3. Molecular biological techniques

3.1 Competent Cells

In molecular biology, competence is the ability of a cell to take up extra cellular ("naked") DNA (which is not associated with any cell or proteins) from its environment. Competence is distinguished into *natural competence*, a genetically specified ability of bacteria that is thought to occur under natural conditions as well as in the laboratory, and *induced* or *artificial competence*, arising when cells in laboratory cultures are treated to make them transiently permeable to DNA. Artificial competence is also known as *Transformation* in molecular biology field. Artificial competence is not encoded in the cell's genes. Instead it is induced by laboratory procedures in which cells are passively made permeable to DNA, using conditions that do not normally occur in nature (Chen 2004).

Chilling cells in the presence of divalent cations such as Ca^{2+} (in $CaCl_2$) prepares the cell membrane to become permeable to pDNA (pDNA) . Cells are incubated on ice with the DNA and then briefly heat shocked (eg 42^0C for 30-120 seconds), which causes DNA to enter the cell. This method works very well for circular pDNAs. An excellent preparation of competent cells will give ~10^8 colonies per microgram of plasmid. A poor preparation will be about 10^4/μg or less. Good non-commercial preps should give 10^5 to 10^6 transformants per microgram of plasmid (Sambrooke 2001 a).

Electroporation is another way to make holes in bacterial (and other) cells, by briefly shocking them with an electric field of 10-20kV/cm. Plasmid DNA can enter the cell through these holes. This method is amenable to use with large pDNA. Natural membrane-repair mechanisms will rapidly close these holes after the shock.

3.2 Preparation of competent cells

Most methods for bacterial transformations are based upon the observation of Mandel and Higa (1970), who showed that bacteria treated with ice cold solution of calcium chloride and then briefly heated could be transfected with bacetriophage- λ DNA. The same method was subsequently used to transform bacteria with pDNA (Cohen 1972)

and *E.Coli* chromosomal DNA. Apparently, the treatment induces a transient state of competence in the recipient bacteria, during which they are able to take up DNAs derived from a variety of sources.

Bacteria treated according to the original protocol of Mandel and Higa yield 10^5-10^6 transformed colonies/μg of super coiled DNA. There are two ways to obtain stocks of competent *E.Coli* cells. The first option is to purchase frozen competence cells from a commercial source (eg. Invitrogen, USA). These products are very reliable and yield transformants at frequencies $\geq 10^8$ colonies/μg of super coiled pDNA. However, they are many more times more expensive than competent cells prepared in lab. The second option is to prepare stocks of competent bacteria in the lab using bacterial cell lines (Sambrooke 2001 a).

3.3 Application of Competent cells

For the given studies, two different strains of competent *E. Coli* cells (DH5-α and BL-21 DE3 pLysS) were used. DH5-α was used for making multiple copies of pDNA, the original pDNA encoded with proteins (antigens) of interest and obtained from Colorado state university (CSU), USA as part of NIH, NIAID Contract, entitled "Tuberculosis Vaccine Testing and Research Materials", (contract no. HHSN266200400091C), While *E. Coli* BL-21 (DE3) pLysS was used for expression of protein encoded in the pDNA.

3.4 Competent E. Coli- DH5-α and BL-21 (DE3) pLysS

3.4.1 DH5 α

Genotype:

*sup*E44 Δ*lac* U169 (Φ80*lac* ZΔM15) *hsd*R17 *rec*A1 *end*A1*gyr*A96 *thi*-1*rel*A1

A recombinant-deficient suppressing strain used for plating and growth of plasmids and cosmids (Low 1968, Meselson and Yuan 1968, Hanahan 1983). This strain has higher transformation efficiency.

3.4.2 BL-21 (DE3) pLysS

Genotype *hsd*S *gal* (λcI*ts*857 *ind*1Sam7*nin*5*lac*UV5-T7gene*1*)

A strain employed for high-level expression of genes cloned into expression vectors containing bacteriophage T7 RNA polymerase, bacteriophage is λ DE3 and it is integrated into the chromosome of BL 21 (Studier and Moffatt 1986).

Although the BL21 cell series is engineered for protein expression, these strains have two significant problems that limit their use as primary transformation hosts. Because BL21 cells are derived from the *E. coli* B cell line rather than the *E. coli* K-12 cell line, their efficiency is relatively low (1 x 10^6 cfu/μg of *pUC18* DNA). The other problem causing *BL21* competent cell lines to be inadequate for initial transformation experiments is that they contain the *EndA* gene, which encodes an endonuclease that rapidly degrades miniprep DNA. This degradation makes restriction mapping and DNA sequencing (procedures usually performed to confirm that the protein expression construct is correct) from $EndA^+$ cells very difficult. To retrieve and confirm the plasmid before performing protein expression studies, investigators first transform the ligation mix of the protein expression construct into a high-efficiency, $EndA^-$ cell line, such as XL1-Blue competent cells. Then, a second transformation into BL21 cells is performed for protein expression (Studier and Moffatt 1968, Studier 1990).

3.5 pDNA transformation

In order to persist and be stably maintained in the cell, a pDNA molecule must contain an origin of replicon, which allows it to be replicated in the cell independently of the chromosome. Because transformation usually produces a mixture of rare transformed cells and abundant non-transformed cells, a method is needed to identify the cells that have acquired the plasmid. Plasmids used in transformation experiments will usually also contain a gene giving resistance to an antibiotic that the intended recipient strain of bacteria is sensitive to. Cells able to grow on media containing this antibiotic will have been transformed by the plasmid, as cells lacking the plasmid will be unable to grow. **For pDNA transformation DH5- α strain of *E. Coli* is widely used** (Sambrooke 2001 a).

them from chromosomal DNA and proteins.

3.5.1.1 Chemicals and Reagents

SE-Coli **DH5- α** competent cells were purchased from Invitrogen, USA, Luria broth (LB) from Hi-media Laboratories Pvt Ltd, Mumbai, RNase I (DNase free) from Genei, Bangalore, Sodium Dodecyl Sulphate (SDS), Glucose, EDTA, Tris.Cl Sodium Hydroxide, Potassium Acetate, Sodium Acetate, Glacial Acetic Acid were of Molecular biology grade and purchased from Sigma Aldrich, USA.

Magnetic stirrer,

Reagents:

Solution I

The following composition was used for solution I for miniprep.

50 mM glucose

25 mM Tris.Cl (pH=8.0)

10mM EDTA (pH=8.0)

Preparation of 1 M stock solution for Glucose:

180.2 gms of glucose was added in 800 mL of Milli Q water, the final volume was made to 1000 mL.

Stock solution: 1M Tris Cl

121.1 gm of Tris base was dissolved in 800 ml of water. pH was adjusted to the desired value by adding concentrated HCl. For adjusting to pH 8, 42 ml of concentrated HCl was added.

Stock solution 0.5 M EDTA (pH 8.0)

186.8 gm of di-sodium ethylene diaminetetraacetate, $2H_2O$ was added in 800 ml of water and stirred vigorously on magnetic stirrer; pH was adjusted to 8.0 with sodium hydroxide (approximately 20 gms of pellet). It was dispensed into aliquots and sterilized by autoclaving (121^0C, 15 PSI for 30 minutes).

(Note: To dissolve disodium salt of EDTA, the pH of solution was adjusted to 8.0 by addition of sodium hydroxide).

Solution I was prepared by adding required quantity of above mentioned solution to give concentration of individual components as mentioned in the formula.

Solution I was prepared in batches of approximately 100 ml autoclaved for 15 minutes at 15 PSI and stored at 4^0C.

Solution II

For preparation of solution II, 0.2 N NaOH and 1% SDS stock solution were prepared and mixed just before its use

Solution III : the following formula was prepared for solution III.

5M Potassium Acetate	60 ml
Glacial Acetic Acid	11.5 ml
Water	28.5 ml

Preparation of 5 M Potassium Acetate stock solutions

98.15 gms of potassium acetate was dissolved in 800 mL of milli Q water and final volume was made 1000 mL. It was then sterilized by Autoclaving.

The resulting solution is 3 M with respect to Potassium and 5M with respect to acetate.

Preparation of 3 M Sodium Acetate:

408.1 gm of Sodium Acetate was dissolved in 800 ml of water. pH of the solution was adjusted to 5.2 by adding glacial acetic acid and finally solution was made to the volume to 1000 mL. Then they were dispensed in aliquots and sterilized by autoclaving.

Preparation of RNAse stock solution:

Pancreatic RNAse at a concentration of 10 mg/ml was added in 0.01 M sodium acetate (pH 5.2). This mixture was heated to 100^0C for 15 minutes and allowed to cool slowly to RT. pH was adjusted by adding 0.1 volumes of 1M Tris CL (pH 7.4) and dispensed into aliquots. Aliquots were stored at -20 ^{0}C.

Preparation of LB Medium:

20 gm of LB was added in 1000 mL in milli Q water and then sterilized by autoclaving (121^0C, 15 PSI for 30 minutes). After cooling to RT, calculated quantity of Ampicillin and Chloramphenicol were added aseptically and dispensed in 3 ml aliquots in 15 mL or 25 mL tubes respectively. Tubes were stored at RT.

Preparation of LB agar plates:

20 gm of LB and 10 gm of N. agar was added in 800 mL in milli-Q water, final volume was made to 1000 mL and then sterilized by autoclaving at 121^0C, 15 PSI for 30 minutes. It was then cooled to 50 ^{0}C and calculated quantity of Ampicillin and Chloramphenicol were added aseptically. About 20-25 mL of medium was transferred to sterile petri-plates aseptically and allowed to solidify. Then they were sealed with parafilm and stored at 4 ^{0}C until used.

Preparation of Ampicillin and Chloramphenicol antibiotic solution:

The stock solution of ampicillin and chloramphenicol was prepared as shown in table 3.1 and depending on the volume of LB medium and LB-agar medium, ampicillin and chloramphenicol were transferred in a way to give final concentration of 100μg/ mL and 34 μg/mL respectively.

Antibiotic	Stock Solution		Working Solution
	Concentration	Storing Temp	
Ampicillin	50 mg/mL in Autoclaved water	-20^0 C	100 μg/ mL
Chloramphenicol	34 mg/mL in Ethanol	-20^0 C	34 μg/ mL

Table 3.1 Preparation of Antibiotic Solution

(Note: Antibiotics prepared in Ethanol was not sterilized. Antibiotic prepared in water was sterilized by Filtration (0.22 μ membrane filter).

The following is the procedure for the miniprep.

Plasmids (PMRLB7/Rv3875 ESAT-6 in pET23b and PMRLB47/Rv1886c Ag85B in pET 23b were transferred in thawed cells (*E.Coli*, competent cells, DH5 α), tapped once and kept in ice for 30 minutes. Heat shock was given at 42^{0}C for 90 seconds and again it was put back in ice for 5 minutes.

The thawed cells containing plasmids were placed in 1 ml of LB medium (sterile) and kept at 37^{0}C for 60 minutes in shaking incubator, spun at 5000 rpm for 5 minutes, re-suspended pellet in 50-100 μl LB and plated on LB agar plates. Plates were kept inverted and placed in the 37^{0}C bacterial incubators (Bacteriological incubator, Classic Scientific, India) overnight.

