CHAPTER I

Kinetic regulation of repertoire discrimination and antibody optimization for epitope
Materials and Methods

ANIMALS AND IMMUNIZATIONS

Female BALB/c (6-8 weeks old) were obtained from the small animal facility at the National Institute of Nutrition (Hyderabad, India). Immunizations with either peptide PS1CT3 or CysCT3 or AibCT3 were of i.p. at a dose of 50 \( \mu \)g/mouse as an emulsion in Complete Freund's Adjuvant (CFA). The experiments where CT3 pre-priming was done, the peptide was given at a dose of 50 \( \mu \)g/mouse in CFA at the base of tail 7 days prior to immunisation with peptide CysCT3.

COLLECTION OF SERUM

Mice were bled from the retro-orbital venous plexus, and the blood was collected in tubes. The tubes were then incubated at 37°C for 20 minutes. The clotted blood was then kept on ice for 30 minutes. The tubes were given a spin at 5000 rpm for 5 minutes and the serum was collected into fresh tubes.

GENERATION OF MONOCLONAL ANTIBODIES (mAbs)

For early primary IgM mAbs, a group of three female BALB/c mice were immunized with PS1CT3 (i.p.). Four days latter, mice were bled individually and sera was checked for anti-PS1CT3 antibody titres by ELISA. The mouse with highest titre (1:500) was choosen for fusion. The spleen from the selected mice was harvested aseptically and made into single cell suspension by teasing the spleen by squeezing with
angled forceps or by chopping with fine tipped dissecting scissors. The debris was 
removed and the cells were further dispersed by passage through a fine-mesh metal 
screen. The cells are given a wash in sterile complete serum free medium RPMI 1640 
(Gibco Brl) for 5 minutes (1500 rpm) at room temperature. RBCs were lysed with RBC 
lysis buffer (Sigma) by suspending 5X10^7 cells/ml of lysis buffer for 5 minutes at room 
temperature). The lysis buffer was diluted with complete serum free media and followed 
by 2 washes. In parallel, the SP2/O cells in their log phase were harvested, and given two 
washes with complete serum free media (CSFM). After counting the cell number, 
splenocytes and SP2/O are mixed in a ratio of 2:1 and given a wash. The pellet is broken 
by hitting the base of the tube against the hood platform. This was followed by 0.5 ml of 
pre-warmed 50% PEG (added drop-wise over a period of 10 seconds). The cells were left 
in PEG for additional 1 minute in continuous stirring condition. Then 1 ml of pre-warmed 
(37°C) CSFM was added over a time period of 1 minute. This step was repeated again. 
Then 7 ml of media (CSFM) was added drop-wise over a period of 3-4 minutes. Cells 
were pelleted down at 500g at room temperature (RT) and dissolved in HAT media (20% 
FCS) so that 100μl of suspension contains 1X10^6 cells. Aliquote 100μl of cell 
suspension was added per well (24 well culture plates having feeder cells in 1 ml HAT 
media in humified 37°C, CO₂ incubator). The cells were selected on HAT media for 
 atleast 10 days, discarding 50% of the media on day 2, 3, 4, 5, 7 and 9 and feeding with 
media at the same time. Then the clones were selected on hypoxanthine-thymidine (HT) 
media from day 10 onwards. The screening for positive clones were done on day 15 by 
ELISA. This was followed with 3 steps of subcloning to select for stable hybridomas 
secrating antibodies specific for the antigen.
Materials and Methods

For mature IgG mAbs, mice immunised with the antigen (peptide PS1CT3 / CysCT3 / AibCT3) and were given a soluble antigen boost (50 μg of antigen in PBS) intravenously on day 24. On day 4 post soluble boost, mice were screened for Ab titre and the mice giving the highest titre was used for fusion as described above.

ANTIBODY ISOTYPE SEPARATION AND GENERATION OF IMMUNE COMPLEXES

Immune sera collected at various time points were individually resolved for the IgM and IgG components by passing over a protein G - sepharose column (Pharmacia, Uppsala, Sweden). The flow through contained IgM, whereas bound IgG - after a thorough washing of the column - was eluted with glycine-HCl buffer of pH 2.7. The eluate was immediately neutralized, concentrated, and then dialyzed against PBS prior to use. Both, verification of absence of the unwanted isotype and estimation of antigen-specific IgG and IgM was determined by quantitative ELISA.

