CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

Restriction enzymes, Taq polymerase and Hibond membranes were procured from Amersham Pharmacia Biotech Asia Pacific, Hong Kong. Primers were synthesised from Integrated DNA Technology Inc., (IDT), Lexington, USA. Chemicals of analytical grade like Agarose, Acrylamide, bisacrylamide, Urea, PEG-8000 and others were purchased from Sigma Chemical Company, St Louis, USA and Merck, Germany. Antibiotics like Ampicillin was purchased from Ranbaxy, Delhi, India and Kanamycin was from Allembic Chemicals, Ltd., Baroda, India. Chemicals from Himedia, Mumbai, India were used for media preparation for E. coli culture. For large-scale purification of plasmids Gigaprep kits were purchased from Qiagen, USA or Genomed, Germany. For endotoxin removal Pierce Deotoxi gel was used. For endotoxin assay E-toxate kit (Limulus amebocyte lysate assay, Sigma, USA) was used.

Most of the immunological reagents used in this study were either from Pierce, USA or from Sigma, USA. Complete and incomplete adjuvant used for immunisation study were purchased from Pierce, USA. The microtitre plate and reagents like secondary antibody were purchased from Pierce, USA. Some of the immunological reagents were also procured from Sigma, USA and
Bangalore Genei, India. The isotype ELISA was done with Pierce isotyping kits (Pierce, USA).

For Splenocyte proliferation assay, NUNC 96 wells flat bottom sterile tissue culture plates were used. DMEM, RPMI 1640 and fetal calf bovine sera were obtained from ATCC, USA and Gibco BRL, USA. For splenocyte proliferation assay, CellTiter 96® Aqueous non-radioactive cell proliferation assay, manufactured by Promega, USA was used. For estimation of Cytokine concentration Pierce Endogen Kit was used.

For micropore chamber protection study 3-mm ring, Millipore Filtration (MF) cement and durapore membranes were purchased from Millipore, USA.

2.2 BACTERIAL STRAINS, PLASMIDS

The T7 expression vector, pRSETB, and *E. coli* strains DH5α, BL21 (DE3) and BL21 (DE3) pLysS were from Invitrogen, Sandiego, USA. BLT 5403 *E. coli* was obtained from Novagen USA. The DNA vaccine vector Vrl020 (Vical, CA, USA) was a kind gift from Dr. J. S. McCarthy, University of Western Australia, Perth, WA, Australia. Genotypes of the *E. coli* strains used in the present study are given in Appendix 1. Maps of the plasmid vectors pRSETB and Vrl020 and T7 phage display vector are shown in Appendix 2.

2.3 ANIMALS USED

Animal experiments to study the immunoprophylactic efficacy of protein and DNA vaccines were carried out in collaboration with University of Illinois, Rockford. Male outbred Mongolian gerbils (jirds) weighing 35-40 gms and six weeks old inbred BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Animals were moved to the laboratory on the
day of the experiment and maintained under standard conditions with food and water at the animal house facilities of College of Medicine, University of Illinois, Rockford.

2.4 PARASITES

L3 larvae of *Brugia malayi* were obtained from Filarial Repository, Universities of Georgia, Athens, GA, (WHO supply). The L3 larvae were washed extensively and resuspended in RPMI 1640 and incubated in 37 °C CO₂ incubator. The viability of L3 larvae was checked by their mobility under microscope.

2.5 CULTURE MEDIA

Luria-Bertani (LB) broth was the most commonly used media for growing *E. coli*. To prepare LB broth, 10g of tryptone, 5g of sodium chloride and 5 g of yeast extract were dissolved in 1L of distilled water and the pH was adjusted to 7.2 with 1N NaOH. 1.5% agar was added to the liquid broth to prepare solid medium. Media was supplemented with 100mg/ml of ampicillin or 50mg/ml of kanamycin or 35mg/ml of chloramphenicol wherever required.

2.6 RECOMBINANT CLONES USED IN THE PRESENT STUDY

The *B. malayi* ALT-2 gene was identified by immunoscreening the *B. malayi* L3 cDNA library using sera from putatively immune individuals residing in an area endemic for Onchocerciasis (James McCarthy, Personal communication). The recombinant plasmid containing BmALT-2 gene in pBLUESCRIPT vector was kindly provided by Dr. James McCarthy, Australia. Dr. Sabarinathan used the above plasmid as template for subcloning the gene in T7 expression vector (pRSETB) and DNA vaccine vector (1020) (Sabarinathan 2000). The pRSET-B containing the ALT-2 for expressing the recombinant
protein is designated as pRSBmALT-2 and the DNA vaccine construct of ALT-2 was designated as 1020BmALT-2. The nucleotide sequence of BmALT2 is given in the appendix 5.

Dr. Raja Rao and Dr. Gnanasekar cloned the *Brugia malayi* L3 λ ZAP cDNA library in T7 bacteriophage display system (Gnanasekar 2001). Biopanning of the library with endemic normal (EN) sera led to the identification of three antigens 'BmALT-2, BmVAH and BmTPX-2' displayed on the surface of the bacteriophage. These clones were of partial length as the genes had an internal Hind III site. The T7 Phage displaying partial ALT-2 with N terminal 72 amino acids was used for the vaccination study.

2.7 EXPRESSION OF THE RECOMBINANT PROTEINS IN *E.COLI*

The recombinant gene constructs pRSBm-ALT-2 was maintained in *E. coli* host DH5α that is deficient of T7 RNA polymerase and hence does not express the foreign protein. A T7 gene expression vector system, pRSET B based on T7 RNA polymerase (Studier and Moffatt 1986) was employed in the present study to clone and express the recombinant constructs in T7 expression host BL21 (DE3) pLysS. Transcription by T7 RNA polymerase is selective and 5 times faster than *E. coli* RNA polymerase thus leading to higher expression of genes cloned under T7 promoter. The vector also contains a nucleotide sequence that encodes a metal binding domain, a series of six consecutive histidine amino acids expressed as N-terminal fusion to the protein of interest. The histidine tag facilitates the easy purification of the recombinant protein by metal affinity chromatography (Crowe et al 1995). Moreover the histidine tag is found to be non-immunogenic due to its small size. Such histidine tagged recombinant proteins can be used in immunological studies. The other
advantages of the vector include its small size, presence of multiple cloning site and ampicillin resistance marker. The availability of these vectors in three possible reading frames A, B, C gives the researcher the choice of selecting vector that maintains the right reading frame of their insert.

BL21 (DE3) *E. coli* strain contains a chromosomal copy of T7 RNA polymerase gene under the control of *lac* UV5 promoter and hence expression of genes under the control of T7 promoter can be induced with the gratuitous inducer IPTG at 1 mM concentration. Further, BL21 (DE3) being a *lon* protease deficient strain protects the expressed heterologous proteins from proteolytic cleavage.

