CHAPTER 2: MATERIALS & METHODS

A. IMMUNOPHENOTYPING

2.1 MATERIALS

2.1.1 Bone Marrow and Blood Source
Bone marrow aspirates or peripheral blood were collected from adult and paediatric leukaemic patients in University Hospital and other hospitals when available.

2.1.2 Chemicals and Reagents
The monoclonal antibodies were obtained commercially. Negative control, HLA-DR, B4 (CD19), T2 (CD7), T3 (CD3), T4 (CD4), T6 (CD1a), T8 (CD8) and normal rabbit serum were obtained from Dakopatt, Denmark. CALLA, T1 (CD5), T11 (CD2) and TCR-1 (WT-31) were purchased from Becton Dickinson, USA. My7 (CD13) and My9 (CD33) were purchased from Coulter Corporation, USA. The immunoperoxidase staining kit was a product of Cambridge Research Laboratory (CRL), USA. Ficoll-Paque was from Pharmacia Molecular Biologicals, Sweden. Phosphate buffered saline, Dulbecco 'A' (PBSa), was purchased in the form of tablets from Oxoid Limited, England. RPMI Medium was obtained from Gibco Laboratory, USA. Fetal calf serum was from Seralab. Dimethylsulphoxide (DMSO) was from Merck, Germany. Glutaraldehyde, glycerol and trypan blue were purchased from Sigma Chemical Co., USA. Mayer's haematoxyl-
in was a product of BDH Chemicals Limited, England.

2.1.3 Preparation of Solutions

Phosphate Buffered Saline a (PBSa)

PBSa was prepared by dissolving 10 tablets in a liter of distilled H₂O. The pH was verified to be 7.3.

Freezing Mixture

Freezing mixture consisted of 17.5 ml of RPMI Medium 1640 fortified with L-glutamine, 22.5 ml of DMSO and 5.0 ml of heat inactivated fetal calf serum (FCS). FCS was heat inactivated at 56°C for 30 min. It was then dispensed into 5 ml aliquots and stored at -20°C. The solution was mixed and the pH calibrated between 7.3 - 7.6 with 1 M HCl or 1 M NaOH. Solution was filtered through a 0.22 micron minisart cellulose acetate disposable filter and stored at 4°C.

Preparation of Monoclonal Antibodies

Monoclonal antibodies from Becton Dickinson arrived in a ready to use form. A 20 ul volume was pipetted out for each test. Dakopatts antibodies were packed in a concentrated form, thus a 20X dilution was carried out before 200 ul was taken out for each test. Both the above antibodies were stored at 4°C. Coulter’s products were received in a lyophilized form. The monoclonal antibodies were reconstituted in sterile distilled H₂O before use. Twenty ml of the reconstituted form was required for each test. Coulter’s antibodies were stored at -70°C.
2.2 METHODS

2.2.1 Immunoperoxidase

Immunoenzyme methods such as immunoperoxidase assay, the method used here, have the advantage over the traditional immunoflourescent method of not needing access to sophisticated and expensive facilities such as fluorescent activated cell sorter or even a fluorescence microscope. Living cells are not required, therefore samples need not be tested within 24 hours. The staining is stable making permanent preparations available for later review or reference. Cell morphology is discernible so that positive and negative cells can be differentiated. Furthermore, intracytoplasmic antigen can be labelled, allowing a higher proportion of actual positive cells to be identified. The peroxidase antiperoxidase (PAP) method is also much more sensitive (Marsden et al., 1987). Thus, it is most suitable for small clinical laboratories.

Immunoperoxidase staining involves the use of antibody and the enzyme peroxidase. Peroxidase is commonly used for several reasons. Its small size will not hinder the binding of antibody to adjacent sites. It is easily obtainable in a highly purified form so chances of contamination is minimized. It is very stable, therefore it will remain unchanged during manufacture, storage and application. Only small amounts are present in tissue specimens and this endogenous peroxidase activity is easily quenched. Also
there is a wide availability of chromogen which can be acted upon by peroxidase to form a coloured end product that will precipitate at the site of the antigen to be localized. Finally it is inexpensive (Bourne, 1983). There are several methods for immunoperoxidase staining. The one used in this project is the PAP (peroxidase anti-peroxidase) method, which utilizes three reagents, primary and secondary antibodies and a PAP complex comprising the enzyme peroxidase and an antibody against peroxidase. The primary antibody or monoclonal antibody is specific to the antigen. The secondary or "link" antibody, which is capable of binding both the primary and the PAP complex, is produced in the same animal species. The link antibody is added in excess. One of its Fab sites binds to the primary antibody leaving the other Fab site free to bind to the antibody in the PAP complex. The peroxidase enzyme is visualized via a substrate-chromogen reaction. The PAP method is important here for its application in identifying specific antigens the cell produces (Bourne, 1983).