Single bacterial colony was transferred in to 2 mL of LB medium containing appropriate antibiotic in loosely capped 15 mL tube and inoculated the culture overnight at 37^{0}C with vigorous shaking. 1.5 mL of culture then poured into an

eppendorff and centrifuged at 5000 rpm for 5 minutes at 4^0C.The medium was removed by aspiration leaving the bacterial pellet as dry as possible.

Lysis by alkali

This procedure was modification of the protocol developed by Birnboim and Doly (1979). Bacterial pellet was suspended in 100 µL of ice-cold solution I and 1 µL of 10 mg/mL RNase (DNase free), kept in ice for 5 minutes and mixed by vortexing. 200 µL of freshly prepared solution II was then added. (Note: Close the tube tightly and mix the contents by inverting the tube rapidly five times. Make sure that the entire surface of tubes comes in contact with solution II. Do not VORTEX. Do not TAPE). 150 µL of ice-cold solution III was added. The tube was closed and vortexed gently in an inverted position for 10 seconds to disperse solution III through the viscous bacterial lysate. The tubes were placed on ice for 3-5 minutes. Then these tubes were centrifuged for 5000 rpm 15 minutes at 4^0C and supernatant was transferred to a fresh tube. To the supernatant, $1/10^{th}$ volume of Sodium Acetate (3M) (50 µL) and double the volume of absolute alcohol (1 mL) was added. The tubes were put in liquid nitrogen for 5 minutes and then they were spun at 12000 rpm for 10 minutes at 4^0C. 1 mL of 70% ethanol was added and again spun at 12000 rpm for 10 minutes at 4^0C.
The pellet was suspended in 20 µL of autoclaved water and stored at -20^0C.
2 µL was transferred on gel for Agarose Gel-Electrophoresis.

3.5.2 Midi prep

The standard way to remove proteins is to extract with phenol: chloroform and then with chloroform. This procedure takes advantage of fact that de-proteinization is more efficient when two different organic solvents are used instead of one. Although phenol denatures protein efficiently, it does not inhibit RNase activity and it is solvent for RNA molecules that contain long tracts of poly (A) (Brawerman 1972). Both of these problems can be solved by using mixture of phenol: chloroform and subsequent extraction with chloroform removes lingering traces of phenol from nucleic acid preparation.
Sodium Acetate (3M, pH 5.2) is used for most routine precipitation of DNA.
The procedure for midi prep is described as below:

PMRLB7/Rv3875 (ESAT-6 in pET23b) and PMRLB47/Rv1886c (Ag85B in pET 23b) were transferred in thawed *E. Coli* competent cells (DH5-α), tapped once and kept in ice for 30 minutes. Heat shock was given at 42^0C for 90 seconds and again put back in ice for 5 minutes.

The thawed cells containing plasmids were placed in 1 ml of sterile LB medium and kept at 37^0C for 60 minutes in shaking incubator, spun at 5000 rpm for 5 minutes, the pellet was re-suspended in 50-100 µl LB medium and plated on LB agar plates containing antibiotics (Ampicillin 100 µg/ mL and Chloramphenicol 34 µg/ mL). Plates were kept inverted and placed in incubators at 37^0C (bacteriological incubators, Classic Scientific, India) overnight.

Single colony was transferred to 3 ml LB and kept at 37^0C in shaking incubator (Orbital shaker Incubator, Classic Scientific, India) till optical density reached to 0.6 when measured at 600 nm using spectrophotometer ($OD_{600} > 0.6$). Then it was spun at 5000 rpm for 5 minutes, pellet was re-suspended in 50-100 µL. To the suspension, 5 mL of solution I was added, votexed and kept in ice for 5 minutes. 10 mL of solution II was then added mixed gently and kept in ice for 5 minutes. (DO NOT Vortex. Mix gently by inverting 5 times. After mixing with solution II, the contents are clear).7.5 mL of solution III was added and again kept in ice for 5 minutes. (Proper mixing of solution was ensured). Solution then spun down at 12000 RPM at 4^0C for 20 minutes and supernatant was transferred into new tubes and again spun at 12000 rpm at 4^0C for 10 minutes. Supernatant was collected in a new autoclaved tube. 0.6 volumes (approximately 13.5mL) of isopropanol was added and kept at RT for 30 minutes and then spun at 12000 rpm for 10 minutes at 4^0C. The pellet was rinsed with absolute ethanol and dried after discarding ethanol and keeping the pellet at 37^0C under hood. Finally it was suspended in 500 µL of autoclaved water.

Equal volume of 5M Lithium chloride was added and kept in ice for 20 minutes. Then it was spun at 12000 rpm for 10 minutes at 4^0C (here the contents need to be split into 2 eppendorf). Supernatant was transferred in a new tube and equal volume of iso-propanolol was added. It was kept at RT for 20 minutes. Then it was centrifuged at 12000 rpm for 20 minutes at 4^0C. Again pellet was rinsed with absolute ethanol.

Ethanol was discarded and pellet was made dry at 37^{0}C under aseptic hood. The pellet was re-suspended in 100 µL of water.

10 µL of RNase A was added and kept at 42^{0}C for 1 hour. The volume was made up to 500 µL with autoclaved water. 500 µL of phenol was added, mixed well and spun at 12000 rpm for 10 minutes at 4^{0}C. The aqueous upper layer was taken, transferred into another tube and (1:1) phenol: chloroform mixture was added. It was then spun it at 12000 rpm for 10 minutes at 4^{0}C. Again the upper layer was taken and 500 µL of chloroform was added. It was then centrifuged at 12000 rpm for 10 minutes at 4^{0}C. Upper layer was taken again and transferred into another tube and 1/10th volume of 3 M Sodium acetate (50 µL) and double the volume of absolute ethanol (1 mL) was added. It was kept in liquid nitrogen for 5 minutes. Then it was centrifuged at 12000 rpm for 10 minutes at 4^{0}C, 70 % ethanol (1mL) was added and centrifuged again at 12000 rpm for 10 minutes at 4^{0}C. Ethanol was removed; pellet was dried at 37^{0}C under aseptic hood. Depending upon the pellet size, autoclaved water (50-100 µL) was added and stored at -20^{0}C.

3.5.3 Restriction enzyme analysis for the transformed bacterial cells

To determine successful transformation of pDNA to the *E.Coli* DH5- α cells, one from four following method is commonly used,

(1) Restriction enzyme analysis
(2) A- complementation
(3) Insertional inactivation
(4) Screening by hybridization

Restriction enzyme analysis method is more applicable for small-scale preparations of pDNA and hence, we used this method for the research work. In this method, numbers of independently transformed bacterial colonies were picked up and they were analysed by double digestion with restriction enzymes followed by the agarose gel electrophoresis. This procedure is although laborious, is the method of choice when there is a high chances of finding the desired recombinant DNA within a small number of randomly chosen transformed colonies (Cohen 1973, Sambrooke 2001b).

Selection of two enzymes for double digestion restriction enzymes method:

We referred plasmid map (Figure 3.1) provided from CSU, USA along with the materials where name of digestion enzymes between which plasmid inserted was given. The map indicated that the plasmid was inserted between digestion enzymes *NdeI* and *PstI* and *XhoI*. That means plasmid can get separated when we use *XhoI* and *NdeI* digestion enzymes or *XhoI* and *PstI* digestion enzymes. For the given experiments we have used digestion enzymes *Nde I* and *Xho I*. When Nde I and Xho I are the enzyme, in this case buffer 'O' is the recommended digestion buffer.

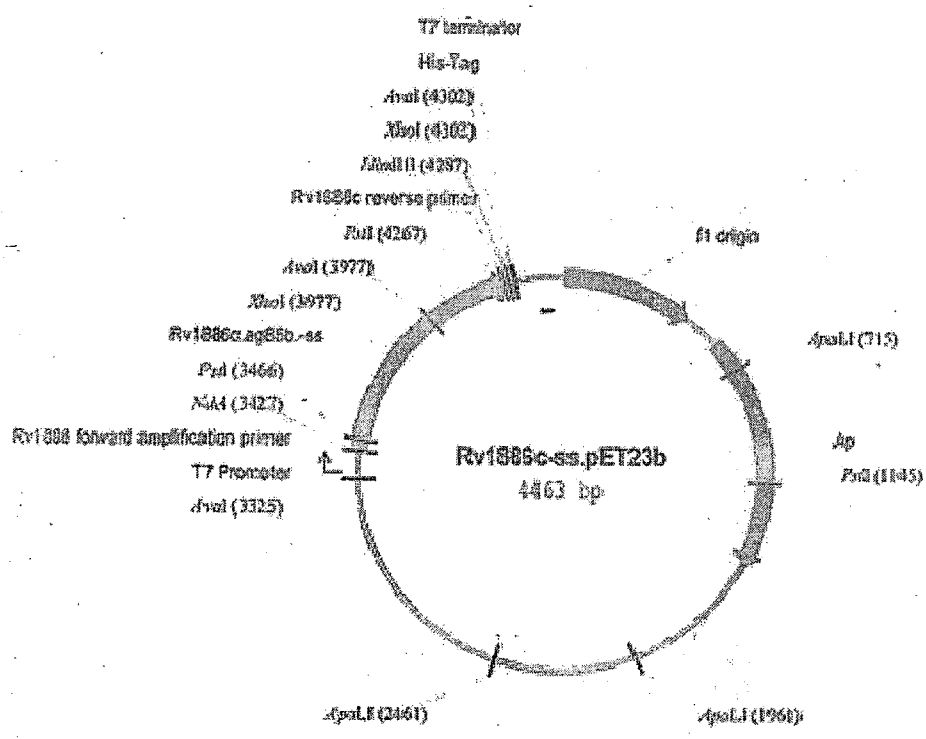


Figure 3.1 Plasmid Map

3.5.3.1 Chemicals, Reagents and procedure

Nde I, Xho I and buffer "O" were purchased from Fermentas, USA.

In an eppendorff the following composition of digestion enzymes, buffer and pDNA was taken and incubated for 6 hours at 37^0C.

PDNA	2 µg
Nde I	3 µL
Xho I	6 µL
Buffer 'O'	2 µL
Milli Q water	2 µL

It was then run on Agarose Gel electrophoresis with DNA Ladder.

3.5.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a method used in biochemistry and molecular biology to separate DNA or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an Agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate farther than longer ones. Agarose gels are cast by melting the Agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the Agarose forms a matrix, the density of which is determined by the concentration of the Agarose. When electric field is applied across the gel, DNA which is negatively charged at neutral pH migrates toward the anode (Brody 1972, Sambrooke 2001 c).

3.5.4.1 Chemicals and Requirements

Minigel gel electrophoresis unit was supplied by Genei Bangalore with power cable; Seakem LE Agarose was supplied by Lonza, Rockland, ME USA, Ethidium Bromide Lichtgeschuzt Lagerm, Boehringer, Germany. Bromophenol blue (#114391, Sigma, USA), Xylene cyanol FF (#95600, Sigma Aldrich, USA). Glycerol, Tris Base, Glacial Acetic Acid, EDTA, Boric Acid were supplied from Sigma Aldrich, USA.

Preparation of Gel running buffer

The composition of gel running buffer (also known as electrophoresis buffer) is shown in the table 3.2.