Briefly the following protocol was used for expression of the recombinant proteins (LB was supplemented with 100 μg/ml of ampicillin and 35 μg/ml of chloramphenicol used for all including O/N cultures).

a) *E.coli* BL21 (DE3) pLysS was transformed with pRSBmALT-2 gene constructs.

b) A single colony of fresh transformant was inoculated into 1.5 ml LB and grown overnight at 37°C in water bath shaker.

c) 200 μl of the overnight culture was inoculated into 200 ml LB in 2000 ml conical flask and grown at 37°C with 150 rpm shaking, till OD\text{600} of the culture reached 0.7.

d) IPTG was added to a final concentration of 1 mM and the culture was grown for 3 hours at 37°C with 150 rpm shaking.
The culture was centrifuged at 10,000g for 5 minutes. The supernatant was discarded and the bacterial pellet containing the recombinant protein was stored at -20°C until further use.

2.8 PURIFICATION OF Bm-ALT2 PROTEINS USING IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC)

The recombinant protein was expressed with 6 histidine residues as an N-terminal fusion peptide. The metal binding domain in the fusion peptide allows simple one step purification of recombinant protein by IMAC. The recombinant proteins was expressed as inclusion bodies, hence the proteins were purified under denaturing conditions (8M Urea).

Briefly the following protocol was adopted for purification:

i) Cells were harvested by centrifugation at 10,000g after induction with IPTG for 3 hrs.

ii) The cell pellet was solubilised with binding buffer (0.1M Phosphate buffer pH 8.0. 0.01 M Tris pH 7.5 and 8M Urea) overnight at 4°C on a rocker.

iii) The column was equilibrated with 3 column volumes binding buffer (pH 8.0). Samples were applied to the column (5mg of recombinant protein per 1 ml of NiNTA column), allowed to bind to the NiCl₂ charged NiNTA column (Pharmacia, USA).
iv) Column was washed with solubilisation buffer (pH 7.5), followed by elution with increasing concentrations of Imidazole (10 - 150 mM) to remove all contaminating proteins.

v) The protein was eluted at 500-mM imidazole concentration. The purity of the protein was checked on SDS-PAGE. After purification the sample was dialysed against 0.1X PBS and then concentrated by Vacuum concentrator. The concentration of the purified recombinant ALT-2 was estimated by Lowry method and stored at −80°C in aliquots till further use. Before vaccination studies the protein concentration was again estimated using BCA Kit (Pierce Chemicals, Rockford, USA).

### 2.9 LARGE-SCALE PRODUCTION OF THE DNA VACCINES

The recombinant *E. coli* containing the DNA vaccine plasmid 1020BmALT-2 were grown in 500ml of Terrific broth media supplemented with 50 μg/ml of kanamycin. A single colony of the recombinant *E. coli* was grown in 50ml at 37°C with shaking till the OD₆₀₀ reached 0.6. This growing culture was used to inoculate the 500ml media in a Haffkine flask.

To circumvent the problem of instability and to produce the DNA vaccine plasmid in large scale the culture was pulsed with 50μg/ml of kanamycin after every three hours. Once the OD₆₀₀ of the culture stabilised, the cells were harvested by centrifugation (7000 rpm 15 minutes) and used for extraction of the plasmid using Genomed gigaprep kit as per manufacturers’ instructions. Briefly the following protocol was used for large-scale isolation of plasmid DNA as per the instructions manual (Genomed, Germany).
a) Harvesting of Bacterial cell: *E. coli* cell from 1 litre culture were pelleted by centrifugation at 7500g for 10 minutes. All the media was removed carefully.

b) Cell Resuspension: 160 ml of E1 buffer (50 mM Tris HCl, 10 mM EDTA + RNase 0.1 mg/ml) was added to the pellet and resuspended until the suspension was homogeneous and no cell clumps were visible.

c) Cell lysis: The bacterial cells were lysed by adding 160 ml of E2 buffer (200 mM NaOH, 1% SDS). The solution was mixed gently but thoroughly until a homogeneous lysate was obtained. Incubated at room temperature for 5 minutes.

d) Neutralisation: The above lysis mix was neutralised by adding 160 ml of E3 buffer (3.2M Potassium acetate, pH5.5), mixed gently but thoroughly until a homogeneous mixture was obtained and centrifuged at 13,000 X g at room temperature.

e) Equilibration: The GIGA catridge was screwed into 45 mm DIN thread (Duran\textsuperscript{R}) bottle and 100 ml of E4 buffer (600 mM NaCl, 100 mM Na Acetate/acetic acid (pH-5.0), 0.15% Triton X100) was filled. The Jetstar 2.0 anion exchanger column material was stirred with a jet stick, until a homogeneous suspension was obtained. The resin was allowed to settle for 2 minutes and the supernatant was sucked off by applying vacuum to the catridge through the side arm with tubing connector. The flow through was discarded.
f) Loading the lysate: The cleared lysate obtained from step D was poured over the resin and the resin was suspended with the jet stick till homogenisation. The resin was allowed to settle for 5 minutes and it was repeated again. Applying vacuum sucked off the whole supernatant.

i) Wash 1: 300 ml of buffer E5 (800mM NaCl, 100mM Na Acetate/ Acetic acid, pH 5.0) was filled into the cartridge and the resin was resuspended homogenously by stirring. The resin was allowed to settle for 3 minutes and was siphoned off. The flow through was checked for the presence of DNA and was discarded as no DNA was present.

ii) Further wash step: The above wash step was repeated to remove impurities like proteins, degraded RNA, metabolites and dyes completely.

iii) Plasmid Elution: The filter cartridge was taken out and screwed on to a sterile 100 ml 45mm DIN thread Duran bottle. The resin was resuspended in 75 ml of buffer E6 (1250mM NaCl, 100 mM Tris-HCl, pH 8.5). The resin was stirred and allowed to settle. The supernatant was sucked off completely by applying vacuum. A second elution was also performed in order to ensure good yield of plasmid.

iv) Plasmid precipitation: The eluate was divided into different centrifuge tubes (Corex tube, Dupont sorvall, USA) and 0.7 volumes of isopropanol was added and mixed thoroughly and kept at room temperatures for 30
minutes. The above solution was centrifuged at 13000g for at least 30 minutes.

v) Washing precipitate: The precipitated DNA was washed with 10 ml of 70 to 80 % ethanol to remove the salt contamination. The pellet was allowed to dry in a 37°C oven.

The dried pellet was resuspended in sterile TE / DD H2O. The DNA was checked by restriction digestion and PCR using gene specific primers for the presence of insert. The concentration and purity of DNA was assessed by checking the ratio of absorption at 260 and 280 nm. The purified DNA was checked for endotoxin contamination by LAL assay. The plasmid DNA was stored in -80°C till the vaccination study.