2.2.2 Sample Collection

Bone marrow or peripheral blood samples were collected in tubes containing the anticoagulant, EDTA. A 1% volume of 15% dipotassium EDTA was added to sterile tubes meant for sample collection. Total volume of blood collected depended on the total white blood cell count of the patient. Volume collected ranged from 5 - 20 ml. Samples were collected for both immunophenotyping and DNA analysis. Samples were
processed immediately or left overnight at 4 C.

2.2.3 Mononuclear Layer Separation

Light density cells such as blast cells, lymphocytes, monocytes and platelets unlike other formed elements in anticoagulated blood are separated as the mononuclear layer on Ficoll-Paque. Ficoll-Paque is a sterile, ready to use solution of Ficoll 400 and sodium diatrizoate with a density of 1.077±0.001 g/ml. While mononucleated cells are trapped on the surface of the medium, red blood cells and polynucleated cells are spun to the bottom of the tube, facilitating easy collection of the mononuclear layer.

Samples were diluted 1:1 or more (depending on the total white blood cell count of the sample) with PBSa (which does not contain calcium and magnesium ions because EDTA anticoagulant requires the use of Ca++ and Mg++ free PBS) which was then layered on top of 4 ml of Ficoll-Paque in a 10 ml polypropylene tube and spun at 2100 rpm for 30 min at room temperature (RT). The separated layer was aspirated out with a glass pipette into another 10 ml tube. This was then diluted with more PBSa and again spun at 2100 rpm for 5 min to wash the sample free of contaminating platelets. The cell pellet was then resuspended in a known volume of PBSa.

2.2.4 Cell Count

Cell count was performed by using an Improved Neubauer
Haemocytometer. For the preparation of $2 \times 10^7$ cells/ml, samples were either diluted further by adding a known volume of PBSa or if the concentration of cells too low, the sample was spun down (2100 rpm, 5 min) and then resuspended with the correct volume of PBSa.

2.2.5 Cryopreservation

Cell samples which were not used immediately or analyzed further were cryopreserved in freezing mixture containing serum (fetal calf serum) and a cryoprotectant, DMSO.

A known volume of a sample with $2 \times 10^7$ cells/ml was spun at 2100 rpm for 5 min. The supernatant was poured off and substituted with an equal volume of freezing mixture. The cell pellet was resuspended gently into the mixture to allow time for a slow penetration of DMSO into the cell. A volume of 0.5-1.0 ml was then transferred into cryovials. The cryovials were attached to aluminium holders and left in a styrofoam box overnight at -70°C for a steady lowering of temperature. The optimal rate differed for various cell types, the average was taken to be 1°C per min. The following day, holders were removed from the boxes and dropped into the vapour phase of liquid nitrogen for long storage.

When needed, vials were removed from liquid nitrogen and immediately submerged in a 37°C bath for rapid warming. As soon as the specimen was liquefied, it was diluted slowly with PBSa to a ratio of 1:10, spun at 2100 rpm for 5 min and
then resuspended with the appropriate volume of PBSa to return the cell concentration to $2 \times 10^7$ cells/ml. Cells were not allowed to stand for too long in freezing mixture because of the cytotoxic effect of DMSO when not in the frozen state (Jewett et al., 1976)

### 2.2.6 Viability Test

A small volume of the sample was diluted 1:1 with PBSa and 0.1% trypan blue. A hundred cells were counted under a light microscope. Percentage of viable cell was the number of cells, in a hundred, which was not coloured by trypan blue. Viability should be greater than 80% (Cambridge Research Lab., 1987).