For preparation of immune complexes, either IgM, IgG or total Ig preparations were incubated with a ten-fold excess of peptide (based on the estimated number of specific binding sites) in PBS for 1 h at 37°C with occasional shaking. The preparation was then dialyzed against PBS (3 changes over 4 h) to remove unbound peptide and concentrated if necessary. Aliquots of 100 μl containing immune complexes corresponding to 500 ng of starting antibody were injected i.v. to recipients. It was first established, in pilot experiments, that this was the optimum antibody concentration for use in GC reconstitution experiments.
ANTIBODY PREPARATION AND GENERATION OF IMMUNE COMPLEXES

For monoclonal antibody preparations, hybridoma culture supernatants were used and the antibodies were concentrated as per the requirement. For polyclonal antibody preparation, antibody of IgM or IgG isotype depending on the requirement were separated from the sera at different time points. For early IgM and IgG isotype antibodies from GL1 treated or untreated mice immunised with the specific antigen, the sera was collected day 7 post immunization and for late IgG response, the sera was passed over a protein-G sepharose column (Pharmacia, Uppsala, Sweden). The flow-through contained IgM whereas the bound IgG antibodies were eluted with glycine-HCl buffer, pH 2.7 after thorough washing of the column. The eluent is immediately neutralised with 1M tris-HCl, pH 9.0. This elute was dialyzed with PBS, pH 7.4, before use. The verification for the purity in the isotype and antigen specific IgM and IgG was done by quantitative ELISA. Wherever necessary, the IgG fractions were purified for peptide specific antibodies by incubating with a PS1CT3-sepharose affinity column (overnight at 4°C). The elution and the steps followed there after are as mentioned above.

For antibody preparation from mice reconstituted with GCs, day 21 post-transfer of primed cells (B&T cells) and appropriate immune complexes, the splenocytes from these mice were transferred to irradiated (550 rad) host (BALB/c mice) on one to one basis. This was followed by a challenge with soluble peptide (50 μg/mouse in PBS, i.v.) sixteen hours later. The sera were collected on day 5 post soluble antigen challenge.

RECONSTITUTION OF GERMINAL CENTERS IN VIVO

A total volume of 200 μl (PBS pH 7.4) containing immune complexes of 500 ng of antibody and 5 x 10⁶ enriched lymph node T cells from CT3 preprimed mice were
transferred (i.v.) into irradiated (550 rads) BALB/c mice. Twenty four hours later these mice were given (i.v.) enriched B cells (1 x 10⁷ in 200 µl/ mouse) derived from splenocytes of mice immunized two days before the day of splenocyte preparation with peptide PS1CT3. Ten days after B cell transfer, spleens were removed from the irradiated hosts and sections prepared on OCT compounds (BDH, England) for cryo sectioning. These spleen sections, embedded in OCT compounds were preserved at -70°C till use.

**IMMUNOHISTOCHEMICAL STAINING OF ANTIGEN-SPECIFIC GERMINAL CENTERS**

6 µm thick sections of frozen spleens were taken on a cryostat microtome and thaw mounted on glass slides. Sections were allowed to dry briefly after which they were fixed in ice-cold acetone for 10 min, air dried for 10 min and stored air tight at -70°C until use. When required, the frozen sections were thawed and rehydrated in PBS for 20 min. Endogenous peroxidase activity was quenched with 0.1% phenylhydrazine (Sigma). After three washes, sections were blocked for non-specific binding with 1:1 (v/v) solution of 3% BSA (in PBS) and mouse non-immune serum for 1 h at 37°C. The slides were rinsed in PBS and then incubated for 90 minutes with a 20 µg/ml of PNA-biotin in HEPES buffer (pH 7.5), washed 93x5 minutes each in PBS, then followed by a second incubation with streptavidin-HRPO (5 µg/ml in PBS) for 45 min. Both incubations were performed at 37°C. Subsequent to this a stock solution of biotinylated tetramer of Tet-PS1 (tetramer of PS1 sequence) was made in PBS at a concentration of 50 µg/ml and the slides incubated overnight at 4°C. After 3 washes, the slides were treated with recommended concentrations of streptavidin-alkaline phosphate conjugate in PBS for 45 min at 37°C. Bound conjugates were then visualized in a sequential manner. The HRPO conjugate was first detected by color development with the AEC staining kit (Vector Labs, USA), where a red color for PNA⁺ cells was obtained. Bound alkaline phosphatase
was revealed with the Blue staining kit (Vector Labs) which detected the presence of antigen-specific cells. A minimum of thirty individual sections - spread longitudinally across the spleen - were examined from each spleen, and each experimental group included between 3 to 5 mice.