Buffer	Working Solution	Concentrated Stock Solution per Litre
Tris- Acetate (TAE)	1 X* : 0.04 M Tris acetate 0.001 M EDTA	50 X : 242 gm Tris Base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH = 8)
Tris Phosphate (TPE)	1X : 0.09 M Tris phosphate 0.002 M EDTA	10 X : 108 gm Tris base, 15.5 mL 85% Phosphoric acid (1.678 g/mL), 40 mL 0.5 M EDTA (pH = 8)
Tris- borate (TBE)	0.5 X : 0.045 M Tris Borate, 0.001 M EDTA	5 X : 54 gm Tris base, 27.5 Boric Acid, 20 mL 0.5 M EDTA pH = 8.

Table 3.2 Gel Running Buffer

Any one from TAE, TBE or TPE buffer can be used as gel running buffer. When TAE is chosen for the work, agarose gel also should be made in TAE buffer.

3.5.4.2 Procedure: Agarose gel electrophoresis

The edges of a clean, dry glass plate were sealed with autoclave tapes so as to form a mold. (Set the mold on horizontal section of the bench, check with a level). The tank was filled with 1 M TAE buffer. Agarose was added to a measured quantity of 1 M TAE buffer in an Erlenmeyer flask or glass bottle with a loose fitting cap. The neck of the Erlenmeyer flask was loosely packed with kimwipes. The slurry was heated in a microwave oven till the agarose dissolves. The slurry was heated for minimum time required to allow all of the grains of agarose to dissolve. Wearing an oven mitt, the bottle or flask was shaken from time to time to make sure that any grains sticking to the walls enter the solution. Care was taken to avoid overheating of agarose solution. The volume of solution was checked to ensure not too much water had evaporated during boiling. The solution was then cooled to 60^0C and ethidium bromide (from stock solution of 10 mg/mL in water to a final concentration of 0.5µg/mL ad mix thoroughly) was added to it (Sambrooke 2001c).

Using pipette, edges of the mold were sealed with a small quantity of agarose solution and the seal was allowed to set. A comb was placed on 0.5 to 1.0 mm above the plates so that a complete well was formed when agarose was added. The remainder of the warm agarose solution was poured into mold. The gel should be between 3 mm and 5 mm thick. After the gel was completely set (30-45 minutes at RT), the comb and autoclave tapes removed carefully and the gel was mounted in the electrophoresis tank. Enough electrophoresis buffer was added to cover the gel to a depth of about 1 mm. DNA was mixed with the gel loading buffer, type III. The mixture was slowly loaded into the slots of the submerged gel using a disposable micropipette.

The lid of the gel tank was closed and attached the electrical leads so that the DNA will migrate toward the anode (red lead). Voltage of 1-5 v/cm (measured as the distance between the electrodes) was applied. (Observation: If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis) and within few minutes, the bromophenol blue should migrate from the wells into the body of the gel). The gel was run until the bromophenol blue and xylene cyanol FF have migrated the appropriate distance through the gel. (Note: During electrophoresis, the ethidium bromide migrates towards the cathode in the direction opposite to that of DNA).

The electric current was turned off and leads were removed. The gel was taken from the electrophoresis tank. Presence of ethidium bromide in the gel and electrophoresis buffer had helped for examining the gel by ultraviolet light, photograph was taken for record.

3.5.5 Results and Discussions

To make multiple pDNA copies, we followed mini prep and midi prep protocol. The mini prep is small scale isolation of pDNA from *E. coli* DH5- α cells, while midi prep is medium scale procedure. The transformation efficiency to DH5- α is very high (~10^6 colonies per 10 μg of the DNA). Although transformation usually produces a mixture of transformed cells and abundant non-transformed cells, a method is needed to identify the cells that have acquired the plasmid. Hence, while performing mini prep, several bacterial colonies were chosen randomly and were analysed for presence of the recombinant DNA by the restriction enzyme analysis. Parallel to this, the same

bacterial colonies were also plated on fresh LB agar plates aseptically and incubated at 37^0C. In the restriction enzyme analysis, the isolated DNA was cut with the help of two enzymes, *Nde I* and *Xho I*, the enzymes between which recombinant DNA was inserted. Then the sample was placed on agarose gel, and run for an hour in mini electrophoresis unit. The DNA ladder, which is a mixture of DNA with known molecular weight, was also run in the same agarose gel. The estimation of DNA size was done on the basis of the fact that distance DNA molecule travelled through agarose gel which is dependent on the size of DNA molecules in electrophoresis. The agarose gel acts as a sieve for DNA molecules so that larger molecules have low diffusivity moving through gel matrix compared to smaller fragments of DNA which are able to migrate further through the gel towards the positive end as DNA is negatively charged. Thus, this process allows the separation of large and small fragments which can be analysed and identified. We used a molecular weight marker which was a mixture of known molecular weight alongside the purified pDNA. This helped us to estimate size of DNA fragment.

Figure 3.2 shows the photograph of agarose gel electrophoresis when gel was exposed to the UV chamber.

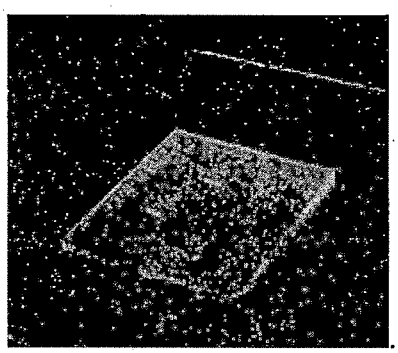


Figure 3.2 pDNA on Agarose Gel Electrophoresis

There are three rows observed in the photograph. The first from the left is the DNA ladder, middle one and right most are the DNA before and after restriction enzyme treatments. The size of pDNA was determined on the basis of relative distance travelled by pDNA with known size of DNA from the DNA ladder. The DNA ladder consists of various sizes of DNA. When we compared with the standard DNA ladder,

we found the size of pDNA 1 KB. Similarly, the size of each fragment was also determined using standard DNA ladder and size of each fragments observed was as follows:

Vector- 3.66 kb (pET 23b + Nde I + Hind III)

Insert 0.860kb RV 1886 C

Other 4.25kb linearized plasmid

Those bacterial colonies which had shown the positive transformation results were further processed for the midiprep, as yield of miniprep is very less compared to the midi prep. But miniprep was done to evaluate the positive transformation and this was way, we could save time and reagents. Each midi prep batch was able to produce around 300 µg of the pladmid DNA and with four such midiprep batches; we could produce around 1 mg of plasmid DNA which was sufficient for our entire protein expression work.

3.6 Induction and Protein Expression

Protein expression is a subcomponent of gene expression. It consists of the stages after DNA has been translated into poly peptide chains, which are ultimately folded into proteins. Protein expression is commonly used by proteomics researchers to denote the measurement of the presence and abundance of one or more proteins in a particular cell or tissue (Sambrooke 2001d).

Escherichia coli (E. coli) is one of the most widely used hosts for the production of heterologous proteins and its genetics are far better characterized than those of any other micro organism. Recent progress in the fundamental understanding of transcription, translation, and protein folding in E. coli, together with availability of improved genetic tools are making this bacterium more valuable than ever for the expression of complex eukaryotic proteins.

Isopropyl β-D-1-thiogalactopyranoside, abbreviated **IPTG** (Chemical formula: $C_9H_{18}O_5S$, Mol Wt: 238.3) is a molecular biology reagent. This compound is used as a molecular mimic of allolactose, a Lactose metabolites that triggers transcription of the *lac operon*. Unlike allolactose, the sulphur (S) atom creates a chemical bond

which is non-hydrolyzable by the cell, preventing the cell from "eating up" or degrading the inductant; therefore the IPTG concentration remains constant.

IPTG induces the transcription of the gene coding for Beta Galactosidase, an enzyme that promotes lactose utilization, by binding and inhibiting the Lac 1 repressor. In cloning experiments, the *lacZ* gene is replaced with the gene of interest and IPTG is then used to induce gene expression (Sambrooke 2001d).

Many regulatory elements of the *lac* operon are used in inducible recombinant protein systems; IPTG is an effective inducer in the concentration range of 100 μM to 1.5 mM.

Protease Inhibitors are the substances that prevent breakdown of protein into small fragments. This substance is added after induction and before cell lysis to prevent protein fragmentations. Phenyl methyl- sulfonyl fluoride (PMSF) was used for the given study. This is active against chymotrypsin and trypsin in the concentration range of 100 μg/ mL. PMSF is inactivated in aqueous solution. The rate of inactivation increases with pH and is faster at 25^0C than at 4^0C. The half life of 20 μM aqueous solution of PMSF can be safely discarded after they have been rendered alkaline (pH> 8.6) and stored for several hours at RT (Sambrooke 2001d).

Protease inhibitors are essential components of most cell lysis and protein extraction procedures. These inhibitors block or inactivate endogenous proteolytic enzymes that are released from subcellular compartments during cells lysis and would otherwise degrade proteins of interest and their activation states (Soliman 2009).

3.6.1 Chemicals and Reagents for Induction and Protein Expression

IPTG was purchased from Novagen, Merck, USA, *BL-21 (DE3) pLysS* from In vitrogen, USA, Protease Inhibitor PMSF (phenyl methyl- sulfonyl fluoride) from Novagen, USA, Sodium chloride, Nickel Sulphate from Sigma Aldrich, USA.

PMR LB 7/RV3875/Esat-6 and PMR LB 47/RV1886c/Ag-85B was provided by the from CSU, USA.

3.6.2 Transformation and Induction

PMR LB 7/RV3875/Esat-6 and PMR LB 47/RV1886c/Ag 85B were transferred in thawed *E. Coli* competent cells (*BL 21 DE3 pLySs*), tapped once and kept in ice for 30 minutes. Heat shock was given at 42^0C for 90 seconds and again put back in ice for 5 minutes. The thawed cells containing plasmids were placed in 1 ml of sterile LB medium and kept at 37^0C for 60 minutes in shaking incubator, spun at 5000 rpm for 5 minutes, the pellet was re-suspended in 50-100 µl LB medium and plated on LB agar plates containing antibiotics (Ampicillin 100 µg/ mL and Chloramphenicol 34 µg/ mL). Plates were kept inverted and placed them in the 37^0C bacteriological incubators overnight.

Meanwhile IPTG solution was prepared by dissolving 2 gms of IPTG in 8 ml of distilled water. The volume of solution was adjusted with distilled water and sterilized by filtration through 0.22 µm disposable filter in bacterial aseptic hood. The solution was dispensed into 1 ml aliquots. The excess IPTG solution was stored -20^0C.

Single bacterial colony was transferred in to 2 mL of LB containing antibiotic (Ampicillin 100 µg/ mL and Chloramphenicol 34 µg/ mL) in loosely capped 15 mL tube. The culture was inoculated overnight at 37^0C with vigorous shaking. 1% of the primary inoculation was transferred to two 3 mL of LB tubes containing antibiotics. It was incubated at 30^0C for 3.5-4 hours till it reached to optical density~0.6 at 600 nm. In one tube sterile 0.5 mM IPTG was added aseptically and in another tube it was not added. For Ag-85B, induction was carried out at 37^0C for 5 hours. Culture was pellet down and treated with 4X SDS dye and checked for induction by SDS-PAGE and followed by coomassie staining.