### 2.2.7 Blocking

Blocking is important to avoid non-specific binding, a positive staining which is not the result of antigen-antibody binding. The most common cause is attachment of protein to highly charged collagen and connective tissues (Bourne, 1983). According to Gadd and Ashman (1983) murine monoclonal antibodies of IgG2a and IgG3 subtypes can bind to human leukaemia cells especially those of the monocytic lineage through the Fc receptor. Fc receptors are present on monocytes, neutrophils and lymphocytes as well as on macrophages. While human T cells have not been demonstrated to carry the Fc receptors, in the mouse, however, activated T cells have been found to express the Fc receptor (Thaler et al., 1977). The presence of Fc receptors in T and B cells
is probably important in feedback regulation of lymphocyte responses (Roitt et al., 1985). Other causes of non-specific binding include inappropriate antibody dilutions, improper rinsing of slides, incorrect substrate incubation, drying up of slides in the middle of staining and the presence of endogenous peroxidase. Also, when red blood cells lyse, their contents are released into the surrounding medium. Even when these cells are subsequently separated, the products of hemolysis which remain react with the peroxidase substrate and give a nonspecific stain (Bourne, 1983). The most effective prevention is to add heat inactivated protein solution to the specimen before the primary antibody.

Reconstitution in 10-20% of heat inactivated, normal rabbit serum was done by spinning down the cells at 2100 rpm for 5 min and resuspending in an appropriate volume of normal rabbit serum and PBSa before proceeding to labelling.

Negative controls, consisting of nonimmune Ig of all subtypes (Mouse IgG 1, 2a, 2b, 3) at the same dilution as other primary moab, were included in all experiments.

2.2.8 Monoclonal Antibody Labelling

A 50 ul volume of 2x10^6 cells/ml cell sample was pipetted into 5 ml polypropylene tubes to give a total of 1x10^6 cells per tube. Monoclonal antibodies of the correct volume were then added into separate tubes. Each tube was vortexed, and
placed in ice and incubated at 4°C for 30 min. Incubation at 4°C was done to prevent capping, that is, the "grouping together" of antigens on the cell surface to form a cap. At intervals, cells were vortexed to break up any cell clumps.

At the end of 30 min, cells were diluted with PBSa and pelleted by spinning at 2100 rpm for 5 min. Excess monoclonal antibodies which remained suspended in solution were removed when the supernatant was poured off. To the tube was then added 1 ml of 0.1% glutaraldehyde to fix the cells (fresh glutaraldehyde was prepared from a 1.0% stock stored at 4°C). Cells were incubated for 10 min at room temperature, after which the cells were again washed, that is, diluted with PBSa and spun at 2100 rpm for 5 min at RT. Another wash was repeated before the final supernatant was poured off, leaving the cell pellet at the bottom of the tube. The moist pellet was then broken up by vortexing, resuspended into the moisture left behind and finally pipetted out and smeared onto the marked circle of an ethanol cleaned microscope slide. Slides were then left to dry overnight (Cambridge Research Laboratory, '1987).

2.2.9 Peroxidase Staining

Staining was done by using either the Cambridge Research Laboratory (CRL) Universal Immunoperoxidase staining kit or Dakopatts staining reagents. The staining kit consisted of a linking reagent (goat antimouse immunoglobulin), a labelling reagent (mouse Ig peroxidase labelled) and a substrate.
solution which was made by the addition of one drop of substrate reagent (1% hydrogen peroxidase) with one drop of substrate chromogen (2% 3-amino-9-ethylcarbazole in formamide) into 2 ml of 0.1% sodium acetate buffer, pH 5.2.

The Dakopatts reagents were goat Ig to mouse Ig solution diluted 1/200-1/250 to replace CRL's linking reagent and Dakopatts peroxidase anti-peroxidase monoclonal antibody diluted similarly to replace CRL's labelling reagent. The same substrate solution was used.

A 50 ul drop of linking reagent was added directly onto the dried cell smear of each slide, making sure the whole smear was fully covered. Slides were left to incubate for 30 min at RT in a "humid chamber" which consisted of a flat tray layered with wet handtowels on top of which the slides were laid. Careful attention was given to avoid solution run-off and drying out of reagents. After 30 min, the incubating solution was rinsed off carefully with PBSa in a wash bottle, so that cells were not dislocated from the slide. The slides were then flooded for 1 min in PBSa. PBSa was then poured off, the slides were rinsed again and the area around the cell smear was wiped dry with clean tissue paper. The specimen must be left moist at all times.