ENRICHMENT OF T AND B CELLS

The spleen (for B cells) or inguinal lymph nodes cells (for T cells) were removed surgically in an aseptic condition. Made into single cell suspension as described before. After a wash in complete media (5% FCS in RPMI 1640), RBCs were lysed with RBC lysis buffer as described before. This was followed by two washings, and then adherent cells were removed by two rounds (1 hour each at 37°C) of panning on culture grade petri plates in 5% complete media. The non-adhering cells were removed by gentle pipetting on the surface with pre-warmed media (37°C). For enrichment of B cells, non-adherent cells were diluted to 5X10⁷ cells/ml in washing buffer (1-2 ml of HBSS having 1% FCS) and T cells were depleted from this population by two rounds of panning with Dynabeads; antimouse Thy1.2 (using 2X10⁸ beads/ml of cell suspension containing 5X10⁷ cells) as recommended by the manufacturer.

In brief, required volume (100 μl of Dynabead suspension contains 4X10⁷ beads) of Dynabeads was pipetted out from the vial and transferred to a 5 ml polypropelene tube. The tube was placed on MPC for 2 minutes, the fluid was pipetted out. Following this, Dynabeads were resuspended in 2 ml of washing buffer after removing the tube from MCP and the previous step is repeated. After 3 washings, the beads were resuspended with equal volume of washing buffer that was originally pipetted from the vial. This was followed with pipetting out the required volume of Dynabeads into cell suspension. This was mixed by inverting, incubating at 4°C for 20 minutes on nutator. The rosetted T cells
were removed by placing the tube on MPC and collecting the cells from the fluid. The beads can again be gently suspended in washing buffer and rosette out the unwanted cells. The collected fluid containing the enriched B cells (upto a purity of 95%) was given a wash in washing buffer and used for further studies.

For enrichment of T cells, anti-B220 coated magnetic beads were used for panning, rest of the remaining steps are same as described for B cells.

**ELISA**

Microtitre plates were coated with 2 µg/ well of antigen in 100 µl of PBS (pH, 7.2) at 37°C for 3.5 hrs or overnight at 4°C. Subsequently, they were blocked with 300 µl/ well of 5% solution of fat-free dry milk powder in PBS at 37°C for 1 hr. Plates were washed thrice with the washing buffer (PBS-Tween-20). 100 µl of the appropriate dilution of mouse antiserum was made in the dilution buffer (PBS-Tween-20 containing 0.1% blocking agent) and added to the coated wells. Plates were incubated at 37°C for 1 hr. After 3 washes, bound antibody was detected with horseradish peroxidase - labeled secondary antibody (100 µl /well, 37°C, 1h), followed by the addition of the substrate (O-phenylenediamine, 0.5 mg /ml) in substrate buffer (0.1M citric acid, 0.2M Na₂HPO₄, 0.1% H₂O₂). Reaction was quenched with 50 µl 2N sulphuric acid. Absorbance was measured at 490 nm. The concentrations of specific antibodies were calculated on the basis of affinity-purified mouse Ig standard curves run in parallel assays.

**COMPETITIVE INHIBITION ELISA**

Inhibition/competitive ELISA assays were done in a similar manner with the exception that prior to addition of sera in the antigen-coated plate, graded doses of
Materials and Methods

soluble antigen were mixed with a fixed dilution of the serum sample that yielded half-maximal absorbance in prior experiments. This was incubated at room temperature for 10 minutes. The specific inhibition obtained was expressed either as a percent value or as molar inhibition.