3.6.3 Determination of Level of Induction by SDS-PAGE

Almost all analytical electrophoresis of protein is carried out in poly acrylamide gels under conditions that ensure dissociation of the proteins into their individual poly peptide sub-units and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded on the gel. The denatured poly-peptides bind SDS and become negatively charged. Because the amount of SDS bound is always proportional to the molecular weight of poly peptide and is independent of its

sequence, SDS-poly-peptide complex migrate through poly acrylamide gels in accordance with the size of poly peptide. At saturation, approximately 1.4 gm of detergent is bound per gram of poly peptide. By using marker of known molecular weight, it is therefore possible to estimate the molecular weight of the polypeptide chain (Sambrooke 2001d).

In most cases, SDS-PAGE is carried out with discontinuous buffer system in which the buffer in the reservoir is of a different pH and ionic strength from buffer used to cast the gel. The SDS-poly peptide complexes in the sample that is applied to the gel are swept along by a moving boundary created when electric current is passed between electrodes. After migrating through a stacking gel of high porosity; the complexes are deposited in a very thin zone on the surface of the resolving gel. The ability of discontinuous buffer systems to concentrate the entire sample into a very small volume greatly increases the resolution of SDS-polyacylamide gels.

Discontinuous buffer system that is most widely used was originally devised by Ornstein (1964). The sample and stacking gel contain Tris. Cl (pH 6.8) and the upper and lower buffer reservoir contain Tris-glycine (pH 8.3) and the resolving gel contains Tris. Cl (pH 8.8). All components of the system contain 0.1% SDS. The chloride ions in the sample and stacking gel from the leading edge of the moving boundary, and the trailing edge is composed of glycine molecules. Between leading and trailing edges of boundary is a zone of lower conductivity and steeper voltage of gradient which sweeps the poly peptides from the sample and deposits them on the surface of the resolving gel. There, the higher pH of the resolving gel favours the ionization of glycine, and the resulting glycine ions migrate through the stacked poly peptides and travel through the resolving gel immediately behind chloride ions. Freed from the resolving boundary, the SDS-poly peptide complexes move through the resolving gel in a zone of uniform voltage and pH and are separated according to size by sieving (Sambrooke 2001d).

Polyacrylamide gels are composed of chains of polymerized acrylamide that are cross linked by a bifunctional agent such as N, N' – methylenebisacrylamide. The effective range of separation of SDS-PAGE depends on the concentration of polyacrylamide used to cast the gel and on the amount of cross linking. Polymerization of acrylamide in the absence of cross linking agents generates viscous solutions that are of no

practical value. Cross links formed from bisacrylamide add rigidity and tensile strength to the gel and form pores through which the SDS-polypeptide complex must pass. The size of these pores decreases as the bisacrylamide: acrylamide ratio increases, reaching a minimum when the ratio is approximately 1:20. Most SDS-polyacrylamide gels are cast with a molar ratio of bisacrylamide: acrylamide of 1:29 which has been shown empirically to be capable of resolving poly peptide that differ in size by as little as 3%.

The sieving properties of the gels are determined by the size of the pores which is a function of the absolute concentration of acrylamide and bisacrylamide used to cast the gel. Depending on the molecular weight of proteins evaluated, the composition of acrylamide in the gel is defined. The higher the molecular weight of the protein, lesser the quantity of acrylamide is added. The table 3.3 shows the suggested % of acrylamide added to the gel while casting the gel for the determination of the molecular weight of the protein

Acrylamide Concentration Separation (%)	Linear range of (KD)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

Table 3.3 Effective range of separation of SDS-PAGE

3.6.3.1 Chemicals and Reagents

SDS-PAGE teaching kit was purchased from Bangalore Genei, India. Acrylamide, N, N' methyleneacrylamide, Ammonium persulphate, Tris Cl, glycine, TEMED and SDS were purchased from Sigma Aldrich, USA. Molecular weight marker was purchased from Fermentas, USA.

Preparation of 30% Acrylamide solution

A stock solution containing 29% w/v acrylamide and 1% w/v N, N' methylene bisacrylamide was prepared in deionized warm water (to assist the dissolution of bisacrylamide).

Preparation of 10% Ammonium per Sulphate

10 gm of Ammonium persulphate (which is hygroscopic in nature) was transferred to 80 mL of de-ionized water and the final volume was made to 100 with water.

Preparation of 10 % SDS

10% stock was prepared in de-ionized water and stored at RT.

Preparation of 1.5 M Tris Cl, pH 8.8

181.65 gms of Tris Cl was added in 800 mL of water, pH was adjusted to 8.8 by adding HCl and the volume was made to 1L with de-ionized water.

Preparation of 1 M Tris Cl, pH 6.8

121.1 gms of Tris Cl was added in 800 mL of water, pH was adjusted to 6.8 by adding HCL and final volume was made to 1L with de-ionized water.

5 M stock solution of Tris-Glycine SDS-PAGE gel running buffer can be prepared by dissolving 15.1 gms of Tris base and 94 gms of glycine in 900 mL of de-ionized water. Then 50 mL of 10% w/v SDS stock solution added and final volume was adjusted to 1000 mL with de-ionized water.

3.6.3.2 Procedure for SDS-PAGE

The plates were assembled, a spacer was placed between two and clips were kept at both the end. The bottom of the plates was sealed with agarose solution. Resolving and stacking solution were prepared as per the following formula table 3.4:

Quantities in mL	Resolving Solution	Stacking Solution
Water	1.15 mL	3.0 mL
30% Acrylamide	2.5 mL	0.5 mL
Tris Cl. 1.5 M (pH 8.8)	2.25 mL	---
Tris Cl 1M pH 6.8	---	0.5 mL
10% SDS	50 µL	50 µL
10% Ammonium Persulphate	50 µL	50 µL

Table 3.4 Preparation of SDS-PAGE plates- resolving and stacking solution

TEMED was added just before pouring the gel (as polymerization starts as soon as TEMED is added).

Assembled plates were kept vertically. Resolving solution was poured between the gap of the plates and a minimum quantity of butanol was also poured to ensure even layer. It was then allowed to polymerize. (It took almost 30 minutes). The upper layer of resolving gel was cleaned by washing with water and then stacking gel solution was poured directly onto the surface of the polymerized resolving gel. Immediately a clean Teflon comb was inserted into stacking gel solution, being careful to avoid trapping air bubble. More stacking gel solution was added to fill the spaces of the comb completely. The gel was kept vertical at RT. While stacking gel was polymerizing (it takes almost 45 minutes), the samples were prepared by heating them to 100 ^{0}C for 3 minutes in 1X SDS-PAGE loading buffer to denature proteins. The composition of SDS-PAGE lading buffer is as given in table 3.8

1 X SDS-PAGE loading buffer
50 mM Tris Cl (pH 6.8)
100 mM dithiothreitol**
2% SDS (electrophoresis grade)
0.1% bromophenol blue
10% glycerol

Table 3.5 SDS-PAGE Gel Loading Buffer

(Note: **1X SDS PAGE gel loading buffer lacking dithiothreitol can be stored at RT. Dithiothreitol was added, just before the buffer used from 1M stock).

After polymerization was complete, teflon comb was removed carefully. Wells were washed with deionized water to remove any un-polymerized acryl amide. The gel was mounted in the electrophoresis apparatus. Tris-glycine electrophoresis buffer was added to the top and bottom reservoir. The composition of gel running buffer is as shown in table 3.6.

Tris Glycine Electrophoresis Buffer***
25 mM Tris Chloride 250 mM glycine (electrophoresis grade pH 8.3 SDS 0.1%

Table 3.6 Gel Running Buffer

15 µL of each of the sample was loaded in a pre-determined order into the bottom of the wells. After each sample was loaded, the syringe was washed with the buffer from the bottom reservoir. The electrophoresis apparatus was attached to an electric power supply and voltage of 50 mV was applied. The gel was run until bromophenol blue moved into resolving gel and the voltage was increased to 120 mV and run until bromophenol blue reached the bottom of the resolving gel (it took about 4 hours). The power was turned off.

Glass plates were removed from the electrophoresis apparatus and placed on a paper towel. The plates were separated by applying gentle pressure with spatula. The orientation of the gel was marked by cutting a corner from the bottom of the gel that is closest to the leftmost well. (Note: do not cut the corner from gels that are to be used for western blotting). The gel was then stained with Coomassie Brilliant blue or silver salts.

3.6.3.3 Staining SDS-PAGE with Coomassie Brilliant Blue

Polypeptide separated by SDS-PAGE can be fixed with methanol: glacial acetic acid and stained with Coomassie Brilliant blue R-250. The gel was immersed for several hours in a concentrated methanol: acetic acid solution of the dye and the excess dye was then allowed to diffuse from the gel during a prolonged period of de-staining.

Procedure for Coomassie Brilliant Blue Staining

0.25 gm of Coomassie Brilliant Blue R-250 was dissolved in 100 ml of the methanol: water: acetic acid mixture (30: 60: 10) by stirring on a magnetic stirrer for about 3 hours and solution was filtered through whatman filter no.1 to remove any particulate matter. This is staining solution. The gel was then immersed in at least 5 volumes of staining solution and placed on a slowly rocking platform for minimum 4 hours at RT. The gel was then removed from staining solution (Sambrooke 2001d).

The gel was de-stained by soaking it in the de-staining solution (composition of de-staining solution is methanol: water: glacial acetic acid) on a slowly rocking platform for 4-8 hours, changing the de-staining solution for 3-4 times. (Note: The more thoroughly the gel is de-stained, the smaller the amount of protein that can be detected by staining with Coomassie Brilliant blue. De-staining for 24 hours usually allows as little as 0.1μg of protein to be detected in a single band).

After de-staining, gels may be stored indefinitely in water in a sealed plastic container without any diminution in the intensity of staining. Stained gels should not be stored in de-staining buffer which will cause the stained protein bands to fade. For the record keeping, the gels were scanned by placing between them two wet plastic sheet with HP LaserJet Pro M1536dnf Monochrome Laser (all in one printer/scanner/copier/fax).

3.6.3.4 Staining SDS-PAGE with Silver salts

A number of methods have been developed to stain polypeptides with silver salts after separation by SDS-PAGE. In every case, the process relies on differential reduction of silver ions that are bound to the side chain of amino acids (Switzer 1979, Hempelmann 1984). These methods fall into two category: (1) those that use ammonical silver solutions and (2) those that use silver nitrate.

Although both types of staining are approximately 100- to 1000- fold more sensitive than staining with Coomassie Brilliant R-250 and are capable of detecting as little as 0.1-1.0 ng of poly-peptide in a single band. Silver nitrate solutions are easier to prepare and in contrast to ammonical silver salts, do not generate potentially explosive by- products.

The silver staining of the SDS-PAGE was done under the guidance of Prof. Anjali Karande, at Department of Biochemistry, Indian Institute of Science, Bangalore, India and the following steps (as shown in table 3.7) were performed:

Steps	Solution/treatment	Duration
Fixing	Methanol 50%, Acetic acid 12%, 37% formaldehyde 0.05%	Minimum 1 hour
Washing	Ethanol 50% with shaking on rocking platform	3 x 10 minutes
Pre- treatment	Sodiumthiosulphate 0.02%	1 minute, very crucial
Rinse	Double distilled water	3 x 20 minutes
Impregnation	Silver nitrate 0.2%, 37% formaldehyde 0.075%	1 x 20 minutes
Rinse	Double distilled water	2 x20 seconds
Developing	Sodium carbonate 6%, Sodium thiosulphate 1 mg (1 crystal), 37% formaldehyde 0.05%	Shake well till band appears on the gel. Do not allow the background to get stained
Rinse	Double Distilled water	3x20 seconds
Stopping	Methanol 50%, Acetic Acid 12%	1x10 minutes
Store	Methanol 50%	Store indefinitely.