Incubation with the labelling reagent followed for another 30 min at RT. At the end of this incubation period, a
similar washing was done and the sides of the slides were
wiped dry. Another 10 min incubation with the linking
reagent was carried out followed by washing and drying. A
further 10 min incubation with the labelling reagent was
again carried out. These were done to enhance the effect of
staining. After washing and drying, slides were incubated
for 20 min at RT with the substrate solution prepared just
before use. A large enough volume was flooded onto the cell
smears. Washing was done by pouring off the solution and
then dipping the slides into beakers containing distilled
H O. Slides (but not the cell smears) were then dried.

Counterstaining was done using Mayer’s haematoxylin. A few
drops were added onto the smears and left to stain for 2
min. The counterstain was then poured off and again dipped
into distilled H O. After the slides were wiped dry, the

specimens were mounted with a non-alcoholic mountant,
PBSa:glycerol, V:V (1:9). The cell smears were finally
covered with coverslips before a count was done under a
light microscope. A total of 200 cells per slide were
counted from different fields and the percentage of positive
cells were ascertained. Positive cells are cells with a
reddish brown peroxidase ring around the cell. Interpretation
was always done together with the negative control.
B. DNA ANALYSIS

2.3 MATERIALS

2.3.1 Bone Marrow and Blood Source

Bone marrow aspirates or peripheral blood were collected from adult and paediatric leukaemic patients in University Hospital and other hospitals when available.

2.3.2 Chemicals and Reagents

All basic chemicals including salts were obtained from either Sigma Chemical Co., USA or BDH Chemicals Ltd., England. Agarose, Ficoll 400, bovine serum albumin, dextran sulfate (MW 500,000), bromophenol blue, proteinase K, ethidium bromide, salmon sperm DNA, ampicillin sodium salt and tetracycline HCl were purchased from Sigma. Tryptone, yeast extract and agar were from Oxoid Limited, England. Phenol was from Merck, Germany. All three deoxyribonucleoside triphosphates were obtained in the solution form from Pharmacia Molecular Biologicals, Sweden. Restriction enzymes were purchased from Pharmacia and New England Biolabs, USA. Random primers and the DNA Polymerase I large fragment (Klenow) were obtained from New England Biolabs. Nylon membrane was from Hoeffer and deoxycytidine 5’ (alpha-32P) triphosphate triethylammonium salt (approx. 50 uCi at 3000 Ci/m mole) was from Amersham International, England.

2.3.3 Probes/cDNA

The human alpha, beta, gamma and delta cDNAs were gifts
from Dr. Tak W. Mak of the Dept. of Medical Biophysics, University of Toronto. Clones were received in the form of stab cultures. Details of each probe were shown in Table 5.

2.3.4 Preparation of Materials

Solutions
Acidic phenol-chloroform, buffered phenol-chloroform, chisam (24 parts chloroform : 1 part isoamyl alcohol), phenol-chisam, Denhardt’s solution, electrophoresis loading buffer in TE (ELB) (10X) were prepared following procedures in Sambrook et al. (1989).

Buffers
TBE (10X), TE, 20X SSC and Tris-HCl, pH 8.0 were mixed as described in Sambrook et al. (1989).

Treatments
Fragmented salmon sperm and dialysis tubing were treated according to the methods in Sambrook et al. (1989).

LB Broth and Agar
LB broth consisted of 10 g of Tryptone, 2 g of yeast extract and 5 g of NaCl in 1 liter of distilled water. LB agar was prepared by adding 15 g of agar to 1 liter of LB broth.

Antibiotics
1. Tetracycline HCl. From a stock solution of 4 mg/ml in
Table 5: Description of probe/cDNA

<table>
<thead>
<tr>
<th>No.</th>
<th>Clone</th>
<th>Detects (chain)</th>
<th>Source</th>
<th>Sequences Present</th>
<th>Length (bp)</th>
<th>Plasmid</th>
<th>Insertion Site</th>
<th>Ref.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>pY14</td>
<td>alpha</td>
<td>Jurkat cDNA</td>
<td>MDJC</td>
<td>1100</td>
<td>pUC8</td>
<td>EcoRI</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>JurB1</td>
<td>beta</td>
<td>Jurkat cDNA</td>
<td>JCBeta1</td>
<td>770</td>
<td>pBR322</td>
<td>PstI</td>
<td>283</td>
</tr>
<tr>
<td>3.</td>
<td>HGP O2</td>
<td>gamma</td>
<td>PB cDNA</td>
<td>VJGammal</td>
<td>1600</td>
<td>pUC12</td>
<td>EcoRI</td>
<td>4</td>
</tr>
<tr>
<td>4.</td>
<td>CXHVO</td>
<td>delta</td>
<td>Thymocyte cDNA</td>
<td>C</td>
<td>1500</td>
<td>pUC18</td>
<td>EcoRI</td>
<td>5</td>
</tr>
<tr>
<td>5.</td>
<td>Jdelta</td>
<td>delta</td>
<td>? probe</td>
<td>J1</td>
<td>700</td>
<td>pUC18</td>
<td>EcoRI-HindIII</td>
<td>6</td>
</tr>
</tbody>
</table>

