving and stored at room temperature.
- **10 X Sodium Chloride – Tris – EDTA (STE) buffer (pH 7.4):** 100 mM of Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0) and 1 M NaCl in water was prepared and the solution was sterilized by autoclaving and stored at room temperature.
- **Alkaline Lysis I:** 25 mM of Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0) and 50 mM of Glucose in water was prepared and the solution was sterilized by autoclaving and stored at 4^0 C.
- **Alkaline Lysis II:** Freshly 0.2 M of sodium hydroxide and 1 % w/v of sodium lauryl sulphate in freshly autoclaved water was prepared and discarded after use.
- **5 M Potassium Acetate:** 490.5 g of Potassium Acetate was dissolved in 500 ml of autoclaved water and the volume was made up with water to 1 liter and stored at 4^0 C.
- **Alkaline Lysis III:** 60 ml of 5 M potassium acetate and 11.5 ml of glacial acetic acid were mixed and the volume was made up with autoclaved water to 100 ml and the solution was stored at 4^0 C.
- **50 X Tris-Acetate-EDTA buffer (TAE):** 242 g of Tris base was dissolved in 500 ml of autoclaved water and was mixed with 100 ml of 0.5 M EDTA (pH 8.0) and 37.1 ml of glacial acetic acid and the volume was made to1 liter with autoclaved water and was stored at 4^0 C

- **Phenol pH 8:** 500 gm of crystalline phenol is initially after melting is mixed with 0.1 % of hydroxyquonoline and is vacuum distilled. This distilled phenol is to be stored in dark container with tight closure. The phenol for DNA purification is prepared by equilibrating the melted pure phenol with Tris Buffer pH 10.0 (200 ml) for 2 times followed by equilibration with Tris buffer pH 8.0, until pH of phenol is near 8.0. The liquid phenol is kept under the layer of Tris buffer until further used.
- **Phenol Chloroform Isoamyl Alcohol:** The solution is freshly prepared before use by mixing Phenol : Chloroform : Isoamyl Alcohol in the ratio of 25:24:1 (v/v). The solution is used for purifying the pDNA from soluble proteins and chromosomal DNA.
- **Ethidium Bromide:** 10 mg of Ethidium Bromide was dissolved in 1 ml of sterile water. The solution was covered in eppendorf tube by aluminum foil and stored in cool and dark place.
- **Gel Loading Dye (Bromophenol Blue):** The gel loading dye is prepared by preparing 0.25 % w/v solution of Bromophenol Blue in 30 % v/v glycerol in water.
- **10 mM Tris (pH 8.0):** 1 ml of 1 M Tris buffer was diluted to 100 ml with sterile water with pH maintained to 8.0.
- **70 % Ethanol:** Absolute ethanol diluted to 70% v/v by water.
- **Absolute Isopropanol**
- **Lithium chloride 5 M:** 21.2 g of LiCl was dissolved in 100 ml of water sterilized by passing it through a 0.22 μm filter and stored at 4°C.
- **Polyethylene Glycol 8000 (PEG 8000) (% w/v):** Appropriate concentration of PEG 8000 was dissolved in sterile water with warming if necessary. Sterilized by passing it through a 0.22-μm filter and the solution was stored at room temperature.
- **Sodium Acetate 3.0 M:** 408.3 g of sodium acetate.3H2O was dissolved in 800 ml of water pH was adjusted to 5.2 with glacial acetic acid or to pH 7.0 with dilute acetic acid final volume was adjusted with water to 1 liter and sterilize by autoclaving.
- **Lysozyme (10 mg/ml):** Lysozyme solution (10 mg/ml) was prepared in 10 mM Tris-HCl buffer pH 8.0. The solution was freshly prepared before every use.
- **DNase-free RNase-A (1 mg /ml)** : DNase-free RNase-A solution (1 mg/ml) was prepared in 10 mM Tris-HCl buffer pH 8.0. The solution was freshly prepared before every use, headed to 60^0C, for 1 hr to destroy DNAse , allowed to cool and then used.