Table 3.7 Laboratory standardized Protocol for Silver Staining of SDS-Gel

3.6.4 Reagents preparation and procedure for induction and protein expression

IPTG, Imidazole, Tris. Cl, Sodium Chloride, Disodium Hydrogen Phosphate, Potassium Dihydrogen Phosphate, LB medium were purchased from Sigma Aldrich, USA.

Probe sonicator Vibra-Cell VCX 750 from Sonics & Materials, Inc., USA was used for the experiments.

3.6.4.1 Reagent Preparation

IPTG 100 mM Solution: 0.238gm of IPTG was dissolved in 10 mL milli Q water and sterilized by 0.22 µm disposal filter unit in a sterile tube under aseptic condition.

1M Imidazole: Accurately weighed 68.08 gm of Imidazole was dissolved in 1L of ultra pure water.

1M Sodium Chloride: 58.5 gm Sodium Chloride (NaCl) was dissolved in 1 L of ultra pure water.

200 mM Tris.HCl: 1.211 gm of Tris base was dissolved in 50 mL of ultra pure water.

10X Phosphate Buffer Saline:

The following quantity was weighed accurately:

80 gm Sodium Chloride, 2gm Potassium Chloride, 14.4 gm of Disodium Hydrogen Phosphate, 2.4 gm of Potassium Dihydrogen Phosphate

They dissolved in 800 mL of ultra pure water and pH of the solution was checked and adjusted to 7.4, final volume was made to 1 L with ultra pure water.

Composition of Various Buffers used for Protein expression and purification:

Binding Buffer: 20mM Tris HCl, 250 mM NaCl, 5 mM Imizadole

Washing Buffer I: 20mM Tris HCl, 250 mM NaCl

Washing Buffer II: 20mM Tris HCl, 250 mM NaCl, 20 mM Imizadole

Washing Buffer III: 20mM Tris HCl, 250 mM NaCl, 40 mM Imizadole

Washing Buffer IV: 20mM Tris HCl

Elution Buffer : 20mM Tris HCl, 600 mM Imizadole

3.6.4.2 Induction for ESAT-6 and Ag 85B

Procedure for ESAT-6

3 mL of 2 LB tubes culture was inoculated overnight. Secondary inoculation was done at 30°C for 3.5 to 4 hours till the OD_{600} ~ 0.6. Induction was done with 0.5 mM IPTG solution in one test-tube and samples were kept at 4°C for about 1 hour. One test-tube, considered as control, where no IPTG was added.

Then the samples were kept at 18°C for 14 hours for the induction and the culture was pellet down.

Procedure for Ag 85 B

3 mL of 2 LB tubes culture was inoculated overnight. Secondary inoculation was done at 30°C for 3.5 to 4 hours till the OD_{600} ~ 0.6. Induction was done with 0.5 mM IPTG solution in one test-tube and samples were kept at 4 °C for about 1 hour. One test-tube, considered as control, where no IPTG was added.

The pellets were then suspended in 100 µL of sterile PBS solution and then they were checked for the protein induction by running them on the SDS-PAGE and then gel was stained with coomassie blue.

3.6.4.4 Protein expression ESAT-6

3 ml of 2 LB tubes culture was inoculated overnight. Secondary inoculation was done at 30°C for 3.5 to 4 hours till the OD ~ 0.6. Induction was done with 0.5 mM IPTG solution in one test-tube and samples were kept at 4°C for about 1 hour. One test-tube, considered as control, where no IPTG was added. Then the samples were kept at 18 °C for 14 hours for induction. The culture was pellet down. To this pellet, approximately 200 µL of binding buffer was added and sonicated with Sonicator (Vibra cell model) with 3 sec on and 3 sec off cycle till clear liquid observed. Centrifuged for 10 minutes at 10000 rpm at 4°C, lysate was collected, SDS-PAGE was run with the lysate by loading 20 µL of the sample (lysate) and stained with coomassie blue.

3.6.4.5 Protein expression Ag 85B

3 ml of 2 LB tubes culture was inoculated overnight. Secondary inoculation was done at 30 °C for 3.5 to 4 hours till the OD ~ to 0.6. Induction was done with 0.5 mM IPTG solution in one test-tube and samples were kept at 4 °C for about 1 hour. One test-tube, considered as control, where no IPTG was added. Then the samples were kept at 18 °C for 16 hours for induction. The culture was pellet down.

To this pellet, approximately 200 µL of binding buffer was added and sonicated with Sonic, vibra cell model with 3 sec on and 3 sec off cycle till clear liquid observed. It was centrifuged for 10 minutes at 10000 rpm at 4°C, lysate was collected, SDS-PAGE was run with the lysate by loading 20 µL of the sample (lysate) and stained with coomassie blue.

3.6.5 Results and Discussion

The proteins (ESAT-6 and Ag85-B) were expressed from pDNA and checked by SDS-PAGE with coomassie staining. We started working with the guidelines given in the protocol provided by the CSU, USA and where necessary we made the changes to get protein expression.

Figure 3.3 shows the gel documents for the ESAT-6 and Ag-85B protein induction.

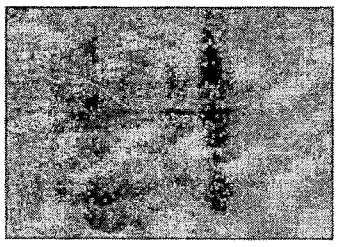


Figure 3.3 IPTG inductions for ESAT-6 protein expression

The description for various bands showed in the figure 3.4 is as follows:

Band 1: no IPTG added in ESAT-6,

Band 2 and 3: IPTG was added in case of ESAT-6,

Band 4: mixture of standard molecular weight marker,

Band 5: no IPTG added in case for Ag 85 B,

Band 6 and 7 where IPTG was added for induction for Ag85 B

Presence of band at around 10kDa size in band 2 and 3 was clear indication of induction and protein expression for ESAT-6 protein. However, bands at 32 kDa in the band 6 and 7 bands were missing for Ag 85 B, indicate that the modification was needed in the protocol.

We changed the time and temperature for Ag-85B protein induction from 30^0C for 3.5 to 4 hours to 16 hours at 18^0C incubation after IPTG addition. Figure 3.4 shows the gel document obtained with modified protocol.

Figure 3.4 IPTG inductions for Ag-85B protein expression

Description of the bands in figure 3.4 is as follows:

Band 1 standard mixture of molecular weight marker,

Band 2 and 3 IPTG added (Ag 85 B)

With modified induction conditions, clear bands were observed at 32 kDa molecular weight in band 2 and 3. This may be due to cell destruction at higher temperature making cells unable to induce protein expression and hence we selected low temparture for this purpose. Ideally low tempareture and high exposure to IPTG induces protein expression form the pDNA and that worked well in our case too.

As indicated in the protocol provided by the CSU, USA, both the proteins are quite stable in nature and therefore, sonication method was the choice of method for the cell lysis. The expressed protein mixture was then placed on SDS-PAGE for the evaluation of proteins. Protocols provided by CSU, USA were followed for this purpose . Figure 3.5 and 3.6 show the protein expression gel documents.

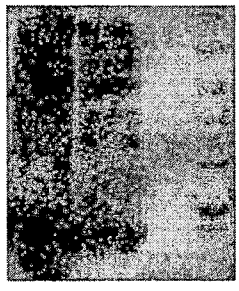


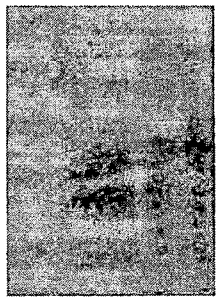
Figure 3.5 ESAT-6 Protein expressions

Description of the bands in figure 3.5 is as follows

Band 1, 2: ESAT-6 protein

Band 3: molecular weight marker

Figure 3.6 for ESAT-6 protein expression indicated over-expression of ESAT-6, the desired protein.



1 2 3

Figure 3.6 Ag 85B Protein expressions

Description of the bands in figure 3 is as follows:

Band 2, 3: Ag 85B protein

Band 1: standard molecular weight marker

Figure 3.6 for Ag85B protein expression indicated that with parameter set above, there was over-expression of Ag 85B, the desired protein.

3.7 Protein Purification by Affinity Chromatography

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. Protein purification is vital for the characterisation of the function, structure and interactions of the protein of interest. The starting material is usually a biological tissues or microbial culture. The various steps in the purification process may free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps exploit differences in protein size, physico-chemical properties and binding affinity (sambrooke 2001d).

The degree of protein purity required depends on the intended end use of the protein. For some applications, a crude extract is sufficient. In order to achieve this, several protein purification methods are typically used:

(1) Size Exclusion Chromatography

(2) Separation based on charge or hydrophobicity

(3) Ion Exchange Chromatography

(4) Affinity Chromatography

- 4.1 Metal Binding
- 4.2 Immuno-affinity chromatography

3.7.1 Affinity Chromatography

Affinity Chromatography is a separation technique based upon molecular conformation, which frequently utilizes application specific resins. These resins have ligands attached to their surfaces which are specific for the compounds to be separated. Most frequently, these ligands function in a fashion similar to that of antibody-antigen interactions. This "lock and key" fit between the ligand and its target compound makes it highly specific, frequently generating a single peak, while all else in the sample is un-retained.

3.7.2 Metal binding

A common technique involves engineering a sequence of 6 to 8 histidine into the C-terminal of the protein. The polyhistidine binds strongly to divalent metal ions such as Nickel and Cobalt. The protein can be passed through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged proteins pass through the column. The protein can be eluted with imidazole, which competes with the polyhistidine tag for binding to the column, which decreases the affinity of the tag for the resin. While this procedure is generally used for the purification of recombinant proteins with an engineered affinity tag (such as a 6xHis tag), it can also be used for natural proteins with an inherent affinity for divalent cations. Figure 3.7 shows the schematic presentation of mechanism of metal binding for protein purification.

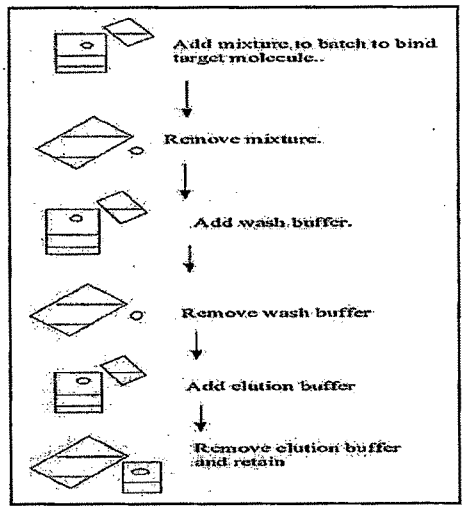


Figure 3.7 Schematic Presentation of Affinity Chromatography

3.7.3 Protein Purification by His Bind columns

3.7.3.1 Chemicals and Reagents

His Bind Resin from Novagen, (Cat # 69630, binding capacity 12.5 mg/ mL of beads. His Bind Resin from Super flow, Quigen, Cat # 1018611, binding capacity 50 mg/ mL of beads). Plastic Columns from Novagen with suitable cap and flow control device. Nickel Sulphate was purchased from Sigma Aldrich, USA.
All buffers like binding buffer, washing buffer and elution buffer were prepared as per procedure shown in section 3.7.4.1.