1. Yanagi et al., 1985  
2. Yanagi et al., 1984  
3. Yoshikai et al., 1984  
4. Yoshikai et al., 1987  
5. Takihara et al., 1988  
6. Tkachuk et al., 1988
distilled water was prepared a 10 ug/ml solution.

2. Ampicillin (sodium salt). A stock solution of 20 mg/ml in distilled water was prepared. The working concentration was 50 ug/ml. Antibiotic stock solutions were stored at -20 C.

2X Sucrose-Triton
For the preparation of 2X sucrose-triton, 219 g of sucrose, 2.42 g of Tris base, 2.03 g of MgCl₂ and 20 ml of Triton X-100 were dissolved in 500 ml of distilled water. The pH was adjusted to 7.6 with 6 N HCl and the total volume then brought up to 1 liter.

Saline EDTA
Saline EDTA was prepared by dissolving 2.19 g of NaCl and 4.47 g of Na₂EDTA in 400 ml of distilled water. The pH of the solution was then adjusted to 8.0 with 6 N NaOH and the total volume brought up to 500 ml.

Denaturing Solution
Denaturing solution was a mixture of 0.6 M NaCl and 0.5 M NaOH.

Neutralizing Buffer
Neutralizing buffer consisted of 1 M Tris base and 0.6 M NaCl. The pH was adjusted to 8.0 with concentrated HCl.
Oligolabelling Buffer

The oligolabelling buffer was made from the following components. Solution O: 1.25 M TrisHCl, 0.125 M MgCl₂ at pH 8.

Solution A: 1 ml of solution O + 18 ul of 2-mercaptoethanol + 5 ul of dATP, 5 ul of dTTP, 5 of ul dGTP (each triphosphate at an initial concentration of 0.1 M). Solution B: 2 M Hepes, titrated to pH 6.6 with 4 M NaOH. Solutions A and B were mixed at a ratio of 100:250 to make the oligolabelling buffer. Solutions O and B were stored at 4°C while solution A and the oligolabelling buffer were stored at -20°C.

Prehybridization Mix I

This solution consisted of 2.5X SSC, 0.1% SDS and 5X Denhardt’s solution.

Prehybridization II

The solution was made up of 2.5X SSC, 5X Denhardt’s solution, 0.1% SDS, 0.1% Na pyrophosphate, 10 ul/ml Poly A, 5% dextran sulfate and 200 ug/ml of denatured salmon sperm DNA.

Deluxe Wash Mix

The deluxe wash mix consisted of 2.5X SSC, 5X Denhardt’s solution, 0.1% SDS, 0.1% Na pyrophosphate and 10 ug/ml of Poly A.

Wash Mix I

Wash mix I consisted of 2.5X SSC, 0.1% SDS and 0.1% Na pyrophosphate.
Deprobing Solution

The solution was made from a mixture of 5 mM EDTA at pH 8 and 0.1% SDS.

2.4 METHODS

2.4.1 Aseptic Methods

All polycarbonate and polypropylene plasticware and glassware were sterilized by autoclaving at 15 psi for 15 min. All solutions were similarly autoclaved whenever necessary and stored at 4°C.

2.4.2 Safety Precautions

Gloves were worn when handling samples and sterile reagents. Used ware were discarded appropriately.

Gloves were also worn when using corrosive substances such as phenol or concentrated acids and mutagens such as ethidium bromide.

Proper shielding was set up with a 1 cm thick perspex whenever radioisotope work was carried out. Radioactive wastes were collected and stored for several half lives before handing over to the appropriate authorities.

2.4.3 Criteria for Sample Collection

Samples sent to the laboratory for immunophenotyping were
classified as T or non-T lineage. T lineage samples were collected for DNA studies. A number of non-T lineage samples were also collected for control studies.