2.10 PREPARATION OF T7 PHAGE DISPLAYED ALT2

Larger amount of phage is grown conveniently and purified from 200 ml of lysate, which can be accommodated in a single polypropylene centrifuge bottle. The Phage displayed antigen preparation was carried out as per the instructions manual of Novagen, USA.

a) BLT5403 E. coli obtained from Novagen, USA was grown overnight in 2.5 ml LB media supplemented with 50 μg / ml of ampicillin.

b) To 200 ml LB media supplemented with 50 μg / ml of ampicillin in a 1 litre Erlenmeyer flask, an inoculum of 1 ml of O/N grown E. coli was added and grown at 250 rpm at 37°C until the OD_{600} reached 0.3 to 0.4.
c) The culture was infected by adding 0.02ml of high titre T7 Phage display lysate and continued to grow at 37°C until lysis was observed. This took around one to one and half hours.

d) 5g of NaCl was added to the centrifuge bottle containing phage lysate and centrifuged at 800 rpm for 10 minutes at 4°C.

e) The supernatant was transferred into another centrifuge bottle containing a stir bar. Slowly 20g of PEG 8000 was added at room temperature till the PEG is dissolved. The lysate-PEG mixture was stored at 4°C O/N.

f) The lysate PEG mixture was centrifuged at 8000 rpm for 10 minutes at 4°C. The supernatant was decanted and the pellet was drained by inverting the bottle on paper towel for few minutes. The inside of the bottle mouth was wiped with tissue paper to remove as much liquid as possible.

g) The PEG pellet was resuspended in 1.5 ml of 1M NaCl, 10mM Tris-Cl, pH8.0, 1mM EDTA.

h) The above phage preparation was dialysed overnight with PBS for removing PEG 8000.

The purified phage protein was passed through polymyxin B-agarose column (Detoxi-gel, Pierce) to eliminate endotoxin. The recombinant Phage was checked for the presence of ALT2 insert by PCR with T7 phage vector specific forward primer and gene specific reverse primer. The protein concentration was estimated by Pierce BCA kit as per the instruction manual, checked for endotoxin contamination by LAL assay, stored at -80°C until the vaccination studies.
2.11 ENDOTOXIN ASSAY

Detection of possible endotoxin contamination in Protein, DNA and T7 phage displayed antigens were performed by a standard E-toxate kit (Limulus amebocyte lysate assay, Sigma).

a) Briefly, standard concentrations of endotoxin (10 pg to 1mg) and working concentration of samples were added to vials followed by the addition of corresponding amounts of endotoxin-free water. 0.1 ml of E-toxate working solution were added to the vials and the contents were mixed gently and incubated for one hour at 37 °C.

b) After incubation the vials were removed and slowly inverted 180 degrees while observing evidence of gelation. A positive test is the formation of a hard gel and all other results were considered negative. All the vaccine preparation in the form of Protein, DNA and T7 phage displayed antigen gave no observable gelation, indicating that the above preparations used in this study were endotoxin-free.

2.12 TRANSIENT TRANSFECTION OF CHO CELL LINE BY DNA VACCINE CONSTRUCT OF BmALT-2

The CHO cell line cryopreserved and maintained by Tissue culture Laboratory, Centre For Biotechnology, Anna University was used for the transient transfection of DNA vaccine construct of BmALT-2 to check for its expression. CHO Cells were transiently transfected using Lipofectamine reagent (GibcoBRL/Life Technologies, Gaithersberg, MD) as described by the manufacturer.
Methods

a) In a six-well or 35 mm tissue culture plate, \( \approx 2 \times 10^3 \) cells were seeded per well in 2 ml of DMEM medium containing 10% FBS and supplemented with 50 \( \mu \)g/ml gentamicin.

b) The cells were incubated at 37°C in a CO\(_2\) incubator until the cells are 70-80% confluent. This usually took around 18-24 h.

c) The following solutions were prepared in sterile 2.0 ml eppendorfs.

Solution A: For each transfection, 2 \( \mu \)g DNA (plasmid) was diluted in 375\( \mu \)l serum-free DMEM medium without gentamicin.

Solution B: For each transfection, 12 \( \mu \)l LIPOFECTAMINE reagent was diluted in 375\( \mu \)l serum-free medium.

d) The above two solutions were mixed gently and incubated at room temperature for 15-45 min.

e) The cells were washed once with 2 ml serum-free medium.

f) For each transfection, 750\( \mu \)l serum-free medium was added to each tube containing the lipid-DNA complexes. Mixed gently and overlaid on the washed cells.

g) The cells were incubated for 5 h at 37°C in a CO\(_2\) incubator.

h) 1.5 ml medium with 20% FBS was added without removing the transfection mixture.

i) Medium was replaced every 18-24 h following start of transfection.

j) The cell extracts were assayed for gene expression by RTPCR during 24-72 h after the start of transfection, depending on cell type and promoter activity.
The transfected cells were harvested after different time points like 24, 48 and 72 hours. The cells were transfected with a positive control plasmid pEGFPN3 (Clonetech, USA), which contains a CMV promoter and expresses green fluorescent protein. RNA was extracted from cells by using RNAZOL and converted into cDNA using MMLV Reverse Transcriptase (Bangalore, Genei) by standard protocols. The cDNA was checked by the presence of message level of BmALT-2 gene in the transfected CHO cell by doing PCR with ALT-2 gene specific forward and reverse primers. The level of β-actin m-RNA (a house keeping gene) was simultaneously assayed as an internal control.

2.13 TISSUE DISTRIBUTION STUDY

100μg of DNA vaccine was injected intramuscularly and the animal was sacrificed by cervical dislocation at different time points viz. 5 hrs, 24 hrs, 7 days and 14 days after DNA vaccine administration. Peritoneal exudate cells were collected by methods described earlier. Different organs and cell types like muscle, spleen, kidney, liver, heart, colon and peritoneal exudate cells were isolated from each mouse and around 100 mg of tissues were taken for isolating the DNA and RNA as described below. The DNA and cDNA from different tissues at different time points were subjected to PCR amplification with BmALT-2 gene specific primers for studying the distribution and \textit{in vivo} expression of DNA vaccine construct of BmALT-2. The level of expression at different time points were evaluated based on the band intensity of the PCR amplified product.

2.13.1 DNA Isolation From Different Tissues

A piece of tissue (100mg) was homogenized with 200μl low salt buffer (10mM Tris HCl, pH 7.6; 10mM KCl, 10mM MgCl$_2$ and 2mM EDTA – TKM1) and transferred to a 1.5ml Eppendorf tube. To this 10μl of Nonidet P-
40 (NP-40, Sigma) was added to lyse the cells and mixed well by inversion several times. The mixture was then centrifuged at 10000 rpm for 10 min at room temperature. The supernatant was discarded and pellet was washed with TKM1 buffer and centrifuged as before. The pellet was resuspended in 200μl of high salt buffer (10mM Tris HCl, pH 7.6; 10mM KCl, 10mM MgCl2 and 2mM EDTA, 0.4 M NaCl – TKM2). To the mixture 15μl of 10% SDS was added. Then mixed the whole suspension thoroughly by pipetting back and forth several times, and incubated for 10 minutes at 55°C, after which 125 μl of 5mM NaCl was added and mixed well. Then the mixture was centrifuged at 12000 rpm for 5 minutes and the supernatant containing DNA was collected. The DNA was recovered by ethanol precipitation and dried. The dried DNA was resuspended in 20 μl TE buffer.