2. **Medium** - LB Media with Ampicillin antibiotic (100 µg /ml of culture)
3. **Culture** – E. Coli culture containing pCMV·SPORT-bgal or pCB6+ ARG p53 pDNA.

Method for Plasmid Amplification and Isolation by Maxi-precipitation:

1. 30 ml of rich medium (LB) was inoculate containing the appropriate antibiotic either with a single colony of transformed bacteria.
2. The culture was incubate at 37°C with vigorous shaking until the bacteria reach late log phase (OD_{600} = approx. 0.6).
3. 500 ml of LB medium containing the appropriate antibiotic in a 2-liter flask was inoculated with 25 ml of the late-log-phase culture. The culture was incubated for approximately 12-16 hours at 37°C with vigorous shaking on a rotary shaker.
4. Bacterial cells from the 500-ml culture were harvested by centrifugation at 5000 rpm for 10 minutes at 4°C. Supernatant was discarded and the centrifuge tubes were inverted on paper towel to remove the last traces of the media.
5. Bacterial cell pellet were resuspended in 200 ml of ice-cold STE buffer and bacterial cells were collected by centrifugation as described in Step 4.
6. The pellet was resuspend in 18 ml of AL- I with gentle vortexing and to that 2 ml of a freshly prepared solution of 10 mg/ml lysozyme and RNase-A (Conc) was added.
7. 40 ml of freshly prepared AL-II was added and the contents were mixed thoroughly by gently inverting the centrifuge tubes several times. Further incubate for 5 minutes at room temperature.
8. 20 ml of ice-cold AL-III was added and the contents were mixed gently by swirling the centrifuge tubes several times until no longer two distinguishable liquid phases were seen. Tubes were placed on ice for 10 minutes.
9. Centrifugation of the bacterial lysate was carried out at 15,000 rpm for 30 minutes at 4°C. After the completion of centrifugation, clear supernatant was decanted into a centrifuge tubes and the pellet was discarded.
10. To the supernatant, equal amount of Phenol : Chloroform : Isoamyl alcohol (25:24:1) was added. The tubes were vortexed for a minute and allowed to separate. The Tubes were centrifuged at 10,000 rpm for 15 min at 4^0C. The supernatant was removed and was similarly

treated with Chloroform alone to remove residual traces of phenol. The supernatant was again separated into the measuring cylinder.

11. Volume of the supernatant was measured. To that, 0.6 volume of isopropanol was added and mixed well and stored for 10 minutes at room temperature.
12. The precipitated nucleic acids were recovered by centrifugation at 15,000 rpm for 15 minutes at room temperature.
13. Supernatant was carefully decanted and the centrifuge tubes were inverted on a paper towel to allow the last drops of supernatant to drain away. The pellet was rinsed with 70% ethanol at room temperature and the ethanol was drained off by inverting the tubes on a pad of paper towels for a few minutes at room temperature and dried in air.
14. The pellet was dissolved in sufficient volume of Tris–EDTA (TE) buffer.
15. The purity of plasmid preparations was determined by 1 % agarose gel electrophoresis.
16. DNA concentration was measured by UV absorption at 260 nm. The purity of pDNA was estimated by measuring the optical density at 260 nm and 280 nm and calculating the ratio of Abs_{260}/Abs_{280} which was generally 1.8 to 2.0.

Plasmid Purification:

A) Polyethylene Glycol 8000 – Lithium Chloride Precipitation: [K. Mukherjee and S. Manniatis (2005)]

1. Crude large-scale plasmid preparation (3 ml) was transferred of to a centrifuge tube and the solution was chilled to 0°C in an ice bath.
2. 3 ml of ice-cold 5M lithium chloride (LiCl) solution was added to the crude plasmid preparation, mixed well, and centrifuged at 14,000 rpm for 15 minutes at 4°C.
3. Supernatant was transferred to a fresh centrifuge tube and an equal volume of isopropanol was added with proper mixing. Precipitate of nucleic acids was recovered by centrifugation at 14,000 rpm for 20 minutes at room temperature.
4. Carefully supernatant was decanted and the tubes were inverted to allow the last drops of supernatant to drain away. Pellet was rinsed with 70% ethanol at room temperature and ethanol was carefully discarded and tubes were inverted on a pad of paper towels for a few minutes.
5. Pellet of nucleic acid was dissolved in 1ml of 1X TE (pH 8.0), to it 40 µL of RNase-A (20 µg/ml) was added and stored it for 30 minutes at room temperature.