2.4.4 Isolation of Single Bacterial Colonies
Clones were streaked onto individual LB agar plates containing ampicillin or tetracycline. Plates were incubated overnight at 37 C.

2.4.5 Alkaline Lysis Method
The alkaline lysis method of Birnboim and Doly (1979) is described in Sambrook et al. (1989). Cells were lysed by SDS at high pH which also denatures DNA. The lysed sample was neutralized with potassium acetate which renatures the plasmid DNA but not the chromosomal DNA. The high salt concentration and cooling process precipitated SDS and the bulk of chromosomal DNA into a protein-DNA-SDS complex which was removed by centrifugation. DNA was then deproteinized with buffered phenol, precipitated with isopropanol before banding in CsCl-dye buoyant density gradients.

2.4.6 Restriction Enzyme Digestion of Plasmid
In a sterile 1.5 ml tube, 50 ug of purified plasmid was digested with 5X or more of its relevant restriction enzyme (in units). One tenth volume of 10X buffer was added and the total digestion volume made up to 200 ul. The reaction was carried out at 37 C overnight. The final result was to achieve two clean bands on a minigel run.
2.4.7 Extraction of Inserts

All equipments were cleaned with ethanol and all solutions sterilized before use. A large gel was prepared for large scale extraction. Into a gel cast (14 x 11 cm) taped at both ends was poured 70 ml of 0.7% agarose in 0.5X TBE and ethidium bromide (EtBr) to a final concentration of 0.5 ug/ml. To make larger wells four teeth (of the comb) were taped into one. In this way, each well could take 0.6-0.8 ml of the digestion. The sample with 10% ELB in TE was then allowed to run overnight at 20V. The next day, under UV light, two ethanol cleaned microscope slides held together, were used to make an oblong mark just above the entire length of the band by pressing on the agar. A sterile scalpel was used to cut out this piece of agar leaving a well above the band. A piece of treated dialysis tubing, cut a bit longer than the well was placed against the cathode end of the wall of the well with the ends of the tubing inserted into the agar which was slit earlier. The well was cleaned and then filled with sterile 0.5X TBE. All other unused wells were also filled with the buffer. The gel was then reversed so that the band was now at the cathode end. The gel was then subjected to 200V for 50 sec to elute the DNA. The current was switched off and DNA from the well was pipetted into sterile 1.5 ml tubes. The well was again filled with buffer and the whole procedure repeated until the whole band had eluted out. The DNA that was collected was extracted twice
with phenol-chisam and twice with chisam. It was then left overnight to precipitate in 0.1 volume of 3M sodium acetate, pH 5.2 and two volumes of ice cold ethanol. The digestion and extraction procedure was repeated until a large enough amount of DNA was collected. The precipitated DNA was then spun at 7000 rpm, dried and dissolved in sterile distilled water. The DNA concentration was determined by using a spectrophotometer (see below). A small volume was run on a minigel to determine the purity and quality of the DNA.

2.4.8 Extraction of Human DNA Samples

Isolation of nuclei

A sample containing approximately 10 nucleated cells (estimated from the WBC value issued by the haematology laboratory) was mixed with an equal volume of ice cold 2X sucrose-triton to disrupt the cell membranes. The mixture was then topped up to 50 ml with ice cold 1X sucrose-triton. The sample was kept on ice for 10 min and inverted several times during this period. It was then spun at 3000 rpm for 15 min at 4°C. The supernatant was decanted and the nuclear pellet stored at -70°C until further treatment.

Proteinase K digestion

The nuclear pellet was agitated overnight at 37°C in 6 ml of saline-EDTA, 0.8 ml of 20% SDS and 75 ul of proteinase K (5 mg/ml).
Extraction of DNA

An equal volume of Tris saturated phenol (Tris-HCl, pH 8.0) was added to the digestion and the tube inverted gently to mix the two layers. The mixture was then spun at 3000 rpm for 5 min. The aqueous phase containing DNA was transferred with a wide bore pipette to a fresh tube. The whole procedure was repeated twice. Extraction with phenol was to remove contaminating protein from the DNA. This was followed by three extractions with chisam to denature residual protein and to remove traces of phenol. The final aqueous phase was then transferred to a 50 ml tube containing 2 ml of ice cold 7.5 M ammonium acetate. The ammonium ions dissociate ionically bound contaminants from the nucleic acid rendering it insoluble in the presence of alcohol. Ice cold 95% ethanol was added to the tube to bring the total volume to 20 ml. The precipitated DNA was removed by spooling onto a sterile glass rod and washed in a 30 sec dip in 70% ethanol. The DNA was air dried for 30 min and then dissolved in 100-400 ul of TE.