2.13.2 RNA Extraction from Tissues

Isolation of RNA was carried out with adequate precautions to eliminate RNase activity. All glasswares and plasticwares were treated with DEPC (Diethyl pyrocarbonate), which inactivates RNase by covalent modification. All the glasswares, plasticwares and solutions were autoclaved and baked at 80°C for three hours. Gloves was used for performing all the experiments. RNA was isolated by following the methods described by Chomczynski and Sacchi (1987). Briefly,

a) 100mg of tissue was homogenized with 600 μl of solution D [5 ml of stock solution [Guanidium isothiocyanate 4M, Sodium citrate (pH 7.0) 25Mm,Sarcosyl 0.5% w/v, β-mercapto ethanol 0.1M, Phenol: chloroform 5:1] and transferred into a micro centrifuge tube. Reagents like 60 μl 2M Sodium acetate, 600 μl of Phenol (water saturated), 80 μl of Chloroform: Isoamyl
alcohol (24:1 v/v) were added to the homogenate one after other by mixing thoroughly by inversion.
b) The above solution was centrifuged at 10,000g for 20 minutes at 4°C.
c) Aqueous phase was removed and 1ml of isopropanol was added and placed at –20°C for one hour to precipitate RNA.
d) RNA pellet was resuspended in 70% ethanol for removing the salt.
e) The above pellet was dissolved in 50μl of 0.5% SDS at 65°C for 10 minutes.
f) This above preparation was dissolved in DEPC treated water and the concentration was estimated by taking the absorbance at 260nm.
g) After the isolation of RNA, 1μg of RNA was converted to cDNA by using Reverse transcriptase.

2.14 IMMUNIZATION OF MICE AND GERBILS

2 μg of protein and 200μg of T7 PDALT2 was immunised intraperitoneally and 100μg of plasmid DNA vaccine was injected intramuscularly for the vaccination studies in the mouse model. 6 mice and 5 gerbils were used per group. For gerbil 10μg of protein, 200 μg of T7PDALT2 was immunised intraperitoneally and 150μg of plasmid DNA vaccine was injected intramuscularly. For the vaccination studies the protein was first emulsified with complete adjuvant and successively with incomplete adjuvant for three times and final dose was given without adjuvant. For T7 PDALT2 and DNA vaccine construct no adjuvant was used. The immunisation and bleeding schedule are described in Figures 2.1 and 2.2.
Figure 2.1 Immunization and bleeding schedule for micropore chamber protection study in mice

Figure 2.2 Immunization and bleeding schedule for micropore chamber protection study in gerbils
2.15 PROTECTION STUDY BY MICROPORE CHAMBER METHOD

Protection study was done by micropore chamber method as described by Abraham et al (1993) and Chenthamarakshan et al (1995). For protection studies, 6 mice or 5 gerbils were taken in each group. The animals were immunised with specified amount of different antigen preparation as mentioned above. The animals were bled by retro orbital puncture one day prior to immunisation. One week following the booster immunisation, mice in all groups were challenged by implanting a micropore chamber intraperitoneal containing 10 *B. malayi* L3 larvae in RPMI 1640 media. The micropore chambers were fashioned out of rings (13 mm outer diameter, 9 mm inner diameter, 2 mm thick), cut from Lucite (poly methyl methacrylate) tube and fire polished. A 13-mm diameter durapore membrane of 5 μm pore size (Millipore, USA) was bonded at each end with MF cement (Millipore). Chambers were sterilised by keeping in 80°C oven for 8 hours. The chambers were loaded, under a dissecting microscope, with larvae in media via a 200μl pipette tip through a hole in the wall of the Lucite ring, which was subsequently sealed by MF Cement (Millipore, USA). Mice were anaesthetised with sodium pentobarbital, and the chambers with live L3 inserted through a small incision of 3 mm in the peritoneum. After placing the micropore chamber the peritoneum was stitched with surgical suturing needle. Strict aseptic conditions were followed for surgical procedures. Betadine and Neosporin were applied to the peritoneum of each animal. Housing, handling and treatment of mice was performed in accordance with NIH guidelines.

For micropore chamber experiments 10 live L3 for mice and 20 live L3 for gerbils were inoculated in the micropore chamber. Each mouse and gerbil was kept in separate cages and after 48 hours the micropore chamber was harvested. Each micropore chamber was placed in a well of 24 well tissue
culture plate containing RPMI media. The numbers of live and dead L3 enumerated by observing movement of larvae under a Nikon microscope. The tissue culture plate containing the micropore chamber was kept at 37°C for 30 minutes and counted again to see the live and dead L3. The data obtained was statistically analysed by performing a chi square test. The % of protection was calculated using the following formula:

\[
\text{% Protection} = \frac{\text{Number of dead larvae}}{\text{Total number of recovered larvae (live + dead)}} \times 100
\]

2.16 ANTIGEN SPECIFIC ANTIBODIES IN ANIMAL MODEL

The 96 wells high binding polystyrene, non-sterile, flat bottom plates were coated with purified BmALT-2 recombinant antigen at a concentration of 250 ng/well in 50μl coating buffer (NaHCO₃/Na₂CO₃ 0.067 M pH 9.6) and incubated at 4°C for overnight.

The plates were washed in PBS containing 0.05% Tween 20 and blocked with 250μl of 5% BSA, incubated at 37°C for 2 hrs.

After further washes, 50μl of different dilutions of sera and peritoneal fluids from different groups of vaccinated mice or gerbils were added to the wells and incubated at 37°C for 2 hours. For protein and PBS immunised mice the dilution of sera used were 1:250, 500, 1000, 2000, 4000, 8000, 16000, 32000 of pre-immune sera. For PD ALT-2 and PD Vector immunised animal the dilution of sera were 1:75, 125, 250, 500, 1000, 2000, 4000, and sera for 1020 ALT2 and 1020 vector alone and DNA Vaccine construct of ALT2 immunised animal a dilution from 1:25, 50, 100, 200, 400, 800, 1600 was used.
For peritoneal fluid also different dilutions were used to find out the antibody titre.

The plates were washed with PBST and incubated with goat anti-mouse IgG horseradish peroxidase for study with mice at 37°C for 2 hrs. For gerbils study Protein-A conjugated to HRP was used as secondary antibody, since there is no commercially available secondary antibody.

The plates were washed with PBST, Diaminobenzidene (Sigma USA) substrate was added and colour developed was measured at 405 nm using a micro plate ELISA reader (BIO-TEK EL311sx).