6. 500 µL of 1.6 M sodium chloride and 13% (w/v) PEG-8000 was added and mixed properly and kept in ice for 30 minutes.
7. Centrifuged at 20,000 rpm for 15 minutes at 4°C, supernatant was decanted carefully and the pellet was dissolved in 600 µL of 1X TE (pH 8.0). 80 µL of 3.0 M sodium acetate and 1.6 ml of cold ethanol was added mixed thoroughly and kept at -20°C for 10 minutes and 0°C for 30 minutes.
8. Centrifuged at 14,000 rpm for 15 min at 4°C, supernatant was discarded and the pellet was rinsed with 70% ethanol tubes were inverted on paper towel to remove excess ethanol, damp pellet was aerial dried.
9. Pellet was dissolved in 600 µL of 1X TE (pH 8.0), keep overnight at room temperature. Absorbance A_{260}, A_{280} and the ratio A_{260} and A_{280} were measured in TE (pH 8.0) and concentration of the plasmid DNA was calculated assuming that

   1 OD_{260} = 50 µg of plasmid DNA/ml.
10. Agarose gel (1%) electrophoresis was carried out to check the purity of the plasmid.
11. Purified pDNA was stored in aliquots at -20°C until further use.

Result and Discussion:

The plasmid DNA was isolated from the working culture of the E. Coli transformed strains using the alkaline lysis method. The plasmid was purified by PEG-LiCl method and was used or liposome formulation development. The purity of the plasmid was ascertained by agarose gel assay (2 bands without smear indicating pure pDNA) and UV spectrophotometry for absorbance detection at 260 and 280 nm. The ratio of absorbance between 1.8-2.0 at 260 and 280 indicated pure plasmid devoid of protein and RNA. The concentration of plasmid is determined by absorbance at 260 nm by comparing with the standard calibration curve or by the equation:

1 OD_{260} = 50 µg of plasmid DNA/ml.

4.4 Plasmid Digestion:

The digestion of the isolated plasmid by alkaline lysis method was carried out by restriction endonuclease enzyme for linearizing the plasmid, which when compared with the molecular marker run alongside the linearized plasmid on 0.7 % agarose gel shows the bands according to molecular weight.

Materials:

1. β gal and p53 purified pDNA
2. Molecular Markers (Provided by Manufacturer)
3. Restriction enzyme (Bam H1 for β gal and EcoRI or p53 plasmid) (Provided by Manufacturer : Fermentas)
4. Bovine Serum Albumin (10 X solution)
5. Restriction Endonuclease Buffer pH 8.0 [The typical restriction endonuclease buffer contains magnesium chloride, sodium or potassium chloride, Tris-HCl, 2-mercaptoethanol (2- ME) or dithiothreitol (DTT), and bovine serum albumin (BSA).] (Provided by manufacturer : Fermentas)

   Method:

   1. To the 2µl of plasmid DNA, 5.5 µl of autoclaved distilled water, 1 µl of 10 X BSA solution, 1 µl of restriction enonuclease buffer and 1 unit (0.5 µl) of restriction endonuclease enzyme was added. (EcoRI for p53 and Bam H1 for β – gal).
   2. The mixture was allowed to incubate overnight at 37^0C.
   3. The above cocktail was loaded along with molecular marker and supercoiled pDNA in three different wells into 0.7 % agarose gel containing ethidium bromide and the fluoroscence was detected under UV light.