Quantitative analysis of DNA

The DNA concentration was measured by using a spectrophotometer. In a quartz cuvette, 10 ul of DNA was added to 1 ml of sterile distilled water, mixed by inverting the covered cuvette and the absorbance value read at 230, 260, 280, and 310 nm. The ratio of the 260 over 280 nm reading provides an estimate for the purity of the nucleic acid. Pure DNA preparation has an OD 260/280 of 1.8 however, a range be-
tween 1.75 to 2.00 is acceptable. The 310 nm value serves as a check for particulate matter and never exceeded 0.02. The 230 nm value was also less than the 260 nm value. An OD 260 of 1 corresponds to approximately 50 ug/ml for double stranded DNA.

The DNA concentration (ug/ml) was calculated as follows:

\[ 50 \text{ ug per OD unit} \times \text{OD at 260 nm} \times \text{dilution factor} \]

(Sambrook et al., 1989).

2.4.9 Restriction Enzyme Digestion

In separate 1.5 ml tubes, 10 ug of DNA samples were digested with 50 units of EcoRI, BamHI and HindIII in the presence of the appropriate buffer. The total volume was made up to 25 ul with sterile distilled water. Tubes were given a quick spin, mixed and left to incubate in a 37 C waterbath for 4 hours to overnight.

2.4.10 Agarose Gel Electrophoresis

A 70 ml volume of 0.8% agarose prepared in 0.5X TBE was poured into a 14 x 11 cm casting tray with a 5 mm teeth comb. The gel was allowed to solidify for half an hour at the end of which, the comb was removed and the gel placed into an electrophoresis tank containing 0.5X TBE with the wells oriented at the cathode end. Lambda phage DNA digested with HindIII was used as a molecular weight standard. The outermost well on the left was filled with 10 ul of
lambda phage-HindIII digest while the remaining wells were loaded with samples. The gel was subjected to 20 V for a period of 16-20 hours until the bromophenol blue tracking dye had travelled 10-12 cm from the cathode end. After electrophoresis, the agarose gel was stained with EtBr (1 ug/ml) for 5 min, destained in water for another 5 min and viewed with a 302 nm UV on a UVP TM transilluminator. A transparent plastic sheet was placed on top of the gel, and the positions of the wells in relation to the bands of the lambda digest were noted and marked for future reference.

2.4.11 Southern Blot Transfer

Prior to transfer, the upper half of the gel which contained fragments larger than 15 kb was depurinated for 5 min in 0.25 M HCl. This improves transfer by nicking the DNAs. After a brief rinse in sterile distilled water, the gel was placed in denaturing solution for an hour and agitated regularly. After another rinse in distilled water, the gel was transferred to yet another tray filled with neutralizing buffer and soaked for another 1 hour.

For Southern transfer the following assembly was set up. SSC, 10X, was used as the transfer buffer. Concentrations above 10X allow DNA to be retained almost completely on the filter (Southern, 1975). Two 12 x 29 cm wicks were cut from 3 MM Whatman paper, soaked in transfer buffer and draped over a plastic support in a tray containing transfer buffer. The gel was laid in an inverted position on the
wick and air bubbles were gently rolled out with a glass rod. A piece of nylon membrane, 0.5 cm longer than the gel, was placed over the gel. The membrane was earlier floated and then submerged in 2X SSC for 5 min. Air bubbles were again rolled out before placing 2 smaller pieces of 3 MM Whatman paper, measuring 11 x 14 cm and previously soaked in buffer, on top of the nylon membrane. Lastly, a stack of precut paper towels (10 x 13 cm), a glass plate and a weight were placed on top of the Whatman paper. The whole assembly was carefully supported so that it did not topple over and left overnight to transfer.