The antibody titre was defined as the lowest dilution of antibodies to bind significantly to antigen, or, alternatively the dilution of antibody that gives a half maximal binding to antigen.

2.17 WESTERN BLOT ANALYSIS BY SUPERSIGNAL CHEMILUMINESCENT KIT

In order to check the immunoreactivity of sera and peritoneal fluid of vaccinated mice with ALT-2 pure protein, 10 μg of pure BmALT-2 Protein was run in a SDS PAGE gel. The gel was transferred to NCP membrane by standard method as described in the general method. For the development of blot, 1% BSA in PBS, 0.1% Tween 20 was added to the blot along with the primary antibody (1:20,000 for protein vaccinated mice / gerbils, 1:25 for DNA vaccinated mice/gerbils, 1: 100 dilution for T7 Phage displayed vaccinated sera of mice/gerbils/crude peritoneal fluids of all the above vaccinated mice/gerbils)
and rocked gently at room temperature for 1 hour. The blot was washed 10 times with PBS for 5 minutes each and was incubated with secondary antibody (1:10,000) in 1%BSA in PBS, 0.1% Tween 20 to hybridise for 1 hour at room temperature. For gerbils protein conjugated to HRP at 1:10,000 dilution was used. Later 10 washes were given with PBS and the membrane was exposed to a mix of solution1 and solution 2 of Super signal kit obtained from Pierce, USA. The membrane was then exposed to Kodak O-mat films and developed in automated X-Ray developer.

2.18 ELISA FOR THE ASSAY OF ISOANTIBODIES IN VACCINATED MICE

The isotype antibody detection was done by Pierce Isotyping kit as per the manufacturer instructions (Pierce, USA). Biotek Elisa reader was used for reading the ELISA plates. For isotype antibody distribution study with the peritoneal fluid, instead of sera peritoneal fluid was used and rest of the steps were similar. Briefly,

a) The flat bottom well of high binding polystyrene ELISA plate (Pierce, USA) was coated with 50 μl of coating buffer containing 250ng of pure recombinant BmALT-2 and incubated at 37°C incubator for 2 hours / at 4°C for O/N.

b) The solution was removed and blocked with 125 μl of the 1x blocking solution.

c) The solution was removed and washed with 4x 125 μl of wash buffer and 50μl of optimal diluted sera from vaccinated mice from different groups / pooled peritoneal fluid were added as per the Table 2.1 and incubated at 37°C for 1 hour.
d) The solution was removed and washed with 4x 125 µl of wash buffer. Separately one drop (50µl) of subclass specific antimouse immunoglobulins (IgG1, IgG2a, IgG2b) was added and incubated at 37°C for 1 hour.

e) The solution was removed and washed with 4 x 125µl of wash buffer. To this 50µl of horse radish peroxidase conjugated goat anti rabbit IgG was added and incubated at 37 °C for 1 hour.

f) The solution was removed and washed with 4x 1x wash buffer. 100 µl of 1X ABTS substrate solution [2,2-azino-di-(3-ethyl benzthiazoline sulfonic acid] was added to each antigen coated well.

Table 2.1 Dilution of sera used for assay of isoantibodies in vaccinated mice

<table>
<thead>
<tr>
<th>Name of Vaccinated sera</th>
<th>Optimal dilution of sera used for isotype ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>1:500</td>
</tr>
<tr>
<td>Recombinant Bm ALT-2 Protein</td>
<td>1:500</td>
</tr>
<tr>
<td>T7 Phage displayed vector</td>
<td>1:250</td>
</tr>
<tr>
<td>T7 phage displayed ALT-2</td>
<td>1:250</td>
</tr>
<tr>
<td>1020 (DNA vaccine vector alone)</td>
<td>1:50</td>
</tr>
<tr>
<td>1020ALT2 (DNA vaccine construct of ALT-2)</td>
<td>1:50</td>
</tr>
</tbody>
</table>

The above plate was incubated at room temperature and colour development was monitored for half an hour. The result was read quantitatively by a ELISA reader at 405nm.
2.19 PERITONEAL FLUID AND CELL PREPARATIONS

After removing the micropore chamber, the peritoneal cavity was washed with 10 ml of RPMI media. The peritoneal wash fluid was then centrifuged at 1500 rpm for 5 minutes in a refrigerated centrifuge and the peritoneal cells were separated. The peritoneal fluid was then kept in -80°C for further analysis by ELISA and western blot for checking the antibody titre. The cell pellet was then resuspended in 500 µl of media. After counting the total cell number using a haemocytometer, cytospin smears (of 50 - 100 µl samples) were prepared in a Cytopro cytocentrifuge (Wescor Inc., Logan, Utah, USA). The cell smears were fixed in methanol and successively stained with eosin and methylene blue using a Leukostat staining kit (Fisher chemicals, Pittsburg, USA). A differential count was made under a Nikon microscope and the percentage of each cell type (neutrophils, lymphocytes, macrophages, eosinophils and mast cells) was calculated using the following formula:

\[
\frac{\text{Total number of each cell type counted}}{\text{Total number of peritoneal cells}} \times 100
\]

2.20 PREPARATION OF SPLENOCYTES

After removing the micropore chamber, peritoneal cells were collected from the peritoneum. Afterwards the spleens were removed aseptically. All the above procedures were performed in strict aseptical conditions under a laminar hood. Briefly following protocol was used for the proliferation study.

a) Spleens were removed aseptically from the mice and gerbils, and passed through a nylon membrane with the head of a syringe plunger to prepare single cell suspension. The cells were
collected in a sterile petriplate containing 1 ml of RBC lysing buffer.

b) The cells were washed once with incomplete RPMI media (RPMI 1640 supplemented with 10 mM HEPES, 1 mM sodium pyruvate 2 mM L-glutamine, 4500 mg glucose/L and 1500 mg sodium bicarbonate / Litre) by centrifugation at 1500 rpm and resuspended in 1 ml of complete RPMI media.

c) After counting the cells number and determining the viability using trypan blue dye exclusion approximately 0.5x 10^6 cells were cultured per well with 200μl of complete RPMI media with 1% glutamine, 1% sodium pyruvate, 0.4%HEPES, 1% Non-essential amino acid mix supplemented with 0.01% β-mercaptopetoethanol and gentamycin.

d) The splenocytes were proliferated with three different conditions stimulated with media alone (Unstimulated), 2.5 μg ConA (ConA stimulated) and 10μg of purified rALT-2 protein (Protein stimulated) for 72 hours at 37°C with 5% CO2 in a CO2 incubator.