3.7.3.2 Handling of column

1. Ni-NTA matrices are stable under a wide variety of conditions and need not be refrigerated, except to inhibit growth of microorganism for long term storage.
2. After use, they should be washed for 30 minutes with 0.5M NaOH.
3. Ni-NTA matrices should be stored in 30% ethanol to inhibit microbial growth.
4. The matrix can be stored up to one week in any of the denaturing buffers.

3.7.3.3 Procedure for Batch purification

3 mL of culture was inoculated overnight. Secondary inoculation was done at 30 °C for 3.5 to 4 hours till the OD ~ 0.6. Induction was done with 0.5 mM IPTG solution in one test-tube and samples were kept at 4 °C for about 1 hour. The samples were kept at 18°C for 16 hours for induction. The culture was pellet down. To this pellet, approximately 200 µL of binding buffer was added and sonicated till clear liquid was obtained. It was then centrifuged for 10 minutes at 10000 rpm at 4 °C and lysate was collected.

200 µL His-Bind matrices were charged with 5 mL of 100 mM of $NiSO_4$, equilibrated with binding buffer and the matrices were loaded with the lysate in an eppendorff. (20 µL of lysate kept as control). Binding of matrices was done in cold room in end to end rotor for about one hour.

Then the matrices were washed with washing buffer 4 times with 200 µL and eluted with 200 µL of elution buffer 3 times (composition of buffers as per suggested in

protocol given by CSU, USA). All fractions were collected. From all the fractions, 20 µL were loaded on SDS-PAGE and gel was later stained with the silver salt as described in protocol for silver staining (3.6.3.4).

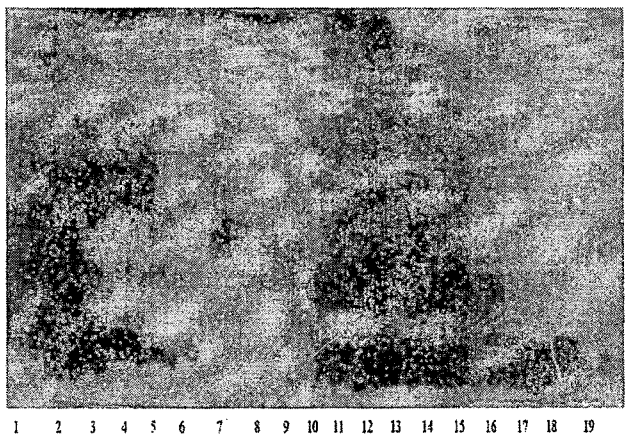


Figure 3.8 Batch purification of Protein ESAT-6 and Ag 85 B

Description of the bands in figure 3.8 is as follows:

Band 1 Ag 85 B after induction,

Band 2 flow through,

Band 3 wash 1, Band 4 wash 2, Band 5 wash 3, Band 6 wash 4,

Band 7 Elute 1, Band 8 Elute 2, Band 9 Elute 3,

Band 10 Molecular weight marker,

Band 11 ESAT 6 after induction,

Band 12 flow through,

Band 13 wash 1, Band 14 wash 2, Band 15 wash 3, Band 16 wash 4,

Band 17 Elute 1, Band 18 Elute 2, Band 19 Elute 3.

The result in figure 3.8 clearly indicated that the protocol provided by CSU, USA was found reproducible at our laboratory for protein purification. The estimated binding capacity of Ni-NTA with the proteins was 12.5mg/ mL of Ni-NTA resin. This was taken into consideration while scaling up the protein production at higher scale.

3.7.3.4 Protein expression from 100 mL culture

The previous section described the protein expression when 3 mL culture was used. To carry out research with the proteins, we estimated at least 200 mg of the proteins. To meet these requirements, we have checked the parameter set for 3 mL culture with the 100 mL culture and then with 400 mL culture. It is always desirable to check all the results again when the same condition was implemented for larger scale. Sometimes it may happen that parameter set for small scale might not work when it is applied for larger scale. Figure 3.9 and 3.10 show the results for ESAT-6 with 100 mL and 400 mL culture when the parameters set for 3 mL culture was implemented and 3.11 showed the results for Ag-85B with 400 mL culture.

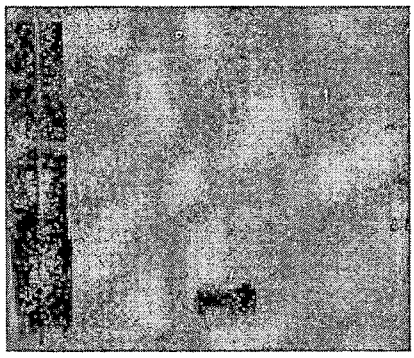


Figure 3.9 ESAT-6 productions with 100 mL culture

Description of the bands in figure 3.9 is as follows:

Band 1: Load,

Band 2: flow through,

Band 3 Wash 1 with 0 mM Imidazole in wash buffer

Band 3 wash 2 with 30 mM Imidazole in wash buffer,

Band 4 Wash 3 with 60 mM Imidazole in in wash buffer 30 mL,

Band 5 Wash 4 with 60 mM Imidazole in wash buffer,

Band 6 Wash 5 25th volume of washing,

Band 7 Elute 1, Band 8 Elute 2, Band 9 Elute 3, Band 10 Elute 4, Band 11 Elute 5, Band 15 Molecular weight marker

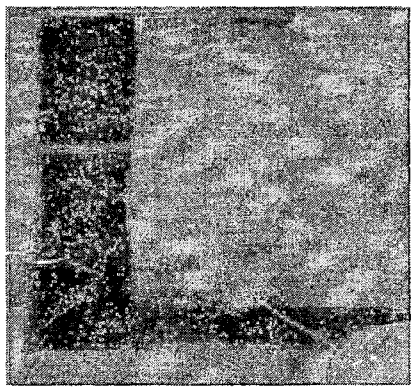


Figure 3.10 ESAT-6 productions with 400 mL culture

The description of the bands is as follows:

Band 1 Molecular weight marker,

Band 2: Load,

Band 3 Flow through,

Band 4 Wash 4,

Band 5 Single Elute 5 mL,

Band 6 Elute 2,

Band 7 Elute 3,

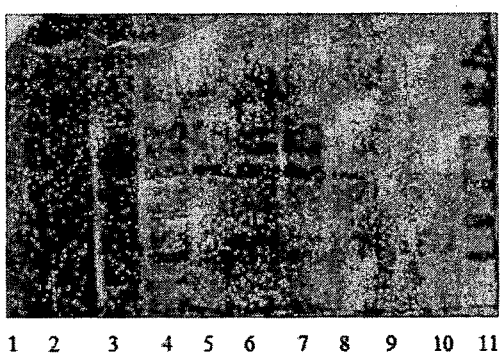


Figure 3.11 Ag85B protein productions with 400 mL culture

The description of the bands is as follows:

Band 1 Load,

Band 2 Flow through,

Band 3 Wash 1 with 00 mM Imidazole,

Band 4 Wash 2 with 20 mM Imidazole in washing buffer,

Band 5 Wash 3 40 mM Imidazole,

Band 6 Elute 1, Band 7 Elute 2, Band 8 Elute 3, Band 9 Elute 4, Band 10 Elute 5,

Band 11 Molecular weight marker

The figure 3.9 and 3.10 showed successful implementation of parameters set for 3 mL culture to 100 mL culture and then with 400 mL culture. Each 400 mL culture was able to give around 35 mg of proteins and hence multiple times, 400 mL culture was handled to get around 200 mg of ESAT-6proteins. Figure 3.11 showed similar results for Ag 85B protein synthesis from 400 mL bacterial culture. However, the loss of desired proteins in washings was found high in concentration which was due to the presence of Imidazole; therefore, we reduced molar concentrations of Imidazole in the initial washings. The yield of Ag-85B protein in 400 mL culture was around 30 mg.

3.8 Removal of Impurities from Protein by Dialysis

Dialysis is the separation of small and large molecules in a solution by selective diffusion through a semi-permeable membrane. Typically a sample containing a protein or nucleic acid will contain unwanted small molecular weight (M.W.) compounds such as a buffer salt (Tris, PBS, etc.), a reducing agent [Dithiothreitol (DTT), b- Mercaptoethanol (BME), etc.] or a preservative (sodium azide, thimerosol, etc.). The sample is placed on one side of the dialysis membrane. The dialysate, which is 200 to 300 times the volume of the sample, is placed on the other side of the dialysis membrane. This creates and maintains a concentration differential across the membrane. Once the liquid-to-liquid interface (sample on one side of the membrane and dialysate on the other) is initiated, all molecules will then try to diffuse in either direction to cross the membrane in order to reach equilibrium. Dialysis (diffusion) will stop when equilibrium is achieved. Generally the rate of dialysis slows as equilibrium approaches, requiring that the dialysate in the beaker be changed after several hours to re-create the concentration differential that drives the dialysis process. The membrane is the key to dialysis. The semi-permeable membrane contains pores of a known size range that are large enough to let small M.W. compounds pass through, but that are small enough that larger M.W. compounds (e.g., proteins and nucleic acids) cannot get through. The ideal membrane is very thin, has

numerous pores of uniform diameter, and is made so proteins and nucleic acids do not bind to it. Unfortunately, the ideal membrane does not exist. What scientists have been using for decades is an extruded regenerated cellulose membrane that has many of the characteristics of an ideal membrane.

However, separation resolution is associated with the membrane's molecular weight cutoff (MWCO), but it is also important that dialysis unit should be in rotation to avoid localized equilibrium.

The figure 3.12 shows dialysis cassette used for the research experiments. These are the ready- to- use dialysis bag with defined MWCO. 10 minutes before the use, they were placed in the PBS buffers. The corners of the cassettes are re-sealable. The capacity of such bags was varied from 5 mL to 30 mL. The samples were injected from one side with the help of syringe. Extra precaution was taken not to pierce the membrane while injecting the samples. The entrapped air was removed from the bag with the help of syringe and then with the help of floating cups, the cassettes were placed in the beakers containing 1 L of 100mM PBS and a magnet bar. Beakers were placed over the magnetic stirrer at speed 1 at 4^0C for 3-4 hours. Then buffer was changed. After changing the buffer, the beakers were left for stirring at 4^0C overnight. The cassettes were removed from the beakers. Extra precautions were taken not to touch the membrane by any means to avoid external contaminations of the proteins. With the help of syringe, some air was injected first to the cassettes and then protein solution was sucked with the same syringe and transferred to a fresh clean tube. Then the proteins were stored until used.

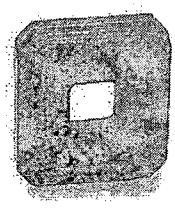


Figure 3.12 Dialysis Cassettes

3.8.1 Dialysis of Ag 85B and ESAT-6

3.8.1.1 Materials and Reagents

For Ag85B, Slide –A-Lyzer dialysis Cassette, MWCO 20 kDa, 0.5-3 mL capacity, Catalog number 66003 and for ESAT-6 , Slide –A-Lyzer dialysis Cassette, MWCO 3.5 kDa, 0.5-3 mL capacity, Catalogue number # 66330 were purchased from Thermo Scientific, USA.