LYMPHOCYTE PROLIFERATION ASSAY

BALB/c mice were immunized with antigen as an emulsion in CFA subcutaneously at the base of the tail with 50µg/mouse. Seven days later, the mice were sacrificed and the inguinal lymph nodes were removed. The lymph nodes were made into single cells suspension in complete media by grinding them between two frosted glass slides. After removing the debris, the cells were given two washes for 10 minutes at 1000rpm at room temperature. 5x10⁵ cells/well were cultured in quadruplicate in 200µl of RPMI 1640 containing 5% FCS, gentamycin (2mg/L) and 2-mercaptoethanol (0.05 mM) in 96 well culture plates. In addition, indicated concentrations of appropriate peptide were included as challenge antigen. Cultures were incubated at 37°C in a humified atmosphere of 5% carbon dioxide for 72 hrs. They were then pulsed with 1µCi/well of [³H]TdR (Amersham, UK) for an additional 18 hrs prior to harvesting. The cells were harvested onto glass-fibre filter mats and counted by liquid scintillation spectroscopy (Betaplate, LKB-Pharmacia, Sweden). Values are presented as stimulation index, which represents the ratio of mean counts obtained at a given concentration of Ag over that obtained in the
absence of any challenge Ag. Background counts generally varied between 1000 to 2500 cpm.

**ELISA ASSAY FOR PIN-BOUND HEXAPEPTIDES**

Hexapeptide sets synthesized on non-cleavable pins were also evaluated for antibody cross-reactivity and epitope mapping by ELISA. For this, the protocol recommended by the manufacturer was strictly followed. The pins were blocked (this step is equivalent to the blocking step in ELISA) with 200 μl of pre-coat buffer (PBS containing 2%BSA, 0.1% v/v Tween-20 and 0.1% w/v sodium azide) for 1hr at room temperature on a shaker (100 rpm). The pins were washed with PBS for 10 minutes on shaker followed by overnight incubation with primary antibody at appropriate dilution in 175 μl pre-coat buffer at 4°C on shaker. Subsequently, the pins were washed four times (10 minutes each) in PBS, pH 7.2 and subjected to a second round of incubation with appropriate dilution of secondary antibody (goat anti-mouse IgM or IgG conjugated to HRPO, Sigma) in 175 μl of antibody conjugate diluent (PBS containing 1% Fetal Bovine Serum, 0.1% Tween-20, 0.1% w/v BSA) at room temperature for 1h with gentle shaking. Following this, the pins were washed in PBS 4 times, 10 minutes with gentle shaking. The chromogen used here for detecting bound antibody was 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium (ABTS, Sigma). 175 μl of ABTS substrate solution was pipetted into ELISA reading plates and pins were placed onto the substrate solution (0.1M citrate-phosphate buffer, 0.5mg/ml ABTS, 0.1% H₂O₂) till
color developed. The reaction was stopped by removing the pins from the substrate solution. The absorbance was measured at 405 nm with subtraction of that at 490 nm.

DETERMINATION OF ON-RATES AND DISSOCIATION CONSTANTS

Equal volumes of mAb and appropriate peptide (PS1CT3 or CysCT3) in PBS were mixed at room temperature and time-dependent Ab binding in terms of quenching of tryptophan fluorescence was continuously monitored over a 100-min period in a Shimadzu RF-1501 spectrofluorimeter (Shimadzu Corporation, Tokyo, Japan). The excitation wavelength used was 280 nm, and emission was recorded at 330 nm. The final Ab concentration employed was between 200 to 300 nM, whereas peptide was maintained at between 10 to 25-fold in molar excess over binding sites (assuming bivalency per antibody molecule) to ensure pseudo-first-order conditions. Extent of fluorescence quenching was used to determine unbound Ab concentrations as a function of time. The log of concentration of unbound antibody was plotted versus time, and the slope, which was obtained by linear regression analysis, was used to determine $k_{\text{app}}$. The $k_{\text{on}}$ value was subsequently calculated by dividing $k_{\text{app}}$ with peptide concentration. Values of $k_{\text{on}}$ presented are the mean ($\pm$SD) of determinations at three independent peptide concentrations.