2.20.1 Splenocyte proliferation assay

After 72 hours cell proliferation was measured by an MTT assay called as CellTiter 96®AQueous non-radioactive cell proliferation assay, manufactured by Promega, USA. The cellTiter 96®AQueous non-radioactive cell proliferation assay is composed of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(0)) and an electron coupling reagents (phenazine methosulphate; PMS). MTS is bio reduced by cells into a formazan product that is soluble in tissue culture medium. Dehydrogenase
enzymes found in metabolically active cells accomplish the conversion of MTS into aqueous soluble formazan. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. Briefly following protocols was used for measuring the cell proliferation by MTT Assay.

a) The MTS solution and the PMS solution were thawed in a 37°C water bath by incubating for 10 minutes.
b) 2ml of the MTS solution from the amber reagent bottle was transferred aseptically to a test tube. To this 100μl of PMS solution was added immediately and gently swirled to ensure the mixing of both the solutions.
c) 100μl of culture were removed from the plate from each well after mixing with a multi-channel pipette. To the rest 100 μl of cell 20 μl of the combined MTS/PMS solution was added by a multichannel pipette into each well of the tissue culture plate.
d) The plate was incubated for around 4 hours at 37°C in a 5% CO₂ incubator and the reaction was stopped by adding 25μl of 10% SDS solution.
e) The absorbance was recorded at 490nm using an ELISA plate reader.

The splenocytes proliferation was performed in triplicate and mean average of the all the O.D. was taken as the mean proliferation O.D.. The background O.D. obtained with media was subtracted from the absorbance of experimental samples. The percentage of splenocytes proliferation was calculated according to the following formula:

Relative cell viability = \( \frac{\text{Absorbance experimental}}{\text{Absorbance control}} \) X 100
The Absorbance control represents the absorbance of unstimulated splenocytes and Absorbance experimental represents the absorbance of splenocytes proliferated by Purified Bm-ALT-2 protein / Con-A. The percentage of splenocyte proliferation was calculated by the above formula and graph was drawn with mean average and standard deviation by Microsoft excel package.

2.20.2 Cytokine analysis by RT-PCR

The splenocytes of vaccinated mice and gerbils were pooled for each group by adding 100 µl of media to each well and were centrifuged for 5 minutes at 1500 rpm in a refrigerated centrifuge (Eppendorf, USA). RNA was isolated from the pellet by RNAZOL (Gibco BRL, USA) as per the manufacturer protocols as described in common methods.

The RNA was converted to cDNA by reverse transcriptase enzyme by using Retroscript kit as per the manufacturer instructions (AMBION, USA). For mouse IL-4 and beta actin commercial primers (AMBION, USA) were used. For mouse IL-5, IL-2 and IFN-γ primers were designed based on a previous publication by Benavides et al (1995) as mentioned in Table 2.2. For gerbil cytokines, primers were designed based on the sequences available in genebank (Table 2.3). For all the cytokines the log linear scale of optimized number of cycles were first determined. Initially the template concentration was adjusted for PCR, so that same level of betaactin profiles was obtained. The RT-PCR data was obtained from three independent experiments.
### Table 2.2 Primer details of mouse cytokines

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Annealing Temperatures and product size</th>
<th>Gene Bank Accession No./Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 Forward</td>
<td>5' AACAGGCACCCACTCTCAAA3' 5' TTAGGATGATGCTTGGACA 3'</td>
<td>55°C, 423bp</td>
<td>K02292 Benavides et al 1995</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4 Forward</td>
<td>Commercial primers, Ambion, USA. - do -</td>
<td>57°C, 388bp</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-5 Forward</td>
<td>5'AGGATGCTTCTGCACCTGA3' 5'ACACCAAGGAACCTTGGACA 3'</td>
<td>54°C, 346 bp</td>
<td>Benavides et al 1995</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ Forward</td>
<td>5' AACGCTACACTGCGACTCTG3' 5'AGCTCATTGAATGCTTGG 3'</td>
<td>55°C, 429 bp</td>
<td>M28621 Benavides et al 1995</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin Forward</td>
<td>Commercial primers, Ambion, USA. - do -</td>
<td>57°C, 294 bp</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.3 Primers details of Gerbils Cytokines

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer Sequences</th>
<th>Annealing Temp &amp; product size</th>
<th>Gene Bank Accession No./Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 Forward</td>
<td>5'CACATCCCTGAGCTGTTAGATT3' 5'TAGGCGTCCTCAGGAAGACTCATT 3'</td>
<td>57°C, 424 bp</td>
<td>L37779</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-5 Forward</td>
<td>5'ATTCTAATCTCGCCTGGGCTCTGG 3' 5'GAACCTGCCTGCTCTGGCATC 3'</td>
<td>58°C, 315 bp</td>
<td>L37780</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ Forward</td>
<td>5' CTTTGGGCCCCTCTGACTCTGTGA 3' 5'TTCCCGTCTCCTTTAGCTGTACT 3'</td>
<td>57°C, 519 bp</td>
<td>L37782</td>
</tr>
<tr>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin Forward</td>
<td>5'GCACACCACTTCTACATGAG3' 5'ATAGCACAGCCTGCTGATAGCAAC 3'</td>
<td>58°C, 163 bp</td>
<td>Takashima et al 2001</td>
</tr>
</tbody>
</table>
2.20.3 Cytokine analysis by ELISA method

The media from the mice splenocyte culture was collected and stored at -80°C for analysing the level of cytokines like IL-4, INFγ by ELISA method. Cytokine kits obtained from Endogen, USA was used as per the manufacturer protocol for measuring the concentration of soluble cytokines at the protein level as follows.

2.20.4 Mouse IL-4 ELISA

Following materials were available along with the Endogen kit. 1. Precoated Strip well plate; 2 vials of lyophilised standard; 1. Standard diluent; Plate reagent; Wash buffers; Prediluted Conjugate Reagent; Premixed TMB substrate; Stop solution; Adhesive Plate covers 9. Detailed Instruction booklet.

Procedure

1. 50 µl of Plate reagent was added to each well and afterwards 50 µl of standard in different dilutions as per the instruction or samples were added.
2. The plates were washed for 5 times with wash buffer.
3. 100 µl of conjugate reagent was added. The plate was covered and incubated in a 37°C humidified incubator.
4. The plate was washed 5 times with wash buffer.
5. 100 µl of TMB substrate solution was added and incubated at 20 -25°C for 30 minutes.
6. 100 µl of stop solution was added and the plate was read at A₄₅₀ – A₅₅₀ nm.

7. Based on the standard graph the concentration of Cytokine from different samples were analysed.

2.20.5 Mouse INF-γ ELISA

Following materials were included in the Endogen kit. 1. Strip well plate; 2 vials of lyophilised standard, standard diluent, Coating antibody, Assay Buffer, Wash buffers, Biotinylated detecting reagent, Strepavidin HRP reagent, Premixed TMB substrate, Stop solutions, Adhesive Plate covers, Detailed Instruction booklet

Coat the Plate

1. 100 µl of diluted coating antibody was added to each well
2. The plate was covered and incubated overnight at 20-25°C.
3. Coating solution was decanted. 200 µl/well of Assay Buffer was added.
4. The plate was covered and incubated one hour at 20-25°C.
5. The plate was washed 3 times and then blotted thoroughly

Sample Incubation

1. 50 µl assay buffer was added to each well.
2. 50 µl standard or sample was added to each well.
3. The plate was covered and incubated overnight at 20-25°C.
4. The plate was washed 3 times and then blotted thoroughly.
Biotinylated Detecting Antibody Incubation

1. 100 μl/well of diluted Biotinylated Detecting Antibody was added.
2. The plate was covered and incubated for 30 minutes at 20-25°C.
3. The plate was washed 3 times and then blotted thoroughly.