Phosphate buffer Saline was prepared as discussed in the previous section.

3.8.1.2 Procedure for dialysis

The dialysis cassette was hydrated for 10 minutes in ultra pure water and fixed with floater. Protein solution was transferred in bag carefully with the help of syringe and needle (only corners are sealable), no leakage was ensured and then placed this packing to 1 L of PBS buffer and kept it in cold room under slow stirring. After 3 hours the buffer was changed and kept for stirring overnight. One more time the buffer was changed and the proteins were collected in cleaned tubes.

Both the proteins were then estimated for their concentration by BCA assay.

3.9 Estimation of Protein Concentration

There are various methods based on different principles applicable for protein concentration estimation. They are as follows:

Absorbance Assays

Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Secondary, tertiary, and quaternary structure all affect absorbance, therefore factors such as pH, ionic strength, etc. can alter the absorbance spectrum.

Absorbance assays are fast and convenient, since no additional reagents or incubations are required. No protein standard need to be prepared. The assay does not consume the protein. The relationship of absorbance to protein concentration is linear. Because different proteins and nucleic acids have widely varying absorption

characteristics *there may be considerable error*, especially for unknowns or protein mixtures. Any non-protein component of the solution that absorbs ultraviolet light will interfere with the assay. Cell and tissue fractionation samples often contain insoluble or colored components that interfere (Layne 1957).

Hartree-Lowry and Modified Lowry Protein Assays

The Lowry assay (1951) is an often-cited general use protein assay. The bicinchoninic acid (BCA) assay is based on the same princple and can be done in one step, therefore it has been suggested that the 2-step Lowry method is outdated.

Biuret assay

The principle of the Biuret assay is similar to that of the Lowry, however it involves a single incubation of 20 min. There are very few interfering agents (ammonium salts being one such agent), and Sargent (1987) reported fewer deviations than with the Lowry or ultraviolet absorption methods. However, the Biuret assay consumes much more material. The Biuret is a good general protein assay for batches of material for which yield is not a problem. The Bradford assay is faster and more sensitive.

Bradford assay

The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis.

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change (Bradford 1976, Zor 1996).

Bicinchoninic acid (BCA) assay

The bicinchoninic acid (BCA) (Smith 1985) assay is available in kit form from Pierce (Rockford, Ill.). This procedure is very applicable to micro-titer plate methods. The BCA is used for the same reasons the Lowry is used. It has been suggested that the

BCA assay will replace the Lowry because it requires a single step, and the color reagent is stable under alkaline conditions.

It is equally applicable to both for concentrated proteins and a micro assay for dilute protein solutions.

3.9.1 BCA assay

The macromolecular structure of protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA (Wiechelman. 1988). Studies with di-, tri- and tetra-peptides suggest that the extent of color formation is caused by more than the mere sum of individual color-producing functional groups.

3.9.1.1 Materials and Reagents

Micro BCA Protein Assay Kit, Catalogue number #23235 from Pierce, USA

Kit contains:

Micro BCA Reagent A (MA), 240 ml, Micro BCA Reagent B (MB), 240 ml, Micro BCA Reagent C (MC), 12 ml, Albumin Standard Ampules, 2 mg/ml, 10 × 1 ml ampoules containing bovine serum albumin (BSA) at 2.0 mg/ml in a solution of 0.9% saline and 0.05% sodium azide.

SpectraMax® $Plus^{384}$Absorbance Microplate Reader from Molecular Device, USA was used to read the microplate.

3.9.1.2 Procedure for Standard Curve

Manufacturer's guideline was followed for the preparation of standard curve for protein estimation. The BCA assay reagent was prepared freshly by adding 25 parts of Reagent A: 24 part of Reagent B and 1 part of Reagent C, as per the manufacturer's instruction.

The known concentration of Bovine Serum Albumin (BSA) was prepared as per the dilution shown in table 3.8. From these solutions, 150 µL of BSA solution was transferred in 96 well plate in triplicate order and in one well 150 µL of PBS was transferred (Blank). 150 µL of reagent was transferred to each well. Plate was kept on shaker incubator for 30 seconds to facilitate mixing of protein solution with reagent. Then plates were incubated at 37 °C for 2 hours.

Plates were removed from incubator and allowed to cool to RT and absorbance was measured at 562 nm with micro-plate reader.

Readings are shown as table 3.9 and graph for standard curve is shown in figure 3.13.

Vial	**Volume of Diluents**	**Volume and Source of BSA**	**Final BSA Concentration**	**Solution**
A	4.5 ml	0.5 ml of Stock	200 µg/ml	A
B	8.0 ml	2.0 ml of vial A dilution	40 µg/ml	B
C	4.0 ml	4.0 ml of vial B dilution	20 µg/ml	C
D	4.0 ml	4.0 ml of vial C dilution	10 µg/ml	D
E	4.0 ml	4.0 ml of vial D dilution	5 µg/ml	E
F	4.0 ml	4.0 ml of vial E dilution	2.5 µg/ml	F
G	4.8 ml	3.2 ml of vial F dilution	1 µg/ml	G
H	4.0 ml	4.0 ml of vial G dilution	0.5 µg/ml	
I	8.0 ml	0	0 µg/ml = Blank	H

Table 3.8 Preparation of BCA curve with Bovine Serum Albumin for standard solution

Concentration µg/ mL	Reading I Absorbance	Reading II Absorbance	Reading III Absorbance	Absorbance ± SD
200	2.5358	2.5016	2.657	2.56± 0.08
40	0.8764	0.8562	0.8608	0.86±0.01
20	0.5722	0.5539	0.5267	0.55± 0.02
10	0.3167	0.3993	0.3403	0.35± 0.04
5	0.2254	0.2464	0.2394	0.24± 0.01
2.5	0.1934	0.1825	0.1894	0.19± 0.005
1	0.161	0.1584	0.1544	0.16± 0.003
Blank	0.1414	0.147	0.1377	0.142± 0.004

Table 3.9 Calibration curve- Protein concentration estimation by BCA method

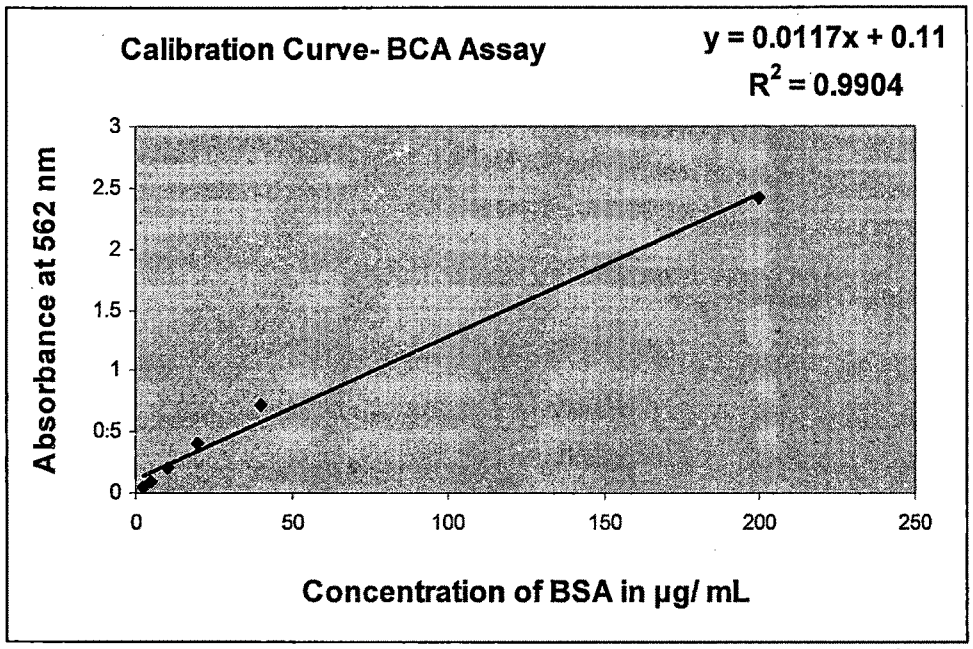


Figure 3.13 Calibration Curve- Micro BCA Assay for Protein Quantization

3.9.1.3 Results and Discussions

MicroBCA assay kit is useful for estimating protein concentration in microgram range. SDS-PAGE gel documents were indicating higher concentration of protein and hence 10 fold dilution was done before Micro BCA assay.

Once proteins were purified by dialysis, they were collected in a clean eppendorff. From each eppendorff, 15 µL of proteins were transferred in each well in triplicate manner and volume was adjusted to 150 µL with PBS. In three wells, PBS was added which was considered for blank. 150 µL of freshly prepared Micro BCA reagent was added and mixed well by keeping the plates on a rotor at 37^0C for 1 minute and allowed to incubate for 2 hours at 37^0C. Plates was removed from the incubator and allowed to cool to room temp. Absorbance was measured at 562 nm using microplate reader (Biotek, USA).

Table 3.10 shows the mean corrected absorbance and final concentration for each protein samples.

Unknown concentration of protein sample	Mean Corrected Absorbance ± SD*	Final Concentration (µg/ mL)
ESAT-6 Sample 1	0.940 ± 0.0677	700.9 ± 9.33
ESAT 6 Sample 2	0.445 ± 0.0276	286.6 ± 5.37
ESAT 6 Sample 3	0.970 ± 0.0412	735.0 ± 9.36
Ag 85 B Sample 1	1.530 ± 0.0312	1213.6 ± 10.45
Ag 85 B Sample 2	1.629 ±0.0712	1298.2 ± 9.53
Ag 85 B Sample 3	0.702 ± 0.0322	505.9 ± 9.87

*N = 3

Table 3.10 Estimation of Protein by BCA method.

The above protein solutions were divided in such a way that upon lyophillization they gave 1 mg of protein in dried form per eppendorff.

3.10 Protein (Antigen) activity determination

Enzyme-linked immunosorbent assay, also called **ELISA, enzyme immunoassay** or **EIA**, is a biochemical technique used mainly in immunology to detect the presence of an antigen or an antibody in a sample. In simple terms, in ELISA an unknown amount of antigen is affixed to a surface, and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal. Performing an ELISA involves at least one antibody with specificity for a particular antigen (Lequin 2005).

Types of ELISA	Specific Use
In Direct ELISA	Determine Antibody concentration
Sandwich ELISA	Detect and Estimate Antigen concentration
Competitive ELISA	Determine antigen/ antibody in crude/ impure samples
Reverse ELISA	simultaneous detection of different antibodies and different antigens for multi-target assays

Table 3.11: Types and specific use of ELISA

3.10.1 Materials and Reagents

96 well plates (Maxisorp) were purchased from Nunc, Denmark. Primary antibodies were gift sample from CSU, USA. Gelatine was purchased from Sigma, USA, Tetramethyl Benzidine (TMB substrate for ELISA) and Goat anti- rabbit IgG- HRP (secondary anti-antibody) from Bangalore Genei, India.

SpectraMax® Plus^{384}Absorbance Microplate Reader from Molecular Device, USA was used to read the microplate.