For dissociation constants, mAbs (final concentration between 100-150 μM) were incubated alone or with either peptide PS1CT3 or CysCT3 at concentrations ranging from $5 \times 10^{-5}$ to $1 \times 10^{-9}$ M at room temperature for 1h. Subsequent to this extent of quenching of tryptophan fluorescence was determined, from which concentration of peptide bound was calculated assuming bivalency for each IgG molecule at saturation. $K_d$ values were subsequently obtained from a Scatchard analysis of the resulting data.
RADIOIODINATION

Either 2 mg of peptide PS1CT3 or 1 mg each of mAbs PC287 and PC7bM were iodinated with Na$^{125}$I in the presence of Iodobeads (Pierce Chemical Co., Rockford, IL) in 400 μl of 10 mM phosphate buffer (pH, 7.2). The reaction time was 10 min. at 4 °C. After this the solution was collected, and the free Iodine removed first by gel filtration followed by exhaustive dialysis against PBS. Specific activity of iodination was calculated from the known molecular mass of peptide (4029 Da) and taking an approximate MW of 150,000 Da for the monomer Ig unit.

RNA ISOLATION

For isolation of RNA from B cell hybridomas, the cells were grown in 25 cm$^2$ flask to log phase almost reaching the confluent stage and to confirm, the flasks were visualized under the inverted light microscope. The media was removed completely. Then 300 μl of RNAZol B (Wak-Chemie Medical, Hamburg, Germany) was added in three steps, pipetted inside to lyse the cells and then transferred to eppendorf tubes. This was followed by addition of 120 μl of chloroform (mol.bio. grade, Amersham) and mixed by inverting few times. Stored on ice for 15 minutes. Centrifuged at 14,000 rpm for 15 minutes at 4°C, the aqueous phase was transferred to a fresh tube. Equal volume of chilled isopropanol was added, mixed by inverting and stored at -70°C for 3 hours or at -20°C for 8 hours. The tube was centrifuged at 14,000 rpm at 4°C, the fluid was decanted and the pellet was given a 70% ethanol (prepared with DEPC treated water) wash. The pellet was air-dried, dissolved in DEPC treated water. The relative purity of RNA was evaluated at $\lambda_{260}$ and $\lambda_{280}$. An approximate quantitation of RNA was done from the absorbance value at $\lambda_{260}$. 

98
POLISHING OF PURIFIED PCR PRODUCTS

The end filling or polishing of the PCR fragment was carried out with the help of stratagene PCR polishing kit. Briefly, the total reaction was carried out in a volume of 10 μl containing 5 μg of PCR product, 10 mM dNTP mix (25 mM each), 1.4 μl of 10x polishing buffer and 0.5 unit of pfu DNA polymerase (Stratagene). The reaction mixture was overlayed with mineral oil (20 μl) and the polishing reaction was done at 72°C for 30 minutes. This was stored at 4°C till further use.

INSERTING THE PCR PRODUCT INTO PCR-SCRIPT Amp SK (+) CLONING VECTOR

Cloning reaction was performed using Stratagene pCR-script Amp SK (+) cloning kit (Stratagene). The reaction was carried out in 10 μl volume reaction mixture containing 10 ng of pCR script Amp SK (+) cloning vector, 1 μl of 10x reaction buffer, 0.5 μl of 10 mM rATP, 500 ng of blunt end PCR product, 5 U of Srf I enzyme and 5 units of T 4 DNA ligase. The reaction mixture was gently mixed taking care not to introduce any air bubble. The reaction mixture was incubated at 22 °C for 3 hrs and then the enzymes were inactivated by incubating at 65 °C for 10 minutes. This was stored on ice until ready to use for transformation.

TRANSFORMATION

Transformation was carried out using Episurian Coli XL1-blue MRF kan supercompetent cells from Stratagene. The cells were thawed on ice, mixed gently by pipetting and 40 μl were transferred into a pre-chilled 15 ml Falcon 2059 polypropylene
Materials and Methods

tube. 0.7 μl of β-mercaptoethanol was added to the tubes containing the cells to yield a final concentration of 25 mM. The tubes were kept for 10 minutes on ice with gently swirling every 2 minutes. 2 μl of cloning reaction was added to the transforming reaction and swirled gently. This was left on ice for 30 minutes. The cells were given a heat pulse (42 °C) for 45 seconds followed by incubation on ice for 2 minutes. This transformation mixture was added with 500 μl of LB and incubated at 37 °C for 1 hr with shaking (225-250 rpm). Following this, the transformation reaction was plated on LB ampicillin (100 μg/ml) agar plates containing X-gal and IPTG and incubated at 37 °C overnight (16 hrs). The transformant bacterial colonies were selected (white colonies with no white blue or with a blue in the center) as inoculum for isolation of plasmids.