The transfer can be stopped when the gel thickness has decreased to approximately 1 mm, since the gel concentration at this point is sufficiently high to prevent further transfer (Berger & Kimmel, 1987). The paper towels and Whatman paper were removed. The gel, still attached to the filter, was placed right side up and the location of the wells marked onto the filter for future references. The filter was then removed, washed briefly with 6X SSC, air dried on a piece of Whatman paper and then exposed to UV for 2-3 min to form cross links between a small fraction of the thymine residues in the DNA and positively charged amine groups on the surface of the membrane. Over-irradiation results in covalent attachment of a high proportion of thymine with consequent decrease in hybridization signal. The side of the membrane carrying the DNA should face the UV light source.
(Sambrook et al., 1989). To observe the efficiency of transfer, the gel was soaked in EtBr for 5 min, destained in water for another 5 min and then exposed under UV.

2.4.12 Labelling of DNA Fragments
The DNA probes were radiolabelled according to the method of Feinberg & Vogelstein (1983, 1984). To a 1.5 ml sterile microcentrifuge tube was added 2-3 ul of DNA (0.5-1.0 ug) and 10 ul of random primers (150 ng/ml). A prick was made in the cap with a sterile needle and the tube was placed in a beaker of boiling water for up to 15 min. This was immediately followed by chilling in iced water for 5 min. One microliter of 10 mg/ml BSA was added to the tube followed by 2.3 ul of oligolabelling buffer, 3.0 ul of radiolabelled dCTP and 1.0 ul of Klenow (2.5 units). The reaction was made up to a total of 20 ul with sterile distilled water. The tube was spun down, flicked to mix and incubated overnight at 37 C.

2.4.13 Prehybridization
Prehybridization was carried out to block nonspecific attachment of the probe to the surface of the filter. This was achieved with a blocking agent consisting of Denhardt's reagent, SDS and denatured salmon sperm DNA fragments. The blot was first floated and then submerged in 2X SSC for 5 min. It was then placed in a tray of prehybridization solution at 65 C and left to incubate for at least 2 hours. The membrane was then prehybridized further with prehybri-
dization solution II containing denatured salmon sperm DNA 200 ug/ml which was denatured by boiling for 5 min followed by rapid chilling. Prehybridization with salmon sperm DNA was carried out for at least 3 hours or overnight.

2.4.14 Hybridization

After prehybridization, hybridization was carried out with 50 ml of fresh prehybridization solution II with denatured salmon sperm DNA (50 ug/ml) and radiolabelled probe. The probe was denatured by boiling for 5-15 min and chilled on ice for another 5 min. At least three tubes of radiolabelled probes were added into 50 ml of hybridization solution. Three to four blots were hybridized at the same time. Hybridization was done at 65°C for 16-48 hours. The hybridization solution was reused but with an addition of a new tube of radiolabelled probe and salmon sperm DNA. The hybridization solution must cover each blot when hybridization was carried out for long hours.

2.4.15 Washing the Blot

Hybridization stringency has to be observed for the type of probes used. Stability of duplexes formed between strands is dependent upon homology between the DNA and probe. The stringency is adjusted either during the hybridization step or in the posthybridization washes by adjusting the salt and/or by changing the temperature (Berger & Kimmel, 1987). The TCR probes used here were heterogenous cloned sequences
corresponding to mRNA populations. Random addition of nucleotides at the N-region during gene rearrangement (Reis et al., 1989) results in enhanced number of base mismatch.

Posthybridization washes were carried out at \( 50 \, ^\circ \text{C} \). The blots were transferred to a tray containing a solution of prewarmed Deluxe wash mix. The blots were left to incubate for 15 min and agitated every 5 min. This was followed by two 5 min incubations with Wash mix I. The filters were then blotted on Whatman paper and individually wrapped in cling film.

2.4.16 Autoradiography

The wrapped filters were then placed in an x-ray cassette with intensifying screens. An x-ray film was inserted in the darkroom and the cassette was then kept at \( -70 \, ^\circ \text{C} \) to expose for 1-8 days according to the activity of the \( ^{32}\text{P} \). After exposure, the x-ray film was developed.

2.4.16 Deprobing the Blot

The formation of a nucleic acid hybrid is a reversible process. Under appropriate conditions, probes are stripped off the blot (Berger and Kimmel, 1987). Deprobing was carried out at 65 \( ^\circ \text{C} \) for 1-2 hours. Blots were placed into a large volume of prewarmed deprobing solution. At the end of the wash, the filters were dried on Whatman paper. Blots were stored wrapped in cling film at \( -20 \, ^\circ \text{C} \).