Streptavidin-HRP Incubation

1) 100 μl/well Strepavidin-HRP was added to each well.
2) The plate was covered and incubated for 30 minutes at 20-25°C
3) The plate was washed 3 times with wash buffer, then blotted.

Substrate Incubation

1) 100 μl/well of TMB substrate solution was added (Product No. N301) and incubated at 20-25°C for 30 minutes.
2) Incubated for 30 minutes at 20-25°C.
3) The plate was washed 3 times with wash buffer, then blotted.

Stop Reaction/ Read the Plate

1) 100 μl/well of Stop Solution was added to each well.
2) The absorbance was measured on an ELISA reader set at OD_{450} - OD_{550}.

Based on the standard graph the concentrations of Cytokines from different samples were analysed.
2.20.6 Statistical analysis

For comparison of two mean value student paired T test was done and for protection study chi square test was done. A probability value of $p \leq 0.05$ was considered statistically significant.

2.21 GENERAL METHODS

Molecular biology methods such as plasmid DNA preparation, agarose and SDS-PAGE gel electrophoresis, transformation, PCR, RTPCR and western blotting used in this study were adopted (Sambrook et al 1989) with minor modifications as described below.

2.21.1 Plasmid DNA preparation

a) Plasmid DNA isolation from recombinant *E. coli* was based on the method of Birnboim and Doly (1979) (All centrifugation steps in this procedure were performed in a microfuge at 12000 g).

b) A 1.5 ml of overnight grown culture of plasmid bearing *E. coli* was centrifuged for 1 minute and the supernatant was discarded. The residual medium was removed by brief centrifugation followed by aspiration.

c) The cell pellet was resuspended in 100 µl of ice-cold TEG buffer (25 mM Tris HCl, 10 mM EDTA, 50 mM glucose) by vigorous vortexing and incubated at room temperature for 5 minutes.

d) Freshly prepared 200 µl of alkaline-SDS (1% SDS in 0.2 N NaOH) was added to the cell pellet, the tubes was gently inverted 3-4 times and placed on ice.
e) After 5 minutes, 150μl of potassium acetate solution (3 M pH 5.2) was added, mixed by gentle inversion, placed on ice for 15 minutes and centrifuged for 15 minutes at 4°C.

f) The supernatant was carefully transferred into a fresh tube. To this RNase was added to a final concentration of 10μg/ml was added and incubated at 37°C for 1 hour.

g) The sample was extracted once with equal volume of Tris buffered phenol: chloroform: isoamyl alcohol (25:24:1) and once with equal volume of chloroform: Isoamyl alcohol (24:1).

h) The DNA in the aqueous phase was precipitated by adding 2.5 volumes of ethanol on ice for 15 minutes. The DNA was pelleted by centrifugation for 15 minutes at 4°C.

The supernatant was discarded, and the DNA pellet was washed using 70% ethanol. The traces of ethanol were dried by speed-vac concentrator and the DNA was dissolved in appropriate volume of either double distilled water or TE (10 mM Tris-Cl, pH 8.0, 1mM EDTA) and stored at 4°C or -20°C.

2.2.1.2 Agarose gel electrophoresis

Horizontal submerged gels were used to separate the DNA fragments. TBE buffer of pH 8.3 (98 mM Tris, 89 mM Boric acid and 2 mM EDTA) was used. The electrophoresis was performed at 5-8 v/cm at room temperature. The gel loading buffer contained 20% glycerol with 0.01% bromophenol blue and 0.01% Orange-G in TE.
1% agarose gels were employed throughout the present study. Gels were stained with approximately 0.5 μg/ml of ethidium bromide, viewed under UV transilluminator (Fotodyne, Hartland, WI, USA). 100 bp, 1Kb Ladder or lambda Hind III marker (Gibco BRL, MD, USA) (New England Biolabs, MA, USA) was used as molecular weight markers. Photographs were taken with AlphaImager TM, (Alpha Innotech Corporation, USA) using UV light filter.

2.21.3 Transformation of E. coli with plasmid DNA

Transformation of E. coli with plasmid DNA was based on the method of Hanahan (1983) using CaCl₂ for the preparation of competent cells. Briefly the following procedure was used.

a) A single colony of freshly revived E. coli culture was inoculated in 2 ml of LB and grown at 37°C overnight.

b) 100 μl of overnight culture was inoculated into 50 ml LB medium in conical flask and allowed to grow at 37°C till OD₆₀₀=0.6.

c) Culture was chilled on ice for 5 minutes by gentle swirling and centrifuged at 3500g for 5 minutes at 4°C.

d) The cell pellet was resuspended in 10 ml of 100 mM ice-cold MgCl₂ and incubated on ice for 20 minutes.

e) Cells were pelleted as in step c. and the pellet was resuspended in 2 ml of 100 mM ice-cold CaCl₂ and incubated on ice for 1 hour.
f) Approximately 10-20 ng of DNA was added to 100 µl of above cells and further incubated for 1 hour on ice.

g) A heat shock at 42°C was given for 90 seconds and chilled on ice for 5 minutes.

h) To this tube 800µl of LB medium was added, allowed to grow for 1 hour at 37°C and 100µl was plated onto LB agar plates supplemented with appropriate antibiotics.

A positive control plasmid (10 ng of pRSET B and 1020) was used in all the experiments to verify the transformation efficiency. Cells with no DNA added served as negative controls.

2.2.1.4 Polymerase chain reaction

For screening transformants, a small portion of freshly grown transformant colony was picked using a sterile toothpick and resuspended in 50µl of 0.1 X TE (1mM Tris and 0.1mM EDTA, pH 8.0) buffer. The cells were lysed by boiling for 10 minutes, snap-chilled on ice, centrifuged (10,000 rpm for 10 minutes) and 1µl of the supernatant was used as template in PCR. For T7 phage antigen also, above protocol was followed.

A reaction mixture containing 200 µM of each dNTPs; 1X PCR buffer (50mM KCl, 10mM Tris.Cl, pH8.3); 2.5mM MgCl₂ 5 Pm of each primer; 1 unit of Taq DNA polymerase and 20ng of template were mixed. PCR was performed on a MJ Research Minicycler. The optimal annealing temperature of 58°C was used for all the primer sets.
The optimised PCR parameters used are as follows:

a) Initial denaturation 96°C 5 minutes
b) Denaturation 96°C 1 minute
c) Annealing 56°C 1 minute
d) Extension 72°C 1 minute

Step from b to d were cycled for 35 times
e) Final extension 72°C 5 minutes

The amplified PCR products were analysed by agarose gel electrophoresis.