PLASMID DNA ISOLATION (MINIPREPS)

Single white colony was picked up from the plate with the help of sterile tooth pick or sterile pipette tips and inoculated into 5 ml sterile LB medium containing 100 μg/ml of appropriate antibiotics (ampicillin). The bacterial culture was grown at 37°C for 16 h with vigorous shaking i.e. 200-250 rpm. 1 ml of this culture was transferred to a sterile eppendorf tube, and cells were pelleted down at 5000 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 100 μl of TEG buffer containing 25 mM Tris-Cl (pH 8.0), 10 mM EDTA and 50 mM glucose. After 5 minutes of incubation on ice, 200 μl of solution II (0.2M NaOH, 1% SDS) was added and mixed inverting. This was followed by another 5 minutes of incubation on ice and addition of 150 μl of chilled solution III (3M sodium acetate, pH 5.2). The contents were mixed by inverting several times and then incubated on ice for 5 minutes. The tubes were given a spin at 10,000g for 10 minutes at 4°C, the clear fluid was transferred to a fresh sterile eppendorf tube and subjected to RNase treatment (10 μg/ml) at 37°C for 30 minutes. For separating the nucleic acids (plasmid DNA) from proteins, equal volume of...
Materials and Methods

phenol:chloroform:isoamylalcohol at a ratio of 25:24:1 was added and mixed by inverting. The tubes were centrifuged at 10,000g at 4°C for 10 minutes. The clear aqueous phase was transferred to another fresh DNase and RNase free eppendorf tube and added 2.5 volume of chilled absolute ethanol. The contents were mixed by vortexing and stored at -70°C for 3 h or at -20°C for 8 hrs. The tubes were centrifuged at 14000 rpm for 15 minutes at 4°C, the fluid was discarded and the pellet was washed with 70% ethanol (equal volume). The pellet was air-dried, mixed with appropriate volume of TE buffer.

RT-PCR

About 10 μg of RNA was taken for each V-gene cDNA synthesis. The primers used for the cDNA synthesis are: 5' - GGC CAG TGG ATA GAC for Cγ; 5' - AGA CGA GGG GGA AGA CAT TT - 3' for Cμ and 5' - GCT TGG ATG GTG GGA AGA TG for Cκ. The reverse transcription reaction was carried out in a reaction volume of 25 μl containing 800 pmol of appropriate primer, 5 μl of 5x RT buffer, 10 mM of dNTP mix (2.5 mM each), 30 units of RNAsin and 40 units of AMV Reverse transcriptase (Promega). This was laid with 20 μl of mineral oil to prevent evaporation. The synthesis was carried out at 42°C for 65 minutes on Perkin Elmer PCR machine.

About 5 μl of this product was taken for PCR amplification of the cDNA. The primers were designed for a nested PCR. For Cγ and Cμ, the variable end primer (5' end primer) that was used are: 5' - AGG T(C/G)(A/C) A(A/G)C TGC AG(G/C) AGT C(A/T)G G - 3' and for VL ; 5' GA(A/C/T) ATT GTG (A/C)T(G/C) AC(A/C) CA(A/G) (A/T)CT CCA - 3'. The 3' primers i.e. from constant region side are: 5' - CAT TTG GGA AGG ACT GAC TC - 3' for Cμ; 5' - GGC CAG TGG ATA GAT AGA C(T/C/A)G A - 3' for Cγ and 5' - GAA GAT GGA TAC AGT TGG TGC A - 3' for Cκ. The reaction was carried out in a volume of 100 μl containing 5 μl of cDNA synthesis product, 200 ng of
Materials and Methods

each primer, 8 μl of 10 mM dNTP mix, 10μl of 10x PCR buffer (50 mmol/L MgCl₂, 0.01% gelatin) and 2.5 units of Taq polymerase (Stratagene). The volume was made up with ddH₂O (sterile). This was overlayed with 30 μl of mineral oil (sterile) to prevent evaporation. The PCR reaction was performed on a Perkin Elmer thermocycler using following programme: one cycle at 95°C for 3 minutes, followed by 30 cycles at 94°C for 1 minute, 58°C for 1.5 minutes, 72°C for 1 minute and finally a 10 minute extensions at 72°C. A 10 μl aliquote of the reaction was analysed on 1.5% agarose gel.