Preparation of Reagent

Antibody dilution was done in Radio Immuno Assay buffer (RIA).

RIA buffer was prepared by adding Bovine Serum Albumin to Phosphate buffer Saline (pH 7.4). Primary antibody was diluted in 1:1000 concentration (1 part in 1000 part of diluents) while secondary antibody was diluted in 1:2000.

0.5% Gelatin in 100 mM PBS (GPBS) was prepared and stored at RT.

0.025% PBST solution: 0.025% of Tween 80 was added to PBS (100mM and pH 7.4).

3.10.2 Antibody Dilution Assay

Antibody dilution assay was done to determine the efficacy of antigen binding to antibody. Standard calibration curve was prepared with antibody dilution curve. The experiments were conducted under the supervision of Dr. Anjali Karande, Professor, at Department of Biochemistry, IISC, Bangalore and following is the procedure.

In the Microtitre plate, antigen 1 μg in 100 μL PBS was coated and left overnight at RT. Wells were blocked with Gelatin PBS (GPBS) (0.5%) to let the antigen bind strongly and kept aside for 2-3 hours. Wells were washed once with 10 mM PBS to remove unbound antigen if any. Primary antibody (ab) 1:1000 prepared in RIA (Radio-Immuno Assay) buffer was added in a serial dilution and kept aside for 2-3-hours. Wells were washed with PBST for 3 times 3-minutes. Secondary antibody 1:2000 concentration in RIA buffer was added and kept aside for 1 hour (time is very important). Wells were washed with PBST and PBS each for 3 minute 3 times as above (Flood the plate with PBST/PBS taking care that all the wells are filled. After 3 minutes, discard the PBST/PBS, so that all the wells are dry and do not have large

amount of PBST/PBS). Tetramethyl Benzidine (TMB) was added as substrate 1:20 concentration prepared in milli Q that forms a complex with enzyme HRP and form a color (Blue color). The reaction was stopped by the addition of 1N H_2SO_4 (100 µL) (If HRP is the conjugate). The readings were taken in an ELISA reader at 450 nm.

The above procedure was followed for both (ESAT-6 and Ag-85B) the proteins. Respective polyclonal antibodies were used as primary antibody for ESAT-6 and Ag-85B. The data obtained from the antibody dilution are given in table 3.12 for ESAT-6 and for Ag-85B. The graphs plotted from the data are shown as figure 3.14 and figure 3.15.

In this case, we have kept antigen concentration constant in each well and concentration of primary antibodies was changing to 10 fold in each well. The purpose of performing antibody dilution curve is to get idea at which concentration of primary antibodies gave absorbance OD~ 1 for the given antigen concentration (1µg/100µL of PBS). That concentration of the primary antibodies determined this was used for the in-direct ELISA which was performed for the determination of protein activity in the following section. The initial concentration of primary antibodies used for the preparation of antibody dilution curve was 1:1000, i.e. 1 mg was added in 1 mL of RIA buffer. The absorbance observed at this concentration was defined as B_{max}. This concentration was then diluted serially (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128,1:256, 1:512, 1:1024). As shown in figure 3.14, for ESAT-6, at concentration of 1:1024, we observed OD~1. While for Ag-85B, as shown in figure 3.15, OD~ 1 was observed at 1: 256.

Sr. No.	Concentration of Primary antibody*	Mean corrected absorbance at 450 nm** (for ESAT-6)	Mean corrected absorbance at 450 nm** (for Ag-85B)
1	B max	3.09 ± 0.015	1.66 ± 0.009
2	1:2	2.85 ± 0.018	1.53 ± 0.012
3	1:4	2.67 ± 0.013	1.47 ± 0.011
4	1:8	2.44 ± 0.016	1.41 ± 0.015
5	1:16	2.25 ± 0.018	1.33 ± 0.016
6	1:32	1.98 ± 0.011	1.28 ± 0.014
7	1:64	1.76 ± 0.009	1.19 ± 0.013
8	1:128	1.58 ± 0.01	1.12 ± 0.012
9	1:256	1.39 ± 0.015	1.08 ± 0.009
10	1:512	1.22 ± 0.008	1.01 ± 0.008
11	1:1024	0.98 ± 0.009	-
12	1:2048	0.77 ± 0.008	-

Table 3.12: Antibody dilution curve for ESAT-6 and Ag 85B

*: Initially, the primary antibody was diluted to 1:1000 concentrations

**: Mean corrected value reflected the values after subtracting absorbance of the blank sample (just 100mM PBS).

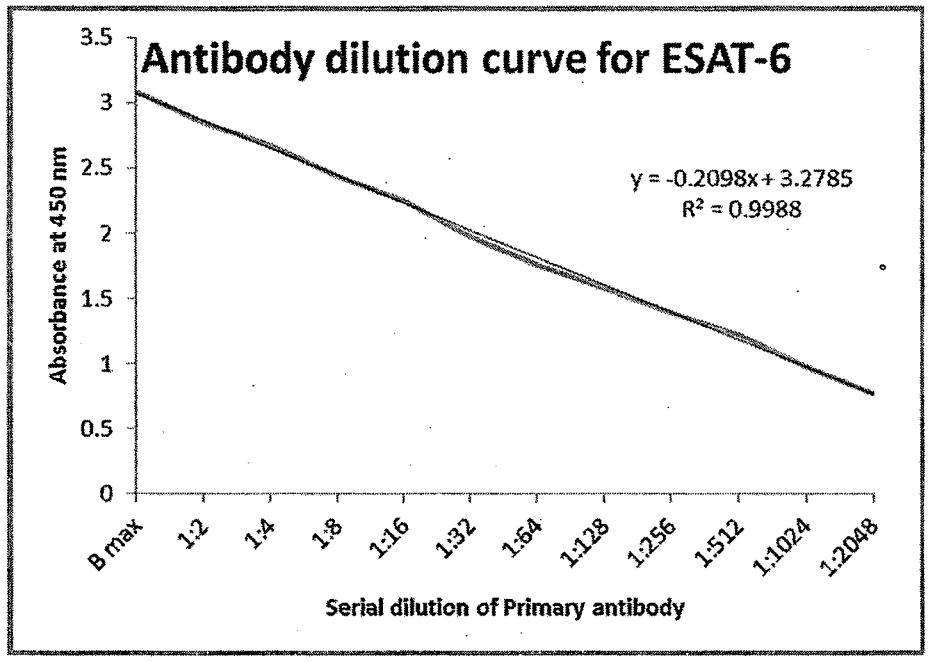


Figure 3.14 ESAT-6 Antibody dilution curve

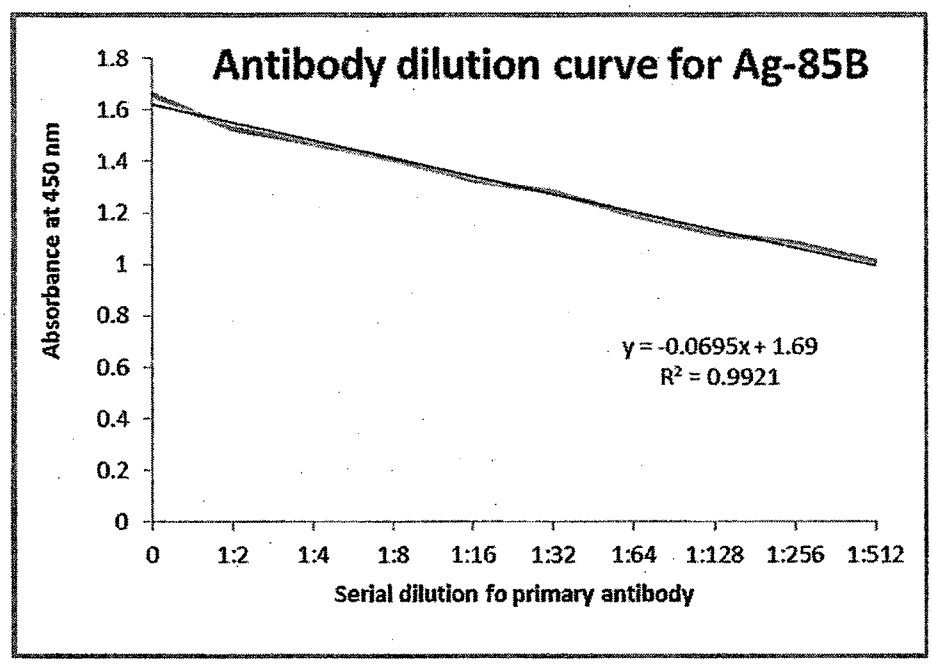


Figure 3.15 Ag-85B Antibody dilution curve

3.10.3 Determination of Protein Activity

The protein collected after dialysis, were estimated for its concentration and antigenicity. The comparison of protein activity was made with the standard proteins provided by CSU, USA.

100 µL solution of 0.1µg/mL in 100 mM PBS buffer (pH 7.4) of both proteins (ESAT-6 and Ag 85B) were transferred to 96 well plates in triplicate. The plates were incubated overnight (about 12-14 hours). The wells were blocked with 0.5% Gelatine in PBS. The plate was left aside for about 2-3 hours to allow antigen to bind with the surface of well. The content was discarded and washed with 100mM PBS solution once. 100 µL of respective primary antibodies were transferred to proteins ESAT-6 and Ag 85 B. The concentration of primary antibodies required here was determined from the antibody dilution curve (as shown in figure 3.14 for ESAT-6 and 3.15 for Ag-85B). It was 1:1024 dilutions of 1:1000 concentration of the polyclonal antibody for ESAT-6 and 1:256 dilutions of 1:1000 concentrations of the polyclonal antibodies of Ag85B.

The plates were left for reaction for about 3 hours. Then the plates were washed 3 times each with PBST and PBS (100mM, pH 7.4) alternately at the interval of 3 minutes.

The wells were treated with 100 µL of secondary antibody (Goat Anti Rabbit HRP 1:2000 dilution) and kept for 1 hour (time for reaction is very crucial. Again the plates were washed 3 times each with PBST and PBS (100mM, pH 7.4) at the interval of 3 minutes. Then TMB (1:20 dilution in ultra pure water) 100 µL was added to each well, the blue colour should develop in 10-15 minutes. To stop the reaction, 100 µL 1M of H_2SO_4 was added, yellow colour developed upon adding this solution and absorbance was measured at 450 nm. Blank was also done where wells were not coated with antigens and remaining all steps was done simultaneously.

3.10.4 Results and Discussions

To know the antigenicity of lab produced protein is very important criteria. Several times, if the conditions are not maintained well during the protein preparation, the protein de-folding occurs and antigenic activity of protein gets compromised. Proteins which have compromised antigenicity have no use in the rest of the research work. Therefore, antigenicity of each protein ESAT-6 and Ag-85B were compared with the standard proteins given by CSU, USA. 10 µg of each of proteins were coated in Nunc Maxisorb ELISA plates in triplicate manner and parallel to this, standard proteins were also coated similarly. The protein activity was determined by Sandwich ELISA as explained in 3.11.3. Average values of absorbance were calculated and percentage of protein activity was calculated with respect to values obtained with standard protein. The antigenicity of individual protein synthesized at laboratory was 96.667% for ESAT 6 and 99.89% for Ag 85 B.

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