2.21.5 Reverse transcription-polymerase chain Reaction (RT-PCR)

2.21.5.1 Extraction of RNA

RT-PCR was carried out as described previously (Hall et al 1998). Cells were lysed in 1 ml total RNA isolation reagent TRIZOL (Life Technologies) after incubating them for required time points and transferred to 1.5 ml centrifuge tubes.
Table 2.4  Sequence of the primers, restriction sites and annealing temperature

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' – 3')</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 Universal promoter</td>
<td>TAATACGACTCACTATAGGG</td>
<td>56°C</td>
</tr>
<tr>
<td>pRSET reverse</td>
<td>TAGTTATTGCCTGAGGTTG</td>
<td>56°C</td>
</tr>
<tr>
<td>VC 5’ (Forward)</td>
<td>CCAAGTGCTGGAGAGAAAACC</td>
<td>58°C</td>
</tr>
<tr>
<td>VC 3’ (Reverse)</td>
<td>GTACTCATCAATGCGATGC</td>
<td>58°C</td>
</tr>
<tr>
<td>BmALT (Forward)</td>
<td>BamHI CGGGATCCAAATGAAACTTTTAATAAGC</td>
<td>58°C</td>
</tr>
<tr>
<td>BmALTR (Reverse)</td>
<td>BamHI CGGGATCCATCTGTGGTTCGTTTGCTTTCG</td>
<td>58°C</td>
</tr>
<tr>
<td>T7 UP primer (Novagen)</td>
<td>GGAGCTGTCGTATTCCAGTC</td>
<td>50°C</td>
</tr>
<tr>
<td>T7 Down primer (Novagen)</td>
<td>AACCCCTCAAGACCCCGTITA</td>
<td>50°C</td>
</tr>
</tbody>
</table>
200 µl of chloroform was added and spun at 12000 r.p.m for 15 minutes at 4°C. The aqueous phase was removed and transferred to a new 1.5 ml centrifuge tube. 500 µl of isopropanol was added and incubated on ice for 15 minutes. Later it was spun at 12000 r.p.m at 4°C for 15 minutes. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol. The pellet was air dried and dissolved in 4.5 µl of deionised autoclaved water.

2.21.5.2 Reverse Transcription Reaction

Reverse transcription reaction was carried out by Reverse transcription was carried out as follows: 200ng of random hexamer was added to 4.5 µl of total RNA and was made up to 10 µl using deionised water. The mix was incubated in a water bath at 72°C for 3 minute and snap chilled on ice.

200 units of avian (AMLV) reverse transcriptase, 2µl of (AMLV) reverse transcriptase, 2.5 pmol of dNTPs, and the reaction mix was made up to 20 µl. The reaction mix was incubated at 42°C in a water bath for about one hour. The reaction mix was then heated at 90°C for a minute to inactivate avian reverse transcriptase. The cDNA synthesized was further used for PCR.

2.21.5.3 Polymerase Chain Reaction of cDNA

For PCR reaction, 1 µl of the cDNA mixture prepared as described earlier was added to a PCR reaction mixture consisting of 1 X PCR buffer, 2.5 pmol dNTP, 5 pmol of paired primers, 1.25 units of Taq polymerase
(Amersham Pharmacia Biotech, U.K), and distilled water in a total volume of 50 μl.

The reaction mixture was overlaid with mineral oil and placed in a PCR thermal cycler for cyclic reactions. The PCR reaction was set up as per the nature of primer and size of amplified product.

The PCR products were run on 1.5% agarose gels stained with ethidium bromide and photographed by gel documentation system.

2.21.6 SDS-Polyacrylamide Gel Electrophoresis

Proteins extracted from recombinant E. coli were analysed by the method of Laemmli (1970) by SDS-PAGE with minor modifications. The various buffers used are as follows.

a) Monomer solution: 20% acrylamide and 0.8% N, N-methylene bis-acrylamide in distilled water. The solution was filtered through whatman filter paper and stored in brown bottles at 4°C.

c) Separating gel buffer: 1.5 M Tris-Cl, pH 8.3

d) Stacking gel buffer: 0.5 M Tris Cl, pH 6.8

e) Electrophoresis buffer: 0.025 M Tris-Cl, 0.192 M glycine, 0.1% SDS, pH 8.3

f) Sample solubilizing buffer (5X) 10% SDS, 10% (v/v) β-mercaptoethanol, 50% sucrose, 0.025% Bromophenol blue in stacking gel buffer.
Depending on the proteins to be separated 10-15% separating gel and 5% stacking gels were used. Stacking gel was approximately 1/5 of the separating gel. Protein estimations were performed (Bradford 1976) and equal amounts of total protein were loaded in each well. Electrophoresis was performed at room temperature with constant current of 20mA for stacking gel and 30 mA for separating gel. Gels were stained with staining solution (0.25 g of Commassie Brilliant Blue R-250 in 45% methanol, 10% acetic acid) o/n and destained with 45% methanol, 10% acetic acid solution until a clear background was obtained. Photographs were taken with ChemiImager Gel Documentation system, Alpha Innotech, USA.

2.2.1.7 Western Blotting

After electrophoresis, the SDS-PAGE gel was incubated for 10 minutes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS). Nitrocellulose membrane (NCP) cut to the exact size of separating gel was also incubated for 10 minutes in transfer buffer. Without trapping air-bubbles the NCP was overlaid on the gel and sandwiched between filter papers and scotch brite pads. Electrophoretic transfer was carried out in the cold room at 300 mA for 3 hours using LKB transphor 2005 electroblotting apparatus. After transfer, the molecular weight marker lane was cut and stained with amido black (100 mg amido block in 45% methanol, 10% acetic acid). The rest of the NCP was stained with Ponceau S (0.2% Ponceau S, Sigma, USA in 0.3% trichloro acetic acid and 0.3% sulfosalicylic acid) staining to ensure the transfer of the proteins. Membrane was washed in PBS and blocked o/n at 4°C with 5% non-fat milk powder in PBS.
The NCP was washed in wash buffer (PBS with 0.05% Tween-20) thrice of 5 minutes duration each, and then incubated with appropriately diluted primary antibody for 3 hours at room temperature. After washing in the wash buffer the membrane was incubated for 1 hour with recommended dilution of secondary antibody conjugated with alkaline phosphatase. After extensive washing, the blot was incubated in pre detection buffer (100 mM Tris Cl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) for 10 minutes. The colour development was carried out by using 60 µl of 5-bromo-5-chloro-inodolyl phosphate (50 mg/ml in diethyl formamide) and 30 µl of Nitroblue tetrazolium (50 mg/ml in 70% diethyl formamide) in 10 ml of detection buffer. The reaction was stopped after 15 minutes by addition of 10 mM EDTA.