RESTRICTION DIGESTION AND ANALYSIS

The plasmid DNA was employed for restriction digestion to analyse the DNA insert they carry. The reaction was carried out in a volume of 10 μl containing 2 μg of plasmid DNA, buffer (compartable buffer choosen from the chart provided by the company which supplied the enzymes), 2.5 units of EcoRI and NotI (Stratgene). The volume was made up with glass distilled autoclaved water. The reaction was carried out in an eppendorf tube at 37°C for 3 h. The digestion product was analysed on 1.5% agarose gel.

SEQUENCING

The plasmid DNA carrying the correct insert was choosen for sequencing. First, DNA was denatured under alkaline conditions. For this, 5 μg of plasmid DNA was taken in a volume of 32 μl of TE buffer or water. This was added with 8 μl of 2N NaOH, mixed by pipetting and left at RT for 10 minutes. This was followed by addition of 7 μl of 3M sodium acetate (pH 4.8) and 4 μl of H₂O. Immediately 120 μl of absolute ethanol was added and vortexed for 2 seconds. The tubes were left at -70°C for 3 h and given a spin
Materials and Methods

for 15 minutes at 14,000 rpm at 4°C. The fluid was pipetted out and pellet was given a 70% ethanol wash. The pellet was air-dried and dissolved in 10 μl of sterile water.

For sequencing reactions, T7- sequencing kit (Pharmacia Biotech) was used and the manufacturers protocol was followed with little modifications. Briefly, in the first step, the primers (T₃ or K₁ primers obtained from Stratagene) were annealed to the template.

<table>
<thead>
<tr>
<th>Template DNA</th>
<th>10 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer (100 pmol)</td>
<td>2 μl</td>
</tr>
<tr>
<td>Annealing buffer</td>
<td>2 μl</td>
</tr>
</tbody>
</table>

Total volume 14 μl

The tube was vortexed gently, spun to get the mixture to the bottom and incubated at 65°C for 5 minutes. After this, the tubes were transferred to 37°C water bath for 5 minutes and then to RT for 5 minutes. In parallel, sequencing reactions were prepared. Fresh eppendorf tubes were marked as A, C, G and T and 2.5 μl of short mix (provided in the kit) was pipetted into respective tubes and kept on ice. Following this, a reaction mixture was prepared as given below.

For two reactions:

<table>
<thead>
<tr>
<th>Labelling Mix</th>
<th>4.0 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labelled ³⁵S dNTP</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Enzyme dilution buffer</td>
<td>3.0 μl</td>
</tr>
<tr>
<td>T7 DNA polymerase</td>
<td>2.0 μl</td>
</tr>
</tbody>
</table>

Total volume 10.0 μl
This reaction mixture was mixed by pipetting, taking care not to introduce any air bubble, and left on ice till use. 5 µl of this mixture was pipetted to the tubes containing the template and primer. The tubes were mixed by pipetting and incubated at RT for 5 minutes. This was followed by transfer of 4.5 µl of this mixture into short mix tubes containing short mix reagent. After proper mixing, tubes were incubated at 37°C for 5 minutes following which the reaction was terminated by adding 5 µl of stop solution. The mixture was spun down and stored at -20°C till use. Before loading onto the sequencing gel (wells), the sample was heated at 75 - 80°C for 2 minutes and chilled on ice.

DENATURING GEL ELECTROPHORESIS

Recipe for 60 ml 6% sequencing gel: Urea - 28.8 g, Acrylamide - 3.6 g, Bis-acrylamide- 0.18 g, 10x TBE - 6 ml and distilled water to make 60 ml. The mixture was heated gently to facilitate the solubilization of acrylamide. The solution was filtered through Whatman 1mm filter paper and when ready to pour, 300 µl of 10% ammonium persulphate (fresh) and 20 µl of TEMED was added. Electrophoresis was carried out at constant power of 60 watts and 50°C using the Sequi-gen GT sequencing cell (38 x 30), BIO-RAD. After run was completed, gel was washed and fixed in 5% acetic acid and 15% methanol to remove excess of urea. Gel was lifted on a Whatman 3 mm paper and covered with a saran wrap. Gel was dried on a gel drier (BIO-RAD) at 80°C and exposed to autoradiographic film (hyperfilm, MP Kodak) for overnight. The autoradiogram was developed.