Result and Discussion:

The digestion of the isolated plasmid was carried out by restriction endonuclease enzyme for confirming the pDNA with the tansformed DNA. The isolated pDNA after linearizing shows a single strong band on the agarose gel corresponding to its molecular weight. *p53* and *β gal* pDNA when transformed, isolated, and digested by enzymes showed the molecular weight similar to their theoretical weight on basis of the map provided by supplier. The molecular weights were confirmed by the molecular weight markers, which were run alongside the plasmids. The p53 plasmid showed a linear band between 5.09 and 9.4 kb corresponding to

molecular weight of 8.4 kb and β gal plasmid showed the linear band between 9.4 and 6.6 kb corresponding to its molecular weight of 7.8 kb (Figure 4 A and B) theirby confirming the plasmid DNA.

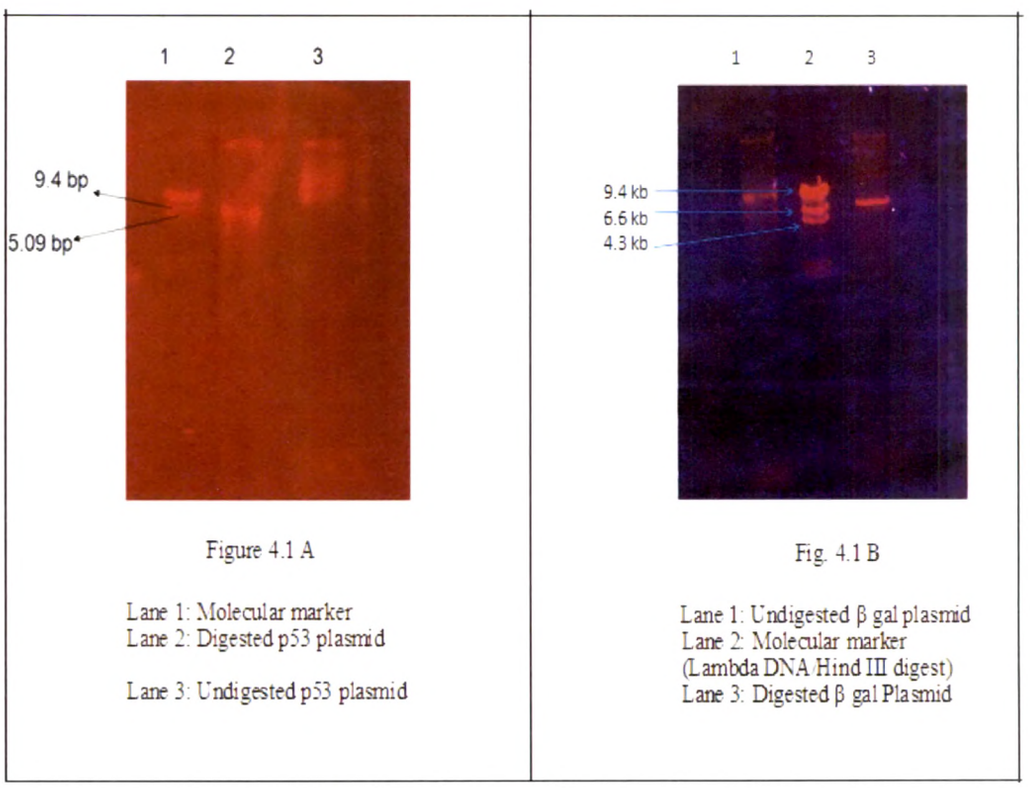

Figure 4.1 A

Lane 1: Molecular marker
Lane 2: Digested p53 plasmid

Lane 3: Undigested p53 plasmid

Fig. 4.1 B

Lane 1: Undigested β gal plasmid
Lane 2: Molecular marker
(Lambda DNA Hind III digest)
Lane 3: Digested β gal Plasmid

Figure 4.1: Digestion of p53 (4A) and β gal (4B) by EcoRI and Bam H1 restriction endonuclease enzyme.

4.5 References:

1. TransformAID TM kit manual
2. Sambrook and Maniatis (2002) Current Protocols in Molecular Biology: 1.0.1-1.0.3 by John Wiley & Sons, Inc.